FLOW CELL DEVICES AND OPTICAL SYSTEMS FOR IN SITU NUCLEIC ACID SEQUENCING

INCORPORATION BY REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 63/469,027, filed May 25, 2023, which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] In typical fluorescence-based genomic testing assays, *e.g.*, genotyping or nucleic acid sequencing (using either real time, cyclic, or stepwise reaction schemes), dye molecules that are attached to nucleic acid molecules tethered on a substrate are excited using an excitation light source, a fluorescence photon signal is generated in one or more spatially-localized positions on the substrate, and the fluorescence is subsequently imaged through an optical system onto an image sensor. An analysis process is then used to analyze the images, find the positions of labeled molecules (or clonally amplified clusters of molecules) on the substrate, and quantify the fluorescence photon signal in terms of wavelength and spatial coordinates, which may then be correlated with the degree to which a specific chemical reaction, *e.g.*, a hybridization event or base addition event, occurred in the specified locations on the substrate. Imaging-based methods provide large scale parallelism and multiplexing capabilities, which help to drive down the cost and accessibility of such technologies. However, detection errors that arise from, for example, overly dense packing of labeled molecules (or clonally-amplified clusters of molecules) within a small region of the substrate surface, or due to low contrast-to-noise ratio (CNR) in the image, may lead to errors in attributing the fluorescence signal to the correct molecules (or clonally amplified clusters of molecules).

SUMMARY

[0003] Described herein are flow cell devices, sequencing systems comprising optical systems, and methods thereof for sequencing nucleic acids. The flow cell devices described herein can include multiple axially-displaced fluidic channels and three, four, or even more axially-displaced surfaces facing the channels so that the surfaces advantageously allow more samples (e.g., increased sample volume and/or sample varieties) to be disposed on a single flow cell than traditional flow cells. The optical systems and methods described herein are capable of imaging two, three, four, or even more surfaces of the flow cell devices that are axially-displaced from each other, therefore advantageously achieving improved sequencing throughput within a set system run time. The optical systems and methods described herein advantageously allow homogenous illumination over an wide field of view (FOV) of at least 10 mm^2 with less than

10% energy variance across the FOV. The systems and methods disclosed herein can enable simultaneous sequencing of morphological, RNA, and/or protein targets within traditional 2D samples or in situ samples. The systems and methods disclosed herein can enable imaging and sequencing of such targets simultaneously within a same sequencing run or even within the same sequencing cycle(s) with significantly reduced hands-on time of a user. As such, the systems and methods herein can increase effectiveness and efficiency of sequencing analysis. The devices, systems, and methods herein can enable imaging of axially-displaced surfaces of the flow cell device without moving any optical compensator into, out of, or along the optical path, therefore providing simpler and more conveniently optical systems that are also less prone to errors due to vibrations. Further, the devices, systems, and methods herein can advantageously allow conveniently switching between imaging traditional flow cells, e.g., with one or dual surfaces, and the multiple surface flow cells herein with three or more axially-displaced surfaces. Such switching does not require adding or removing any optical elements in the system, e.g., an optical compensator. The devices, systems, and methods herein also enable imaging with a numerical aperture (NA) of less than 0.6 with sufficient image qualities for accurate sequencing analysis, allow imaging of the multiple axially-displaced surfaces independently, and allow adjustment of the objective lens to alter the NA to a desired value less than 0.6. The devices, systems, and methods herein can reduce reagent consumption needed for sequencing analysis of a same amount of samples than existing flow cell devices and optical systems.

[0004] In an aspect, the present disclosure provides a system for in situ biomolecule analysis, the system comprising: an imaging system comprising: a flow cell configured to immobilize a cell or a tissue, wherein the cell or the tissue comprises a plurality of analytes that differ in type from each other; a light source configured to illuminate the cell or the tissue, thereby generating a plurality of signals corresponding to the plurality of analytes; and a detector configured to image the plurality of signals; and one or more processors communicatively coupled to the imaging system, wherein the one or more processors is individually or collectively programed to (a) illuminate, using the light source, the cell or the tissue, thereby generating the plurality of signals corresponding to the plurality of analytes; (b) detect, using the detector, the plurality of signals; and (c) determine, using the one or more computer processors, an identity or sequence of the plurality of analytes using the plurality of signals.

[0005] In some embodiments, the cell or tissue is an in situ cell or tissue sample. In some embodiments, the light source is configured to illuminate greater than about 20 square millimeters (mm^2) of the flow cell and the cell or the tissue with a peak-to-valley variation of at most about 5%. In some embodiments, the light source is configured to illuminate greater than

about mm^2 of the flow cell and the cell or the tissue with a RMS wavefront error of at most about 0.09λ. In some embodiments, the imaging system has a composite root mean square error of less than about 0.05. In some embodiments, the cell or the tissue is a whole cell or whole tissue. In some embodiments, the imaging system does not comprise an objective disposed within an optical path of the light source or the detector. In some embodiments, the imaging system does not comprise an objective. In some embodiments, the imaging system does not comprise a tube lens. In some embodiments, the illumination has an irradiance of at least about 40 milliwatts per square meter. In some embodiments, the cell or the tissue has been permeabilized. In some embodiments, the plurality of signals are a plurality of fluorescent signals. In some embodiments, the plurality of signals are detected with a Q-score of at least about 30. In some embodiments, the cell or the tissue is illuminated with at most about 10 illumination fields in a plane perpendicular to an optical axis of the imaging system. In some embodiments, a field of view of the detector is at least about 10 mm^2. In some embodiments, the cell or the tissue is imaged with a resolution of at least about 1 micrometer. In some embodiments, the flow cell is configured to permit the flow of one or more reagents into contact with the cell or the tissue. In some embodiments, the cell or the tissue is a cultured cell or a cultured tissue. In some embodiments, the cell or the tissue is an isolated cell or an isolated tissue. In some embodiments, a fidelity of imaging of the cell or the tissue is at least about 0.1 micrometers. In some embodiments, the plurality of analytes comprise a nucleic acid molecule. In some embodiments, the nucleic acid molecule is a deoxyribonucleic acid molecule. In some embodiments, the nucleic acid molecule is a ribonucleic acid molecule. In some embodiments, the plurality of analytes comprise a protein. In some embodiments, the plurality of analytes comprise a carbohydrate.

[0006] In another aspect, the present disclosure provides a method for imaging an in situ sample, comprising: (a) providing the in situ sample comprising a plurality of different types of analytes; (b) illuminating the plurality of different types of analytes to generate a plurality of signals related to the plurality of analytes; and (c) imaging the plurality of signals.

[0007] In some embodiments, the illuminating the plurality of different types of analytes is a sequential illumination of the plurality of different types of analytes. In some embodiments, the illuminating the plurality of different types of analytes is a simultaneous illumination of the plurality of different types of analytes. In some embodiments, the plurality of different types of analytes are selected from the group consisting of deoxyribonucleic acid molecules, ribonucleic acid molecules, proteins, and phosphorylated proteins. In some embodiments, the method further comprises applying a plurality of sequencing reagents on the in situ sample, each configured to sequence a different analyte of the plurality of different types of analytes. In some

embodiments, the illuminating is over an area of the flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%. In some embodiments, the illuminating is over at least about 1 mm^2 of the flow cell with a RMS wavefront error of at most about 0.09λ. In some embodiments, the illuminating is over an area of the flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%. In some embodiments, the illuminating is over at least about 1 mm^2 of the flow cell with a RMS wavefront error of at most about 0.09λ. In some embodiments, the method further comprises (d) using a computer processor operatively coupled to the detector to analyze the plurality of signals. In some embodiments, the analyzing the plurality of signals comprises determining a sequence of a nucleic acid molecule within the in situ sample. In some embodiments, the sequence of the nucleic acid molecule is determined with an accuracy, sensitivity, or specific of at least about 95%. In some embodiments, the sequence of the nucleic acid molecule is determined in an absence of destroying the in situ sample. In some embodiments, the in situ sample has a length, width, or height of at least about 10 micrometers. In some embodiments, the in situ sample comprises a tissue. In some embodiments, the in situ sample comprises a plurality of cultured cells. In some embodiments, the in situ sample comprises a plurality of isolated cells. In some embodiments, the in situ sample is imaged with at most about 10 images in a plane perpendicular to an optical axis of the optical assembly. In some embodiments, the plurality of signals are a plurality of fluorescent signals. In some embodiments, the plurality of signals are detected with a Q-score of at least about 30. In some embodiments, the in situ sample comprises a nucleic acid molecule. In some embodiments, the nucleic acid molecule is a deoxyribonucleic acid molecule. In some embodiments, the nucleic acid molecule is a ribonucleic acid molecule. In some embodiments, a field of view of the optical assembly is at least about 10 mm^2. In some embodiments, the in situ sample is imaged at a resolution of at least about 1 micrometer. In some embodiments, the in situ sample is imaged within at most about 24 hours. In some embodiments, a fidelity of imaging a plurality of images of the in situ sample is at least about 0.1 micrometers.

[0008] In another aspect, the present disclosure provides an optical assembly for in situ imaging, comprising: a flow cell configured to contain an in situ sample; a light source configured to illuminate the in situ sample in the flow cell, thereby generating a signal related to a property of the in situ sample; and a detector configured to image the signal.

[0009] In some embodiments, the illumination over an area of the flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%. In some embodiments, the illumination has a root-mean-square (RMS) wavefront error of at most about 0.09λ over an area of at least about 1 square millimeter (mm^2). In some embodiments, the

optical assembly further comprises a processor configured to analyze the signal to determine the property of the in situ sample. In some embodiments, the in situ sample has a length, width, or height of at least about 10 micrometers. In some embodiments, the optical assembly does not comprise an objective. In some embodiments, the system does not comprise an objective. In some embodiments, the optical assembly does not comprise a tube lens. In some embodiments, the system does not comprise an objective. In some embodiments, the in situ sample comprises a tissue. In some embodiments, the in situ sample comprises a plurality of cultured cells. In some embodiments, the in situ sample comprises a plurality of isolated cells. In some embodiments, the in situ sample is imaged with at most about 10 images in a plane perpendicular to an optical axis of the optical assembly. In some embodiments, the signal is a fluorescent signal. In some embodiments, the signal is detected with a Q-score of at least about 30. In some embodiments, the in situ sample comprises a nucleic acid molecule. In some embodiments, the nucleic acid molecule is a deoxyribonucleic acid molecule. In some embodiments, the nucleic acid molecule is a ribonucleic acid molecule. In some embodiments, a field of view of the optical assembly is at least about 10 mm^2. In some embodiments, the in situ sample is imaged at a resolution of at least about 1 micrometer. In some embodiments, the in situ sample is imaged within at most about 24 hours. In some embodiments, a fidelity of imaging a plurality of images of the in situ sample is at least about 0.1 micrometers.

[0010] In another aspect, the present disclosure provides a method for imaging an in situ sample, comprising: (a) providing the in situ sample in a flow cell comprised within a system comprising an optical assembly comprising a light source and a detector; (b) illuminating the in situ sample and generating a signal related to an analyte of the in situ sample; and (c) imaging, using the detector, the signal.

[0011] In some embodiments, the illuminating is over an area of the flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%. In some embodiments, the illuminating is over at least about 1 mm^2 of the flow cell with a RMS wavefront error of at most about 0.09λ. In some embodiments, the method further comprises (d) using a computer processor operatively coupled to the detector to analyze the signal. In some embodiments, the analyzing the signal comprises determining a sequence of a nucleic acid molecule within the in situ sample. In some embodiments, the sequence of the nucleic acid molecule is determined with an accuracy, sensitivity, or specific of at least about 95%. In some embodiments, the sequence of the nucleic acid molecule is determined in an absence of destroying the in situ sample. In some embodiments, the in situ sample has a length, width, or height of at least about 10 micrometers. In some embodiments, the optical assembly does not comprise an objective. In some embodiments, the system does not comprise an objective. In

some embodiments, the optical assembly does not comprise a tube lens. In some embodiments, the system does not comprise an objective. In some embodiments, the in situ sample comprises a tissue. In some embodiments, the in situ sample comprises a plurality of cultured cells. In some embodiments, the in situ sample comprises a plurality of isolated cells. In some embodiments, the in situ sample is imaged with at most about 10 images in a plane perpendicular to an optical axis of the optical assembly. In some embodiments, the signal is a fluorescent signal. In some embodiments, the signal is detected with a Q-score of at least about 30. In some embodiments, the in situ sample comprises a nucleic acid molecule. In some embodiments, the nucleic acid molecule is a deoxyribonucleic acid molecule. In some embodiments, the nucleic acid molecule is a ribonucleic acid molecule. In some embodiments, a field of view of the optical assembly is at least about 10 mm^2. In some embodiments, the in situ sample is imaged at a resolution of at least about 1 micrometer. In some embodiments, the in situ sample is imaged within at most about 24 hours. In some embodiments, a fidelity of imaging a plurality of images of the in situ sample is at least about 0.1 micrometers.

INCORPORATION BY REFERENCE

[0012] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference in its entirety. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The novel features of the inventive concepts are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0014] FIGS. 1A-1B schematically illustrate non-limiting examples of imaging multiple surface support structures for presenting sample sites for imaging by the imaging systems disclosed herein. FIG. 1A: illustration of imaging front and rear interior surfaces of a flow cell. FIG. 1B: illustration of imaging front and rear exterior surfaces of a substrate.

[0015] FIGS. 2A-2B illustrate a non-limiting example of a multi-channel fluorescence imaging module comprising a dichroic beam splitter for transmitting an excitation light beam to a sample, and for receiving and redirecting by reflection the resultant fluorescence emission to four detection channels configured for detection of fluorescence emission at four different

respective wavelengths or wavelength bands. FIG. 2A: top isometric view. FIG. 2B: bottom isometric view.

[0016] FIGS. 3A-3B illustrate the optical paths within the multi-channel fluorescence imaging module of FIGS. 2A and 2B comprising a dichroic beam splitter for transmitting an excitation light beam to a sample, and for receiving and redirecting by reflection a resultant fluorescence emission to four detection channels for detection of fluorescence emission at four different respective wavelengths or wavelength bands. FIG. 3A: top view. FIG. 3B: side view.

[0017] FIG. 4 is a graph illustrating a relationship between dichroic filter performance and beam angle of incidence.

[0018] FIG. 5 is a graph illustrating a relationship between beam footprint size and beam angle of incidence on a dichroic filter.

[0019] FIGS. 6A-6B schematically illustrate an example configuration of dichroic filters and detection channels of a multi-channel fluorescence imaging module wherein the dichroic filters have reflective surface tilted such that the angle between the incident beam (*e.g.*, the central angle) and the reflective surface of the dichroic filter is less than 45. FIG. 6A: schematic illustration of a multichannel fluorescence imaging module comprising four detection channels. FIG. 6B: detail view illustrating the angle of incidence (AOI) of a light beam on a dichroic reflector.

[0020] FIG. 7 provides a graph illustrating improved dichroic filter performance corresponding to the imaging module configuration illustrated in FIGS. 6A and 6B.

[0021] FIG. 8 provides a graph illustrating improved dichroic filter performance corresponding to the imaging module configuration illustrated in FIGS. 6A and 6B.

[0022] FIGS. 9A-9B provide graphs illustrating reduced surface deformation resulting from the imaging module configuration of FIGS 6A and 6B. FIG. 9A illustrates the effect of folding angle on image quality degradation induced by the addition of 1 wave of PV spherical power to the last mirror. FIG. 9B illustrates the effect of folding angle on image quality degradation induced by the addition of 0.1 wave of PV spherical power to the last mirror.

[0023] FIGS. 10A-10B provide graphs illustrating improved excitation filter performance (*e.g.*, sharper transitions between pass bands and surrounding stop bands) resulting from use of s-polarization of the excitation beam. FIG. 10A: transmission spectra for an example bandpass dichroic filter at angles of incidence of 40 degrees and 45 degrees, where the incident beam is linearly polarized and is p-polarized with respect to the plane of the dichroic filter. FIG. 10B: changing the orientation of the light source with respect to the dichroic filter, such that the incident beam is s-polarized with respect to the plane of the dichroic filter, results in a substantially sharper edge between the passband and the stopband.

[0024] FIGS. 11A-11B illustrate the modulation transfer function (MTF) of an example multiple surface imaging system disclosed herein having a numerical aperture (NA) of 0.3. FIG. 11A: first surface. FIG. 11B: second surface.

[0025] FIGS. 12A-12B illustrate the MTF of an example multiple surface imaging system disclosed herein having an NA of 0.4. FIG. 12A: first surface. FIG. 12B: second surface.

[0026] FIGS. 13A-13B illustrate the MTF of an example multiple surface imaging system disclosed herein having an NA of 0.5. FIG. 13A: first surface. FIG. 13B: second surface.

[0027] FIGS. 14A-14B illustrate the MTF of an example multiple surface imaging system disclosed herein having an NA of 0.6. FIG. 14A: first surface. FIG. 14B: second surface.

[0028] FIGS. 15A-15B illustrate the MTF of an example multiple surface imaging system disclosed herein having an NA of 0.7. FIG. 15A: first surface. FIG. 15B: second surface.

[0029] FIGS. 16A-16B illustrate the MTF of an example multiple surface imaging system disclosed herein having an NA of 0.8. FIG. 16A: first surface. FIG. 16B: second surface.

[0030] FIGS. 17A-17B provide plots of the calculated Strehl ratio for imaging a second flow cell surface through a first flow cell surface. FIG. 17A: plot of the Strehl ratios for imaging a second flow cell surface through a first flow cell surface as a function of the thickness of the intervening fluid layer (fluid channel height) for different objective lens and/or optical system numerical apertures. FIG. 17B: plot of the Strehl ratio as a function of numerical aperture for imaging a second flow cell surface through a first flow cell surface and an intervening layer of water having a thickness of 0.1 mm.

[0031] FIG. 18 provides a schematic illustration of a dual-wavelength excitation / four channel emission fluorescence imaging system of the present disclosure.

[0032] FIG. 19 provides an optical ray tracing diagram for an objective lens design that has been designed for imaging a surface on the opposite side of a 0.17 mm thick coverslip.

[0033] FIG. 20 provides a plot of the modulation transfer function for the objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface on the opposite side of a 0.17 mm thick coverslip.

[0034] FIG. 21 provides a plot of the modulation transfer function for the objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface on the opposite side of a 0.3 mm thick coverslip.

[0035] FIG. 22 provides a plot of the modulation transfer function for the objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface that is separated from that on the opposite side of a 0.3 mm thick coverslip by a 0.1 mm thick layer of aqueous fluid.

[0036] FIG. 23 provides a plot of the modulation transfer function for the objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface on the opposite side of a 1.0 mm thick coverslip.

[0037] FIG. 24 provides a plot of the modulation transfer function for the objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface that is separated from that on the opposite side of a 1.0 mm thick coverslip by a 0.1 mm thick layer of aqueous fluid.

[0038] FIG. 25 provides a ray tracing diagram for a tube lens design which, if used in conjunction with the objective lens illustrated in FIG. 19, provides for improved multiple-side imaging through a 1 mm thick coverslip.

[0039] FIG. 26 provides a plot of the modulation transfer function for the combination of objective lens and tube lens illustrated in FIG. 25 as a function of spatial frequency when used to image a surface on the opposite side of a 1.0 mm thick coverslip.

[0040] FIG. 27 provides a plot of the modulation transfer function for the combination of objective lens and tube lens illustrated in FIG. 25 as a function of spatial frequency when used to image a surface that is separated from that on the opposite side of a 1.0 mm thick coverslip by a 0.1 mm thick layer of aqueous fluid.

[0041] FIG. 28 provides ray tracing diagrams for tube lens design (left) of the present disclosure that has been optimized to provide high-quality, multiple-side imaging performance. Because the tube lens is no longer infinity-corrected, an appropriately designed null lens (right) may be used in combination with the tube lens to compensate for the non-infinity-corrected tube lens for manufacturing and testing purposes.

[0042] FIG. 29 illustrates one non-limiting example of a single capillary flow cell having 2 fluidic adaptors.

[0043] FIG. 30 illustrates one non-limiting example of a flow cell cartridge comprising a chassis, fluidic adapters, and optionally other components, which is designed to hold two capillaries.

[0044] FIG. 31 illustrates one non-limiting example of a system comprising a single capillary flow cell connected to various fluid flow control components, where the single capillary is compatible with mounting on a microscope stage or in a custom imaging instrument for use in various imaging applications.

[0045] FIG. 32 illustrates one non-limiting example of a system that comprises a capillary flow cell cartridge having integrated diaphragm valves to reduce or minimize dead volume and conserve certain key reagents.

[0046] FIG. 33 illustrates one non-limiting example of a system that comprises a capillary flow cell, a microscope setup, and a temperature control mechanism.

[0047] FIG. 34 illustrates one non-limiting example for temperature control of the capillary flow cells through the use of a metal plate that is placed in contact with the flow cell cartridge.

[0048] FIG. 35 illustrates one non-limiting approach for temperature control of the capillary flow cells that comprises a non-contact thermal control mechanism.

[0049] FIGS. 36A-36C illustrates non-limiting examples of flow cell device fabrication. FIG. 36A shows the preparation of a one-piece glass flow cell. FIG. 36B shows the preparation of a two-piece glass flow cell. FIG. 36C shows the preparation of a three-piece glass flow cell.

[0050] FIGS. 37A-37C illustrates non-limiting examples of glass flow cell designs. FIG. 37A shows a one-piece glass flow cell design. FIG. 37B shows a two-piece glass flow cell design. FIG. 37C shows a three-piece glass flow cell design.

[0051] FIG. 38 illustrates visualization of cluster (or polony) amplification in a capillary lumen.

[0052] FIG. 39 provides a non-limiting example of a block diagram for a sequencing system as disclosed herein.

[0053] FIG. 40 provides a non-limiting example of a flow chart for a sequencing method as disclosed herein.

[0054] FIG. 41 provides a non-limiting example of a schematic for a illumination system as disclosed herein.

[0055] FIG. 42 provides a non-limiting example of a flow chart for acquiring and processing structured illumination images of a flow cell surface as disclosed herein.

[0056] FIGS. 43A-43B provide non-limiting schematic illustrations of a multiplexed read-head as disclosed herein. FIG. 43A: side view of a multiplexed read-head in which individual microfluorimeters are configured to image a common surface, *e.g.*, the interior surface of a flow cell. FIG. 43B: top view of a multiplexed read-head illustrating the imaging paths acquired by individual microfluorimeters of the multiplexed read-head.

[0057] FIGS. 44A-44B provide non-limiting schematic illustrations of a multiplexed read-head as disclosed herein. FIG. 44A: side view of a multiplexed read-head in which a first subset of a plurality of individual microfluorimeters 4401 is configured to image a first surface, *e.g.*, a first interior surface of a flow cell, and a second subset of the plurality of individual microfluorimeters is configured to image a second surface, *e.g.*, a second interior surface of a flow cell. FIG. 44B: top view of the multiplexed read-head of FIG. 44A illustrating the imaging paths acquired by individual microfluorimeters 4401 of the multiplexed read-head.

[0058] FIG. 45 illustrates a non-limiting example of an optical imaging system having multiple imaging sensors configured for transmission imaging a flow cell upon sequential illumination by

multiple light sources, each light source emitting a different color, according to some embodiments herein. Liquid samples are introduced to the flow cell on a hydrophobic pad and flow through the flow cell by a pulling force.

[0059] FIG. 46 provides a non-limiting schematic illustration of a method utilizing an optical system for imaging the surface of a flow cell for nucleic acid sequencing, according to some embodiments herein.

[0060] FIGS. 47A-47B provides optical systems according to various embodiments described herein. FIG. 47A provides a non-limiting cut-away illustration of an optical system for imaging the surfaces of a flow cell, according to some embodiments herein. FIG. 47B provides a comparison of the optical system of FIG. 47A with IDEX instrument core.

[0061] FIG. 48A provides a non-limiting example of a flow cell with 424 individual tiles, imaged by the IDEX instrument core shown in FIG. 47 B. FIG. 48B provides a non-limiting example of a flow cell with < 40 individual tiles, imaged by the optical system described herein (see FIGS. 45, 46, 47A-47B).

[0062] FIGS. 49A-49B provide a non-limiting cut-away illustration of an optical system configured for multiple side imaging of a multiple sided flow cell. The optical system as shown comprises a piezo driven wedge block for rapid focusing. FIG. 49A illustrates the optical system configured to focus on the back-interior surface of the flow cell. FIG. 49B illustrates the optical system configured to focus on the front-interior surface of the flow cell.

[0063] FIG. 50 provides a non-limiting cut-away illustration of an optical system configured for imaging a large area surface. The optical system comprises multiple optical subsystems, wherein the optimized FOV of each subsystem overlaps the FOV of each neighboring optical subsystem, thereby providing a large area FOV.

[0064] FIGS. 51A-51B provides a non-limiting cut-away illustration of a focusing lens assembly. The focusing lens assembly is configured to maintain a fixed position within the optical path (e.g., optical axis) and to allow for relative motion between at least a first lens and second lens contained within a lens housing of the focusing lens assembly. FIG. 51A shows a focusing lens assembly with a first lens and a second lens. FIG. 51B shows the same focus lens assembly with the relative movement of the second lens as compared with FIG. 51A.

[0065] FIG. 52 provides a non-limiting cut-away illustration of an optical system configured for imaging a curved, large area surface. The optical system comprises multiple optical subsystems wherein each system is placed approximately orthogonal to the surface and wherein the FOV of each subsystem overlaps the FOV of each neighboring optical subsystem, thereby providing a system for imaging curved, large area surfaces.

[0066] FIGS. 53A-53B provides a non-limiting cut away illustration of an optical system configured to image a capillary flow cell. In this example, the optical system configured to image curved, large area surfaces are rotated about the x-axis and translated along the x-axis to obtain images of the entire interior surface of the capillary flow cell. FIG. 53A illustrates the optical axis of the center optical subsystem aligned with the z-axis. FIG. 53B illustrates the optical axis of the center optical subsystem rotated 90 degrees to align with the y-axis.

[0067] FIGS. 54A-54B provides a non-limiting cut away illustration of an optical system configured to image a capillary flow cell without the need for a stage to rotate the optical system about the x-axis. The optical system as shown comprises a piezo driven wedge block for rapid focusing. FIG. 54A illustrates the optical system configured to focus on the interior surface of the capillary flow cell closest to the light sources. FIG. 54B illustrates the optical system configured to focus on the interior surface of the capillary flow cell further from the light sources.

[0068] FIG. 55 is a bar graph showing the results of a trapping assay conducted by reacting various fluorescently-labeled multivalent molecules with a corresponding correct DNA template.

[0069] FIG. 56 is a bar graph showing the results of a trapping assay in which increasing concentrations of various fluorescently-labeled multivalent molecules were reacted with corresponding correct DNA templates.

[0070] FIG. 57 presents four graphs showing the results of a trapping assay comparing the signal intensity of fluorescently-labeled multivalent molecules carrying nucleotide arms comprising either an N3-Linker, Linker-6, Linker-8 or propargyl Linker. The multivalent molecules were labeled with CF680 or CF532 fluorophores. Two different concentrations of multivalent molecules were tested (20 and 80 nM). The graphs show trap time in seconds (x-axis) and P90 signal intensity (y-axis).

[0071] FIG. 58 presents four graphs showing the results of a trapping assay comparing the signal intensity of fluorescently-labeled multivalent molecules carrying nucleotide arms comprising either an N3-Linker, Linker-6, Linker-8 or propargyl Linker. The multivalent molecules were labeled with AF647 or CF570 fluorophores. Two different concentrations of multivalent molecules were tested (20 and 80 nM). The graphs show trap time in seconds (x-axis) and P90 signal intensity (y-axis).

[0072] FIG. 59 presents three graphs showing the results of real-time imaging trapping kinetics assays comparing signal intensity of fluorescently-labeled multivalent molecules carrying nucleotide arms comprising one of Linkers 6 or 10-16. Three different concentrations of the

multivalent molecules were tested (15, 7.5 and 2.5 nM). The graphs show trap time in second (x-axis) and signal intensity (y-axis).

[0073] FIG. 60 is a graph showing the results of a binding kinetic study of fluorescently-labeled multivalent molecules carrying nucleotide arms comprising one of Linkers 6 or 10-16. The graph shows multivalent molecule concentration (x-axis, nM) and rate (y-axis). The legend shown in FIG. 60 is also applicable to FIG. 59.

[0074] FIG. 61 is a bar graph showing the binding constant (K) determined for fluorescently-labeled multivalent molecules carrying nucleotide arms comprising one of Linkers 6 or 10-16.

[0075] FIG. 62 generally shows an example of a combined sequencing by avidity system, according to some embodiments.

[0076] FIG. 63 shows a computer system that is programmed or otherwise configured to implement methods provided herein.

[0077] FIGS. 64A-64F show exemplary embodiments of multiple-surface sample support structure or multiple-surface flow cell in which the multiple surfaces are axially displaced from each other, in accordance with certain embodiments herein.

[0078] FIG. 65 shows a non-limiting example of the illumination system of the optical assembly herein, which includes an illumination subsystem and a light beam delivery subsystem.

[0079] FIG. 66 shows a non-limiting example of the illumination subsystem herein.

[0080] FIGS. 67A-68C show uniformity in the illumination power density by the illumination system of FIG. 65 herein. FIG. 67A shows an example image of an illumination field and the associated illumination intensity. FIG. 67B shows a line trace of the illumination intensity along the long axis of FIG. 67A. FIG. 67C shows a line trace of the illumination intensity along the short axis of FIG. 67A.

[0081] FIG. 68 illustrates a non-limiting example of the illumination subsystem and the light beam delivery system of the optical assembly.

[0082] FIG. 69 illustrates a non-limiting example of the illumination subsystem of the optical assembly.

[0083] FIG. 70 shows a despeckler and its relative position to a collimator of the light beam delivery subsystem.

[0084] FIG. 71 shows a non-limiting example of the optical fiber and the light beam delivery subsystem.

[0085] FIG. 72 shows a non-limiting example of the liquid light guide and the light beam delivery subsystem.

[0086] FIGS. 73A-73D show non-limiting examples of the despeckler, in this case, a mechanical vibration source that is loosely or fixedly attached to at least a portion of the optical

fiber(s). FIG. 73A shows a wound portion of an optical fiber, according to some embodiments. FIG. 73B shows a portion of an optical fiber round around a vibration source, according to some embodiments. FIG. 73C shows a portion of an optical fiber round around a fan vibration source, according to some embodiments. FIG. 73D shows a portion of an optical fiber round around a fan vibration source, according to some embodiments.

[0087] FIG. 74 shows a table of different despeckler configurations in relation to the optical fiber and their corresponding speckle noise levels.

[0088] FIG. 75 illustrates a block diagram of a sequencing system for imaging DNA sample(s) during DNA sequencing reactions, according to some embodiments.

[0089] FIG. 76 is a schematic of various examples of configurations of multivalent molecules. Left (Class I): schematics of multivalent molecules having a "starburst" or "helter-skelter" configuration. Center (Class II): a schematic of a multivalent molecule having a dendrimer configuration. Right (Class III): a schematic of multiple multivalent molecules formed by reacting streptavidin with 4-arm or 8-arm PEG-NHS with biotin and dNTPs. Nucleotide units are designated 'N', biotin is designated 'B', and streptavidin is designated 'SA'.

[0090] FIG. 77 is a schematic of an example of a multivalent molecule comprising a generic core attached to a plurality of nucleotide-arms.

[0091] FIG. 78 is a schematic of an example of a multivalent molecule comprising a dendrimer core attached to a plurality of nucleotide-arms.

[0092] FIG. 79 shows a schematic of an example of a multivalent molecule comprising a core attached to a plurality of nucleotide-arms, where the nucleotide arms comprise biotin, a spacer, a linker and a nucleotide unit.

[0093] FIG. 80 is a schematic of an example of a nucleotide-arm comprising a core attachment moiety, a spacer, a linker and a nucleotide unit.

[0094] FIG. 81 shows the chemical structure of an example of a spacer (top), and the chemical structures of various examples of linkers, including an 11-atom linker, 16-atom linker, 23-atom linker and an N3 linker (bottom).

[0095] FIG. 82 shows the chemical structures of various examples of linkers, including linkers 1-9.

[0096] FIG. 83 shows the chemical structures of various examples of linkers joined/attached to nucleotide units.

[0097] FIG. 84 shows the chemical structures of various examples of linkers joined/attached to nucleotide units.

[0098] FIG. 85 shows the chemical structures of various examples of linkers joined/attached to nucleotide units.

[0099] FIG. 86 shows the chemical structures of various examples of linkers joined/attached to nucleotide units.

[0100] FIG. 87 shows the chemical structure of an example of a biotinylated nucleotide-arm. In this example, the nucleotide unit is connected to the linker via a propargyl amine attachment at the 5 position of a pyrimidine base or the 7 position of a purine base.

[0101] FIG. 88 shows a flow chart of a method of analyzing a biological molecule, according to some embodiments.

[0102] FIG. 89 shows a flow chart of a method for analyzing a biological sample, according to some embodiments.

[0103] FIG. 90 illustrates a perspective view of a non-limiting example of an imaging module or optical assembly.

[0104] FIG. 91 illustrates a cross-sectional view of the non-limiting example of an imaging module or optical assembly.

[0105] FIG. 92 shows a cross-sectional view of the non-limiting example of the single channel time-sequential color imaging module or optical assembly in FIGS. 90-91.

[0106] FIGS. 93A – 93B show examples of an external actuator coupling, according to some embodiments. FIG. 93A shows a detail view of an external actuator and optical assembly, according to some embodiments. FIG. 93B shows a far view of an external actuator and the optical assembly, according to some embodiments.

[0107] FIG. 94 shows an example of the optical elements of an optical assembly and the associated focus paths, according to some embodiments.

[0108] FIG. 95 shows an example of the optical elements of an optical assembly and the associated focus paths, according to some embodiments.

[0109] FIGS. 96A – 96B provide diffraction modulation transfer functions (MTFs) for optical systems, according to some embodiments. FIG. 96A shows an example MTF for an objective based optical system. FIG. 96B shows an example MTF for an optical system not comprising an objective.

[0110] FIGS. 97A – 97B show wavefront analysis calculations for an optical system of the present disclosure, according to some embodiments. FIG. 97A shows an example wavefront analysis calculation at position 1. FIG. 97B shows an example wavefront analysis calculation at position 2.

[0111] FIG. 98 shows a top surface optical performance curve, according to some embodiments.

[0112] FIG. 99 shows a bottom surface optical performance curve, according to some embodiments.

[0113] FIG. 100 shows a plot of an MTF of an optical system, according to some embodiments.

[0114] FIG. 101 shows a plot of a cumulative probability of achieving a given wavefront error, according to some embodiments.

[0115] FIG. 102 is a schematical illustration of a rotatory stage for moving the sample(s) relative to the objective lens of the optical system for imaging sequencing reactions.

[0116] FIG. 103A-103D show exemplary images of identification of morphological targets of in situ cells using the systems and methods disclosed herein.

[0117] FIG. 104 shows an exemplary image of identification of RNA targets of in situ cells using the systems and methods disclosed herein.

[0118] FIG. 105A-105F show exemplary images of identification of protein and/or phosphorylated protein targets of in situ cells using the systems and methods disclosed herein.

[0119] FIG. 106A-106C show exemplary images of identification of protein targets of in situ cells using the systems and methods disclosed herein (FIG. 106B) in comparison to protein localization using immunofluorescence (FIG. 106A).

[0120] FIG. 107 is a schematic of a guanine tetrad (*e.g.*, G-tetrad).

[0121] FIG. 108 is a schematic of an exemplary intramolecular G-quadruplex structure.

[0122] FIG. 109A is a schematic showing an embodiment of a bridge circle complex (1600) comprising a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500).

[0123] FIG. 109B is a schematic showing an embodiment of an analyte detection complex comprising an antibody bridge circle complex (1700) which includes a primary antibody attached to the bridge circle complex (1600) which is shown in FIG. 109A.

[0124] FIG. 110A is a schematic showing an embodiment of a bridge circle complex (1600) comprising a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500).

[0125] FIG. 110B is a schematic showing an embodiment of an analyte detection complex comprising an antibody bridge circle complex (1700) which includes a primary antibody attached to the bridge circle complex (1600) which is shown in FIG. 110A.

[0126] FIG. 111A is a schematic showing an embodiment of a bridge circle complex (1600) comprising a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500).

[0127] FIG. 111B is a schematic showing an embodiment of an analyte detection complex comprising an antibody bridge circle complex (1700) which includes a primary antibody attached to the bridge circle complex (1600) which is shown in FIG. 111A.

[0128] FIG. 112A is a schematic showing an embodiment of a bridge circle complex (1600) comprising a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500).

[0129] FIG. 112B is a schematic showing an embodiment of an analyte detection complex comprising an antibody bridge circle complex (1700) which includes a primary antibody attached to the bridge circle complex (1600) which is shown in FIG. 112A.

[0130] FIG. 113A is a schematic showing an embodiment of a bridge circle complex (1600) comprising a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500).

[0131] FIG. 113B is a schematic showing an embodiment of an analyte detection complex comprising an antibody bridge circle complex (1700) which includes a primary antibody attached to the bridge circle complex (1600) which is shown in FIG. 113A.

[0132] FIG. 114A is a schematic showing an embodiment of a target analyte comprising a first and second epitope.

[0133] FIG. 114B is a schematic showing an embodiment of an antibody bridge circle complex (1700) binding directly to a target analyte. In some embodiments, the antibody bridge circle complex (1700) comprises an antibody having an antigen binding site that binds a first epitope of a first target analyte.

[0134] FIG. 115A is a schematic showing an embodiment of a first antibody bridge circle complex (1700-1) binding directly to a first target analyte. In some embodiments, the first antibody bridge circle complex (1700-1) comprises a first primary antibody having an antigen binding site that binds an epitope of a first target analyte. In some embodiments, the first antibody bridge circle complex (1700-1) comprises a first bridge circle complex (1600-1) attached to the first primary antibody.

[0135] FIG. 115B is a schematic showing an embodiment of a second antibody bridge circle complex (1700-2) binding directly to a second target analyte. In some embodiments, the second antibody bridge circle complex (1700-2) comprises a second primary antibody having an antigen binding site that binds an epitope of a second target analyte.

[0136] FIG. 116 is a schematic showing an embodiment of a first antibody bridge circle complex (1700-1) and a second antibody bridge circle complex (1700-2) binding different epitopes of the same target analyte.

[0137] FIG. 117 is a schematic showing an embodiment of an analyte detection complex comprising a bipartite complex (1800) which includes a secondary antibody attached to a bridge circle complex (1600) and a primary antibody which is bound to the secondary antibody.

[0138] FIG. 118 is a schematic showing an embodiment of an analyte detection complex comprising a bipartite complex (1800) which includes a secondary antibody attached to a bridge circle complex (1600) and a primary antibody which is bound to the secondary antibody.

[0139] FIG. 119 is a schematic showing an embodiment of an analyte detection complex comprising a bipartite complex (1800) which includes a secondary antibody attached to a bridge circle complex (1600) and a primary antibody which is bound to the secondary antibody.

[0140] FIG. 120A is a schematic showing an embodiment of an analyte detection complex comprising a first bipartite complex (1800-1) which includes a first secondary antibody attached to a first bridge circle complex (1600-1) and a first primary antibody which is bound to the first secondary antibody.

[0141] FIG. 120B is a schematic showing an embodiment of an analyte detection complex comprising a second bipartite complex (1800-2) which includes a second secondary antibody attached to a second bridge circle complex (1600-2) and a second primary antibody which is bound to the second secondary antibody. In some embodiments, the second primary antibody can bind a second target analyte.

[0142] FIG. 121 is a table showing several embodiments of target barcode sequences that can be employed for simultaneously detecting and identifying two or more cellular target analytes (e.g., cellular structures) by conducting a single sequencing cycle and employing multi-color imaging. In some embodiments, the target barcode sequences listed in the table in FIG. 121 can be used for cell painting.

[0143] FIG. 122 shows images of fluorescent signals emitted from sequencing the barcode regions of concatemers inside a cellular sample wherein the concatemers were generated from bipartite complexes (1800). Cells were permeabilized and fixed, and reacted with barcoded bipartite complexes under a condition suitable for the bipartite complexes to bind their cognate target analytes, for example C-myc or tubulin. The analyte-bipartite complexes were subjected to rolling circle amplification to generate barcoded concatemers corresponding to tubulin or c-myc. The barcoded concatemers were sequenced using sequencing primers specific for the tubulin concatemers or the c-myc concatemers and a two-stage sequencing workflow employing labeled multivalent molecules and non-labeled nucleotide analogs. The images shown in FIG. 27 represent five consecutive sequencing cycles of the same cells and the same field-of-view using sequencing primers specific for the tubulin barcoded concatemers. The images are rendered in false color. The fluorescent signals emitted during the five sequencing cycles detect and identify tubulin structures inside the cells. The target barcode sequences are listed in the table.

[0144] FIG. 123A shows images of fluorescent signal emitted from sequencing the barcode regions of concatemers inside a cellular sample wherein the concatemers were generated from

bipartite complexes. Cells were permeabilized and fixed, and reacted with barcoded bipartite complexes under a condition suitable for the bipartite complexes to bind their cognate target analytes, for example histone or tubulin. The analyte-bipartite complexes were subjected to rolling circle amplification to generate barcoded concatemers corresponding to histone or tubulin. The histone and tubulin concatemers carried sequencing primer binding sites having different sequences. TOP: The barcoded concatemers were simultaneously sequenced using a mixture of sequencing primers specific for the histone concatemers or the tubulin concatemers and a two-stage sequencing workflow employing labeled multivalent molecules and non-labeled nucleotide analogs. The top image shows fluorescent signals emitted from a single sequencing cycle in which the tubulin barcode emits a green signal and the histone barcode emits a red signal. The top image shows histone (red) and tubulin (green) structures inside the cells. The top image is not a merged image. BOTTOM: The sequencing read products generated from sequencing the tubulin concatemers and histone concatemers were removed from the concatemers by extensive washing. The histone concatemers were sequenced using sequencing primers specific for the histone concatemers and a two-stage sequencing workflow employing labeled multivalent molecules. The bottom image shows fluorescent signals emitted from a single sequencing cycle in which the histone barcode emits a red signal. The bottom image shows histone (red) structures inside the cells. The top and bottom images represent the same cells and the same field-of-view.

[0145] FIG. 123B TOP is the same fluorescent image shown in the top image of FIG. 123A. FIG. 123B BOTTOM: The sequencing read products generated from sequencing the tubulin concatemers and histone concatemers (see FIG. 123A TOP) were removed from the concatemers by extensive washing. The tubulin concatemers were sequenced using sequencing primers specific for the tubulin concatemers and a two-stage sequencing workflow employing labeled multivalent molecules and non-labeled nucleotide analogs. The bottom image shows fluorescent signals emitted from a single sequencing cycle in which the tubulin barcode emits a green signal. The bottom image shows tubulin (green) structures inside the cells. The top and bottom images represent the same cells and the same field-of-view.

[0146] FIG. 124 shows an image of a dividing cell. The image was generated by fluorescent signals emitted from sequencing the barcode regions of concatemers inside a cellular sample wherein the concatemers were generated from bipartite complexes. Cells were permeabilized and fixed, and reacted with barcoded bipartite complexes under a condition suitable for the bipartite complexes to bind their cognate target analytes, for example histone or tubulin. The analyte-bipartite complexes were subjected to rolling circle amplification to generate barcoded concatemers corresponding to histone or tubulin. The histone and tubulin concatemers carried

sequencing primer binding sites having different sequences. The sequencing was conducted using a two-stage sequencing workflow employing labeled multivalent molecules and non-labeled nucleotide analogs. The image shown in FIG. 124 is not a merged image.

DETAILED DESCRIPTION

[0147] There is a need for increased throughput and flexibility in next generation sequencing (NGS) analysis systems. Disclosed herein are flow cell devices and sequencing systems including optical system designs that may provide any one or more of the following advantages: higher system throughput for fluorescence imaging-based genomics applications, compatibility with traditional flow cell devices and/or optical systems, flexibility in analysis or comparison of samples (e.g., larger sample volume and/or increased sample variety), improved optical resolution (including high performance optical resolution), wide FOV with homogenous illumination (e.g., less than 10% variance in excitation energy); simultaneous sequencing and identification of various targets inside cells or tissue; and improved image quality. The disclosed optical illumination and imaging system designs may provide any one or more of the following advantages: improved dichroic filter performance, increased uniformity of dichroic filter frequency response, improved excitation beam filtering, larger fields-of-view, increased spatial resolution, improved modulation transfer, contrast-to-noise ratio, and image quality, higher spatial sampling frequency, faster transitions between image capture when repositioning the sample plane to capture a series of images (*e.g.*, of different fields-of-view), improved imaging system duty cycle, and higher throughput image acquisition and analysis.

Optical systems

[0148] Described herein, in some embodiments, is an optical system 4500 as shown in the non-limiting schematic of FIG. 45, that eliminates a need for dichroics, or corrective optics, such as a tube lens for multiple-side imaging of a flow cell. The multiple-side can be dual-side, three-side, quad-side, or even more sides. The optical system 4500 disclosed herein may be used as components of systems designed for a variety of chemical analysis, biochemical analysis, nucleic acid analysis, cell analysis, or tissue analysis applications. As shown in FIG. 45, the optical system comprises multiple imaging sensors 4501-4504 that are configured for imaging a flow cell 4521, in some embodiments. In some embodiments, an imaging sensor 4501-4504 may be a CCD imaging sensor. In some embodiments, the imaging sensor 4501-4504 may be a CMOS imaging sensor. In some embodiments, pixel shifters 4505-4508 are used to translate the object being imaged relative to a corresponding imaging sensor 4505-4508. In some embodiments the optical system comprises a multi-band bandpass filter 4509. In some

embodiments, the multi-band bandpass filter is a multi-band fluorescence bandpass filter. In some embodiments, the multi-band bandpass filter is a tri-band fluorescence bandpass filter. In some embodiments, the tri-band fluorescence bandpass filter is referred to as a tri-band notch filter. In some embodiments, imaging optics 4510-4513 are positioned between the imaging sensors 4501-4504 and the flow cell 4521. In some embodiments, one imaging optic 4505-4508, also referred to as an imaging optic assembly, focuses light emitted from the flow cell 4521 to one of the imaging sensors, for e.g., 4501, 4502, 4503, or 4504. In some embodiments, the optical system comprises an integrated field flattening assembly. In some embodiments, the optical system comprises aberration correction. In some embodiments, the optical system lacks bandpass filters. In some embodiments, the optical system lacks cutoff filters. In some embodiments, the optical system lacks dichroic mirrors. In some embodiments, a liquid handling system 4514 dispenses a sample 4515 to the flow cell 4521. In some embodiments, the liquid handling system 4514 dispenses a liquid sample to a hydrophobic pad 4516 attached to the flow cell 4521. In some embodiments, the liquid handling system 4514 is a drop dispensing system. In some embodiments, the drop dispensing system 4514 delivers the sample 4515 as a droplet to the hydrophobic pad 4516 of the flow cell 4521. In some embodiments, the liquid sample 4515 is drawn into the interior 4517 of the flow cell 4521 by a pulling force. In some embodiments, the pulling force is initiated by a vacuum pump 4518. In some embodiments, the flow cell 4521 comprises an interior channel 4517 enclosed by a bottom plate 4519 and a top plate 4520. In some embodiments, the top plate 4520 and bottom plate 4519 are transparent. In some embodiments, the top plate comprises a front interior surface 4528. In some embodiments the bottom plate comprises a back interior surface 4529. In some embodiments, the sample, present in the interior channel 4517 of the flow cell 4521 is illuminated by a plurality of light sources 4522, 4523 or 4524. In some embodiments, each of the individual light sources 4522, 4523 and 4524 emit a different color or spectrum of light, 4525, 4526 and 4527 respectively. In some embodiments, the optical system 4500 comprises a heater.

[0149] Although the flow cell 4521 is shown with two interior surfaces in FIG. 45, in other embodiments, the flow cell 4521 can include two or more axially displaced channels and three or more axially displaced interior surfaces.

[0150] In some embodiments, a notch filter refers to a band stop filter. In some embodiments, a notch filter refers to a band stop filter. In some embodiments, the notch of a filter refers to a band stop or stopband. In some embodiments, the notch of a filter refers to a band pass or passband. In some embodiments, a multiband notch filter refers to a multiband bandpass filter. In some embodiments, a multiband notch filter refers to a multiband band stop filter.

[0151] In some embodiments, an imaging optic 4510 of the optical system 4500 comprises a reduction of 1x. In some embodiments, the optical system has a field-of-view (FOV) of greater than 1 mm^2, greater than 2 mm^2, greater than 4 mm^2, greater than 10 mm^2, greater than 20 mm^2, greater than 36 mm^2, greater than 40 mm^2, greater than 60 mm^2, greater than 80 mm^2, or greater than 100 mm^2. In some embodiments, the optical system has a numerical aperture (NA) of less than 0.6. In some embodiments, the NA is between about 0.1 to about 0.50, about 0.20 to about 0.40, or about 0.30. In some embodiments, the NA is 0.25. In some embodiments, the NA is about 0.1, 0.15, 0.20, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, or 0.60. In some embodiments, the plurality of imaging sensors is configured to capture the FOV. In some embodiments, a plurality of light sources comprises: a first light source 4522 configured to emit a first wavelength range 4525; a second light source 4523 configured to emit a second wavelength range 4526; and a third light source 4524 configured to emit a third wavelength range 4527. In some embodiments, a first fluorophore is excited by the first wavelength range 4525 of the first light source 4522. In some embodiments, a second fluorophore is excited by the second wavelength range 4526 of the second light source 4523. In some embodiments, a third fluorophore is excited by the third wavelength range 4527 of the third light source 4524. In some embodiments, a sample comprises a plurality of biological polymers. In some embodiments, the optical system 4500 does not comprise a dichroic filter. In some embodiments, the optical system 4500 does not comprise a tube lens.

[0152] Described herein are various methods for a variety of chemical analysis, biochemical analysis, nucleic acid analysis, cell analysis, or tissue analysis application. FIG. 46 provides a schematic illustration of an imaging method 4601 utilizing the optical system 4500 shown in FIG. 45 for imaging a sample 4515 contained within the flow cell 4521, according to some embodiments herein. In some embodiments, the imaging method may be configured for nucleic acid sequencing. In some embodiments, the sample 4515 is contained within, or flows through, the interior channel 4517 of a flow cell 4521, as shown in FIG. 45. In some embodiments, the sample comprises a biological polymer. In some embodiments, the biological polymer comprises units. In some embodiments, a fluorophore is complementary to a unit of the biological polymer. In some embodiments, the fluorophore is attached to a nucleotide that is complementary to a unit of the biological polymer. In some embodiments, two or more detectably distinct fluorophores are attached to a nucleotide that is complementary to a unit of the biological polymer. In some embodiments, the biological polymer is a nucleic acid sequence. In some embodiments, the unit is a nucleotide complementary to the fluorophore labeled nucleotide. In some embodiments, the multiple light sources emit light, transmitting through the sample.

[0153] Described herein are various methods for sequencing a biological polymer (e.g., nucleic acid molecule). A non-limiting schematic illustration of the sequencing method and instrumentation 4601 and the base calling method 4602 is shown in FIG. 46. In some embodiments, the method comprises: illuminating a sample 4515 using an optical system 4500 comprising a first light source 4522 of a plurality of light sources, wherein the first light source 4522 emits a first wavelength range 4525 exciting a first fluorophore of the sample 4515 and acquiring a first image of the sample 4515, wherein the optical system 4500 comprises a plurality of imaging sensors 4501-4504, further wherein the sample 4515 is disposed in an optical path between the plurality of light sources 4522-4524 and the plurality of imaging sensors 4501-4504; illuminating the sample 4515 using a second light source 4523 of the plurality, wherein the second light source 4523 emits a second wavelength range 4526 exciting a second fluorophore of the sample 4515 and acquiring a second image the sample 4515; illuminating the sample 4515 using a third light source 4524 of the plurality, wherein the third light source 4524 emits a third wavelength range 4527 exciting a third fluorophore of the sample and acquiring a third image of the sample 4515; combining the first image, the second image, and the third image into a composite image; identifying the presence of a first nucleotide via a first signal emitted by the first fluorophore, wherein the first signal is extracted from a first region of interest (ROI) of the composite image; identifying the presence of a second nucleotide via a second signal emitted by the second fluorophore, wherein the second signal is extracted from a second ROI of the composite image; identifying the presence of a third nucleotide via a third signal emitted by the third fluorophore, wherein the third signal is extracted from a third ROI of the composite image; and identifying the presence of a fourth nucleotide via the first and third signals emitted by the first and third fluorophores, respectively, wherein the first and third signals are extracted from a fourth ROI of the composite image. In some embodiments, the optical system 4500 further comprises a flow cell 4521, wherein the flow cell 4521 is disposed in the optical path between the plurality of imaging sensors 4501-4504 and the plurality of light sources 4522-4524. In some embodiments, the optical system 4500 further comprises at least one pixel shifter 4505-4508. In some embodiments, the optical system 4500 further comprises a multi-band bandpass filter 4509 disposed in the optical path between the plurality of imaging sensors 4501-4504 and the flow cell 4521. In some embodiments, the method further comprises imaging optics 4510-4513 disposed in the optical path between the multi-band bandpass filter 4509 and the flow cell 4521. In some embodiments, the optical system 4500 has a reduction of 1x. In some embodiments, the optical system has a field-of-view (FOV) of greater than 1 mm^2, greater than 2 mm^2, greater than 4 mm^2, greater than 10 mm^2, greater than 20 mm^2, greater than 36 mm^2, greater than 40 mm^2, greater than 60 mm^2, greater than 80 mm^2, or greater than 100

mm^2. In some embodiments, the optical system has a numerical aperture (NA) of less than 0.6. In some embodiments, the NA is 0.25. In some embodiments, the FOV is captured by the plurality of image sensors 4501-4504.

[0154] In some embodiments, the sequencing is sequencing-by-avidity. Additional discussion of sequencing-by-avidity is included in U.S. Patent No. 10,768,173 filed on September 23, 2019, which is incorporated herein by reference in its entirety. In some embodiments, the first fluorophore is associated with a first nucleotide conjugate. In some embodiments, the second fluorophore is associated with a second nucleotide conjugate. In some embodiments, the third fluorophore is associated with a nucleotide conjugate. In some embodiments, the first fluorophore and the third fluorophore are associated with a fourth nucleotide conjugate. In some embodiments, the nucleotide conjugate may comprise a polymer-nucleotide conjugate. In some embodiments, the nucleotide conjugate may comprise a particle-nucleotide conjugate.

[0155] In some embodiments, fluorophores which may serve as a first fluorophore, a second fluorophore, and/or a third fluorophore include, but are not limited to fluorescein and fluorescein derivatives such as carboxyfluorescein, tetrachlorofluorescein, hexachlorofluorescein, carboxynapthofluorescein, fluorescein isothiocyanate, NHS-fluorescein, iodoacetamidofluorescein, fluorescein maleimide, SAMSA-fluorescein, fluorescein thiosemicarbazide, carbohydrazinomethylthioacetyl-amino fluorescein, rhodamine and rhodamine derivatives such as TRITC, TMR, lissamine rhodamine, Texas Red, rhodamine B, rhodamine 6G, rhodamine 10, NHS-rhodamine, TMR-iodoacetamide, lissamine rhodamine B sulfonyl chloride, lissamine rhodamine B sulfonyl hydrazine, Texas Red sulfonyl chloride, Texas Red hydrazide, coumarin and coumarin derivatives such as AMCA, AMCA-NHS, AMCA-sulfo-NHS, AMCA-HPDP, DCIA, AMCE-hydrazide, BODIPY and derivatives such as BODIPY FL C3-SE, BODIPY 530/550 C3, BODIPY 530/550 C3-SE, BODIPY 530/550 C3 hydrazide, BODIPY 493/503 C3 hydrazide, BODIPY FL C3 hydrazide, BODIPY FL IA, BODIPY 530/551 IA, Br-BODIPY 493/503, Cascade Blue and derivatives such as Cascade Blue acetyl azide, Cascade Blue cadaverine, Cascade Blue ethylenediamine, Cascade Blue hydrazide, Lucifer Yellow and derivatives such as Lucifer Yellow iodoacetamide, Lucifer Yellow CH, cyanine and derivatives such as indolium based cyanine dyes, benzo-indolium based cyanine dyes, pyridium based cyanine dyes, thiozolium based cyanine dyes, quinolinium based cyanine dyes, imidazolium based cyanine dyes, Cy 3, Cy5, lanthanide chelates and derivatives such as BCPDA, TBP, TMT, BHHCT, BCOT, Europium chelates, Terbium chelates, Alexa Fluor dyes, DyLight dyes, Atto dyes, LightCycler Red dyes, CAL Flour dyes, JOE and derivatives thereof, Oregon Green dyes, WellRED dyes, IRD dyes, phycoerythrin and phycobilin dyes, Malachite green, stilbene, DEG dyes, NR dyes, near-infrared dyes and others

known in the art such as those described in Haugland, Molecular Probes Handbook, (Eugene, Oreg.) 6th Edition; Lakowicz, Principles of Fluorescence Spectroscopy, 2nd Ed., Plenum Press New York (1999), or Hermanson, Bioconjugate Techniques, 2nd Edition, or derivatives thereof, or any combination thereof. Cyanine dyes may exist in either sulfonated or non-sulfonated forms, and comprise two indolenin, benzo-indolium, pyridium, thiozolium, and/or quinolinium groups separated by a polymethine bridge between two nitrogen atoms. Commercially available cyanine fluorophores include, for example, Cy3, (which may comprise 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium or 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium-5-sulfonate), Cy5 (which may comprise 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium or 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate), and Cy7 (which may comprise 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium or 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium-5-sulfonate), where "Cy" stands for 'cyanine', and the first digit identifies the number of carbon atoms between two indolenine groups. Cy2 which is an oxazole derivative rather than indolenin, and the benzo-derivatized Cy3.5, Cy5.5 and Cy7.5 are exceptions to this rule. In some embodiments, the reporter moiety can be a FRET pair, such that multiple classifications can be performed under a single excitation and imaging step. As used herein, FRET may comprise excitation exchange (Forster) transfers, or electron-exchange (Dexter) transfers.

[0156] Described herein are optical systems 4700 for imaging samples in a flow cell where no focusing step is included.

[0157] Described herein are optical systems 4700 for imaging samples in a flow cell for analysis of biological polymers (e.g., nucleic acid sequencing). In some embodiments, such systems 4700 as shown in FIGS. 47A-47B are more compact and have higher throughput than previous optical systems. Table 1 and FIGS. 48A-48B provide a non-limiting example comparing sequencing cycle times for a standard flow cell and optical system versus the optical system as described herein. Table 1 provides cycle and run times and the respective calculations for a standard flow cell with 424 individual tiles (e.g., active area, region of interest, etc.) as shown in

FIG. 48A, compared with a flow cell with < 40 individual tiles optimized for imaging on the optical system described herein is shown in FIG. 48B. In some embodiments, one image is equivalent to one tile in area. In some embodiments, when the flow cell 4521, also shown in FIG. 48B, is imaged by the optical system, each tile is exposed to three sequential pulses of light from three separate LED light sources, where each LED light source emits a different wavelength. In some embodiments, each different wavelength is matched to the excitation spectra of a different fluorophore as described herein. In some embodiments, the imaging sensors of the optical system 4500 are synced with each excitation pulse to generate an image, wherein one image the entire area of one tile, and further wherein the pixels of the image each represent the amount of fluorescence emitted by the fluorophore. In some embodiments, 2 separate surfaces are imaged in one tile by the optical system 4500. In some embodiments, 8 total images having a total exposure time of 0.3 seconds are acquired by the optical system 4500, 4700 comprising 8 imaging modules (e.g., optical subsystems). In Table 1 the row titled "current" and highlighted in blue represent the total time, over 322 cycles, to be 36.17 hours for the standard flow cell shown in FIG. 48A when imaged with the IDEX optical system shown in FIG. 47B. In comparison, the rows titled "Sleq" show total times between 13.63 and 14.28 hours for the Sleq Cell (see FIG. 48B) when imaged with the optical system 4700 as shown in FIGS. 47A-47B. The bottom row of Table 1 displays a total time of 1.11 hours when only 25 cycles are performed. The decreased sequencing times demonstrate the advantage of a larger FOV allowed by the optical system 4700 as described herein.

[0158] TABLE 1. Cycle and run times for the previous system versus the system according to some embodiments.

Flow cell Design	Width	Length	Total Tiles	Imaging Time per Tile (sec)	Imaging Time per Cycle (min)	Number of Cycles	Total Imaging Time (hrs)	Chem-istry Time per Cycle (min)	Total Time (hrs)
Current	35	640	424	0.6	4.24	322	22.75	2.5	36.17
Sleq Cell	7	64	8	0.3	0.04	322	0.21	2.5	13.63
Sleq Cell	7	64	16	0.3	0.08	322	0.43	2.5	13.85
Sleq Cell	7	64	32	0.3	0.16	322	0.86	2.5	14.28
Sleq Cell	7	64	32	0.3	0.16	25	0.07	2.5	1.11

[0159] FIG. 48A provides an illustration of an imaging area of a flow cell described herein with 424 individual tiles. FIG. 48B provides an illustration of an imaging area of a flow cell described herein with less than 40 tiles.

[0160] FIG. 47A provides a non-limiting cut-away illustration of an optical system for imaging the surfaces of a flow cell 4521. In some embodiments, the optical system comprises an LED bank heat sink 4701, light pipe illuminators 4702, a flow cell 4521, sections of imaging optics 4703, one or more pixel shifters 4704, and a plurality of imaging sensors 4705. As shown in FIG. 47B, the optical system 4700 is smaller than a comparable instrument, such as the IDEX instrument core. Advantages of a smaller optical instrument include, but are not limited to reduced cabling requirements, reduction in the number of available failure modes, reduced heat exchange requirements, as well as a reduced benchtop footprint.

[0161] Described herein, in some embodiments, is an optical system 4900, as shown in the non-limiting schematic of FIGS. 49A-49B, configured for multiple side imaging of a flow cell 4905. The multiple-side can be dual-side, three-side, quad-side, or even more sides. The optical system 4900 disclosed herein may be used in systems designed for a variety of chemical analysis, biochemical analysis, nucleic acid analysis, cell analysis, or tissue analysis applications. As shown in FIGS. 49A-49B, the optical system comprises an imaging sensor 4912 that may be configured for imaging a flow cell 4905. In some embodiments, the sample flow is coincident with the x-axis as shown in FIGS. 49A-49B. In some embodiments, there may be a plurality of imaging sensors 4912. The imaging sensor 4912 may be a CCD imaging sensor. In some embodiments, the imaging sensor 4912 may be a CMOS imaging sensor. In some embodiments, the optical system 4900 comprises a pixel shifter 4911. The pixel shifter 4911 may be configured to improve image resolution. In some embodiments, the pixel shifter 4911 translates the object being imaged relative to the imaging sensor 4912. In some embodiments the optical system comprises a filter 4910. In some embodiments, the filter 4910 is a multi-band filter. In some embodiments, the filter 4509 is a multi-band stopband filter. In some embodiments, the filter 4910 is a tri-band fluorescence stopband filter. In some embodiments, the tri-band fluorescence stopband filter is referred to as a tri-band notch filter. In some embodiments, the system comprises imaging optics 4909. In some embodiments, the imaging optics 4909 comprise an objective lens.

[0162] In some embodiments, the filter 4910 is positioned between the imaging sensor 4912 and the flow cell 4905. In some embodiments, the imaging optics 4909, also referred to as an imaging optic assembly, focuses light emitted from the flow cell 4909 to the imaging sensor 4912. In some embodiments, the optical system 4900 comprises an integrated field flattening assembly. In some embodiments, the optical system comprises an aberration correction module.

In some embodiments, the optical system comprises a wedge block 4916, configured for adjusting the pathlength of the optical system. In some embodiments, the wedge block 4916 comprises a first wedge piece 4907, a second wedge piece 4906, or a combination thereof. In some embodiments, the system comprises a piezo drive 4908 configured to move the position of the first wedge piece 4907 and the second wedge piece 4906 relative to each other, therefore adjusting the optical path length of the optical system. In some embodiments the flow cell 4905 is configured for multiple sided imaging (DSI). In some embodiments, the flow cell 4905 comprises a front interior surface 4904, a back-interior surface 4905, or a combination thereof. In some embodiments the front interior surface 4904 and/or the back-interior surface 4903 comprise sample sites 4902. In some embodiments, the optical system comprises an optical axis 4913. In some embodiments, the optical system comprises an optimal imaging volume 4915. In certain aspects, the optimal imaging volume 4915 comprises the field-of-view (FOV), the area of illumination, the area of acquisition, the focal plane, the focal depth, the region and/or volume where sample sites 4902 emit a brightness at or above an acceptable level, or a combination thereof. Typically, in the art of microscopy, the brightness of objects in the center of the FOV may be maximum at the center and decrease toward the corners and/or edges.

[0163] In some embodiments, the optical system lacks bandpass filters. In some embodiments, the optical system lacks cutoff filters. In some embodiments, the optical system lacks dichroic mirrors or dichroic filters.

[0164] In some cases, imaging with multiple tiles can produce registration errors (e.g., errors in overlapping the tiles of the image). The amount of registration error can be a fidelity of the imaging system (e.g., a difference in registration between two images can be a fidelity of the optical system with respect to those two images). The methods and systems of the present disclosure can achieve a fidelity of at most about 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5, 0.1, 0.05, 0.01, or fewer micrometers. For example, a difference in the positioning of a plurality of images of a sample can be at most about 0.1 micrometers.

Wedge-block assemblies

[0165] Described herein are various embodiments of an optical system. In some embodiments, the optical system is an optical system configured for the fluorescent readout of samples. In some embodiments, the optical system comprises a wedge block assembly 4916 as shown in FIGS. 49A-49B. In certain aspects, the wedge block assembly 4916 comprises a first wedge piece 4907 and a second wedge piece 4906. In some embodiments, the wedge block assembly 4916 comprises an adjustable optical path length. In some embodiments, the first wedge piece 4907 is configured to move relative to the second wedge piece 4906. In some embodiments, the relative movement of the first wedge piece 4907 to the second wedge piece 4906 causes the

optical path length of the wedge block assembly 4916 to change due to the change in the physical thickness wedge block assembly 4916 as shown in FIGS. 49A to 49B. In some embodiments, the wedge block assembly 4916 comprises a gap separating the first wedge piece 4907 from the second wedge piece 4906. In some embodiments, the gap maintains a constant distance, regardless of the relative position of the first wedge piece 4907 and the second wedge piece 4906. In some embodiments, the first wedge piece 4907 and the second wedge piece 4906 are comprised of fused silica. In some embodiments, the first wedge piece 4907 and the second wedge piece 4906 are comprised of fused silica having a refractive index of 1.5. In some embodiments, the first wedge piece 4907 is coupled to a piezo drive 4908. In some embodiments, the optical system comprises a housing. In some embodiments, the wedge block assembly 4916 and piezo drive 4908 are contained within the housing. In some embodiments, the wedge block assembly 4916 and piezo drive 4908 comprise a wedge block-piezo drive assembly. In some embodiments, the second wedge piece 4906 of the wedge block assembly 4916 contacts the housing. In some embodiments, the second wedge piece 4906 of the wedge block assembly 4916 contacts the flow cell 4905.

[0166] In some embodiments, the position of the first wedge piece 4907 relative to the second wedge piece 4906 determines the position of the focal plane along the optical axis 4913 (e.g., z axis). In some embodiments, the top wedge piece 4907 is aligned with the bottom wedge piece 4906, as illustrated in FIG. 49A. In such an embodiment, the physical distance of the wedge block assembly 4916 results in the focal plane aligning with the back-interior surface. In this case, the sample sites 4902 of the back-interior surface are in focus. In some embodiments, the piezo drive 4908 moves the top wedge piece 4907 to a position relative to the bottom wedge piece 4906, as illustrated in FIG. 49B, such that the physical thickness of the wedge block 4916 within the optical path is greater than in the aligned state illustrated in FIG. 49A. In such an embodiment, the focal plane is shifted to align with the front-interior surface. In this case, the sample sites 4902 of the front-interior surface are in focus.

[0167] Additional examples of flow cells can be found in International Patent Application No. PCT/US2024/010760, which is incorporated by reference herein in its entirety.

Stages

[0168] Described here are various embodiments of an optical system comprising a stage. The stage may be a tilt stage. The stage may be a tip-tilt stage. The stage may allow for rotation. The stage may be configured to translate in three different axes, simultaneously, wherein all axes are perpendicular to each other. The stage may be configured to translate in three different axes, simultaneously, wherein all axes are perpendicular to each other. The stage may be configured to translate in, and rotate about, three different axes, simultaneously, wherein all axes are

perpendicular to each other. The stage may translate the plurality of optical subsystems 5001 relative to the flow cell 4905 as shown in FIG. 50. The stage may translate the flow cell 4905 relative to the plurality of optical subsystems 5001 as shown in FIG. 50. The stage may translate a single optical subsystem 4914. The stage may rotate the plurality of optical subsystems 5001 about the x-axis of the capillary flow cell 5201 as shown in FIG. 53A-53B. The stage may translate the plurality of optical subsystems 5001 along the x-axis, coincident to the long axis of the capillary flow cell 5201 as shown in FIG. 52A-53B.

Pixel Shifters

[0169] Described herein are various embodiments of an optical system comprising a pixel shifter 4911. In some embodiments, the pixel shifter 4911 enables sub-pixel resolution imaging. In certain aspects, the resolution of the optical system may be increased by use of the pixel shifter 4911 without increasing the actual optical system resolution. In some embodiments, the pixel shifter 4911, effectively multiplies the resolution of the imaging sensor 4912. In some embodiments, a piezoelectric actuator is configured for defined lateral pixel shifts coincident to the image plane (e.g., in the x-y plane). In some embodiments, a piezoelectric actuator is configured for pixel shifting in the optical axis 4913 (e.g., z-axis or z-axis containing planes). In some embodiments, tilt stage is configured for pixel shifts in X-Z or Y-Z or X-Y-Z. In some cases, the tilt stage is configured for pixel shifts in two dimensions. In some embodiments, the optical system comprising the pixel shifter is configured for imaging the 3D sample objects. In some cases, the optical system comprising the pixel shifter is configured for imaging a 2D sample object.

[0170] In some embodiments, the 3D objects may comprise sample sites 4902. In some embodiments, sample sites 4902 are amplified nucleic acids. In some embodiments, a sample site may comprise a polony or multiple polonies.

[0171] The pixel-shifter 4911 may utilize polarization.

Autofocus elements

[0172] Described herein are various embodiments of an optical system comprising an autofocus element.

[0173] FIGS. 51A-51B provides a non-limiting cut-away illustration of a focusing lens assembly. The focusing lens assembly is configured to maintain a fixed position within the optical pathway (e.g., optical axis) and to allow for relative motion between at least a first lens and second lens contained within a lens housing of the focusing lens assembly.

[0174] In some embodiments, the autofocus element is configured for initial focus. In some embodiments, the autofocus element is contained within a lens barrel. In some embodiments, the autofocus element is built into and/or integrated with the lens barrel. In some embodiments, the

autofocus element is contained within the lens barrel of the lens assembly. In some embodiments, the autofocus element is configured to improve reliability, reduces mechanical footprint of the optical system. In some embodiments the autofocus element comprises the wedge block assembly, the piezo drive, the wedge block-piezo drive assembly, or a combination thereof.

Multiple imaging subsystems

[0175] In some embodiments, the optical system as seen in FIG. 50 comprises a plurality of optical subsystems 4914. In some embodiments, each optical subsystem 4914 of the plurality 5001 comprises an imaging sensor 4912, a pixel shifter 4911, a filter 4910, imaging optics 4909, a piezo driven - wedge block assembly, a light source 4901, or a combination thereof. In some embodiments, the imaging sensor 4912 is a cellphone-style camera. In some embodiments, the plurality of optical subsystems 5001 comprises an array of optical subsystems. In some embodiments, the array of optical subsystems may be configured for multiple focal depths, multiple wavelengths, or a combination thereof. In some embodiments, each optical subsystem 4914 of the plurality 5001 is configured for a focal depth, wherein the focal depths of at least two optical subsystems of the plurality are different. In some embodiments, each optical subsystem of the plurality is configured to detect a wavelength, wherein the wavelengths detected by at least two optical subsystems of the plurality are different. In some embodiments, an image sensor 4912 of each optical subsystem 4914 of the plurality 5001 comprise an array of image sensors 4912. In some embodiments, high resolution low-cost cameras are configured to provide imaging, with aberrations compensated by software. In some embodiments, the optical system comprises one optical subsystem 4914, wherein the optical subsystem 4914 comprises one optimal imaging volume as shown in FIGS. 49A-49B. In FIGS. 49A-49B the extent of the optimal imaging volume 4915 along the x axis is limited. Certain factors may affect the width of the optimal imaging volume in the x-y plane (e.g., the focal plane). The x-y plane, or focal plane, comprises a cross section of the optimal imaging volume and may comprise referred to as the area of illumination, area of acquisition, or a combination thereof. Surfaces comprising sample sites 4902 that extend beyond the optimal FOV are not optimally illuminated by the light source, not optimally captured by the imaging sensor, not optimally resolved by the optics, or a combination thereof. Such non-optimal regions of the surface exhibit non-uniform brightness and non-uniform resolution as may be observed in the edges and/or corners of the image in FIG. 38. In FIG. 38 the sample sites become dimmer and less resolved from the center to the edges and/or corners of the image. FIG. 50 illustrates an embodiment where the sample site 4902 covered surface extends beyond the optimal imaging volume 4915 of one optical subsystem

4916 and where overlapping optimal imaging volumes 4915 overlap to provide a composite optimal imaging volume.

[0176] In some embodiments, the optical system has an optimized FOV of 6 mm x 6 mm. In some embodiments, the system has an optimized FOV of about 0.5 mm to about 9 mm. In some embodiments, the system has an optimized FOV of about 0.5 mm to about 1 mm, about 0.5 mm to about 3 mm, about 0.5 mm to about 6 mm, about 0.5 mm to about 9 mm, about 1 mm to about 3 mm, about 1 mm to about 6 mm, about 1 mm to about 9 mm, about 3 mm to about 6 mm, about 3 mm to about 9 mm, or about 6 mm to about 9 mm. In some embodiments, the system has an optimized FOV of about 0.5 mm, about 1 mm, about 3 mm, about 6 mm, or about 9 mm. In some embodiments, the system has an optimized FOV of at least about 0.5 mm, about 1 mm, about 3 mm, or about 6 mm. In some embodiments, the system has an optimized FOV of at most about 1 mm, about 3 mm, about 6 mm, or about 9 mm.

[0177] In some embodiments, the optical system has an optimized area of illumination, of 6 mm x 6 mm. In some embodiments, the system has an optimized area of illumination, of about 0.5 mm to about 9 mm. In some embodiments, the system has an optimized area of illumination, of about 0.5 mm to about 1 mm, about 0.5 mm to about 3 mm, about 0.5 mm to about 6 mm, about 0.5 mm to about 9 mm, about 1 mm to about 3 mm, about 1 mm to about 6 mm, about 1 mm to about 9 mm, about 3 mm to about 6 mm, about 3 mm to about 9 mm, or about 6 mm to about 9 mm. In some embodiments, the system has an optimized area of illumination, of about 0.5 mm, about 1 mm, about 3 mm, about 6 mm, or about 9 mm. In some embodiments, the system has an optimized area of illumination, of at least about 0.5 mm, about 1 mm, about 3 mm, or about 6 mm. In some embodiments, the system has an optimized area of illumination, of at most about 1 mm, about 3 mm, about 6 mm, or about 9 mm.

[0178] In some embodiments, the optical system is configured for rapid imaging of the surface. In some embodiments, the optical system is configured for rapid imaging of the surface of the flow cell. In some embodiments, the optical system is configured for rapid imaging of a first surface and a second surface of the flow cell. In some embodiments, the entire active area (e.g., region of interest, ROI) of the surface 4903 or 4904 of the flow cell 4905 is imaged in 5 imaging steps. In some embodiments, the active area (e.g., region of interest) of the surface 4903 or 4904 is imaged in about 1 imaging step to about 10 imaging steps. In some embodiments, the active area (e.g., region of interest) of the surface is imaged in about 1 imaging step to about 2 imaging steps, about 1 imaging step to about 3 imaging steps, about 1 imaging step to about 4 imaging steps, about 1 imaging step to about 5 imaging steps, about 1 imaging step to about 6 imaging steps, about 1 imaging step to about 10 imaging steps, about 2 imaging steps to about 3 imaging steps, about 2 imaging steps to about 4 imaging steps, about 2 imaging steps to about 5 imaging

steps, about 2 imaging steps to about 6 imaging steps, about 2 imaging steps to about 10 imaging steps, about 3 imaging steps to about 4 imaging steps, about 3 imaging steps to about 5 imaging steps, about 3 imaging steps to about 6 imaging steps, about 3 imaging steps to about 10 imaging steps, about 4 imaging steps to about 5 imaging steps, about 4 imaging steps to about 6 imaging steps, about 4 imaging steps to about 10 imaging steps, about 5 imaging steps to about 6 imaging steps, about 5 imaging steps to about 10 imaging steps, or about 6 imaging steps to about 10 imaging steps. In some embodiments, the active area (e.g., region of interest) of the surface is imaged in about 1 imaging step, about 2 imaging steps, about 3 imaging steps, about 4 imaging steps, about 5 imaging steps, about 6 imaging steps, or about 10 imaging steps. In some embodiments, the active area (e.g., region of interest) of the surface is imaged in at least about 1 imaging step, about 2 imaging steps, about 3 imaging steps, about 4 imaging steps, about 5 imaging steps, or about 6 imaging steps. In some embodiments, the active area (e.g., region of interest) of the surface is imaged in at most about 2 imaging steps, about 3 imaging steps, about 4 imaging steps, about 5 imaging steps, about 6 imaging steps, or about 10 imaging steps.

[0179] In some embodiments, each imaging step includes imaging at least a portion of the entire active area or ROI. In some embodiments, each imaging step includes imaging at least an overlapping portion of the ROI, and the overlapping portion in the ROI can also be imaged in a different imaging step. In some embodiments, the complete ROI can be imaged, a portion at a time in an imaging step (with or without some overlapping area), in multiple imaging steps.

[0180] In some embodiments, the image acquired using the optical systems herein is a flow cell image. The flow cell image can include a FOV that covers at least a portion of the entire active area or ROI on a surface of a flow cell or otherwise a different sample support structure. The flow cell images can be aligned with each other to cover the entire ROI of a surface of the flow cell.

Methods of using the optical systems

[0181] Described herein are various methods for imaging a biological polymer, comprising: providing an optical system comprising: a plurality of optical subsystems, each optical subsystems of the plurality comprising: a light source configured to separately emit a first wavelength and a second wavelength, wherein said first wavelength is different from said second wavelength; a multiband filter configured to reject each of said first wavelength and said second wavelength; an imaging sensor configured to image one or more biological polymers disposed in an optical path between each light source and each imaging sensor; and bringing said one or more biological polymers into contact with a plurality of fluorophores under conditions sufficient to cause a first biological polymer of said one or more biological polymers to bind with a first fluorophore of said plurality of fluorophores and a second biological polymer

of said one or more biological polymers to bind with a second fluorophore of said plurality of fluorophores, wherein said first fluorophore is different than said second fluorophore; imaging said first biological polymer with each imaging sensor, wherein said imaging comprises (i) illuminating said first biological polymer with said first wavelength, thereby exciting said first fluorophore, and (ii) acquiring a first image; and imaging said second biological polymer with each imaging sensor, wherein said imaging comprises (i) illuminating said second biological polymer with said second wavelength, thereby exciting said second fluorophore, and (ii) acquiring a second image, and wherein said one or more biological polymers are disposed on a curved surface, and wherein the optical axis of each optical subsystem of said plurality is orthogonal to said curved surface. In some embodiments, the method further comprises imaging a third biological polymer of said one or more biological polymers comprising (i) illuminating said third biological polymer with a third wavelength, exciting a third fluorophore of said plurality of fluorophores, and (ii) acquiring a third image. In some embodiments, the method further comprises combining said first image and said second image into a composite image. In some embodiments, the method further comprises identifying a unit of said first biological polymer bound by said first fluorophore comprising analyzing a first region of interest (ROI) of said composite image to detect a first signal emitted by said first fluorophore. In some embodiments, the method further comprises identifying a unit of said second biological polymer bound by said second fluorophore comprising analyzing a second ROI of said composite image to detect a second signal emitted by said second fluorophore. In some embodiments, the method further comprises identifying a first unit of said first biological polymer bound by said first fluorophore comprising analyzing a first ROI of said composite image to detect a first signal emitted by said first fluorophore; and identifying a second unit of said second biological polymer bound by said second fluorophore comprising analyzing a second ROI of said composite image to detect a second signal emitted by said first fluorophore. In some embodiments, the method further comprises combining said first image, said second image, and said third image into a composite image. In some embodiments, the method further comprises identifying a third unit of said third biological polymer bound by said third fluorophore comprising analyzing a third ROI of said composite image to detect a third signal emitted by said third fluorophore. In some embodiments, the method further comprises: identifying a first unit of said first biological polymer bound by said first fluorophore comprising analyzing a first region of interest (ROI) of said composite image to detect a first signal emitted by said first fluorophore; identifying a second unit of said second biological polymer bound by said second fluorophore comprising analyzing a second ROI of said composite image to detect a second signal emitted by said first fluorophore; identifying a third unit of said third biological polymer

bound by said third fluorophore comprising analyzing a third ROI of said composite image to detect a third signal emitted by said third fluorophore; and identifying a third unit of said third biological polymer bound by said third fluorophore comprising analyzing a third ROI of said composite image to detect a third signal emitted by said third fluorophore.

[0182] Described herein are various methods of using the optical system as described herein for super resolution imaging. In some embodiments, the method comprises providing a surface further comprising at least one sample site comprising clonally-amplified, sample nucleic acid molecules immobilized to a plurality of attached oligonucleotide molecules, wherein said plurality of immobilized clonally amplified sample nucleic acid molecules are present at distance less than $\lambda/(2*NA)$, wherein λ is the center wavelength of an light source and NA is the numerical aperture of an imaging system; applying a stochastic photo-switching chemistry to said clonally amplified sample nucleic acid molecules at the same time to cause said plurality of clonally amplified sample nucleic acid molecules to fluoresce in on and off events in up to four different colors by stochastic photo-switching; and detecting said on and off events in a color channel for each color in real-time as the on and off events are occurring for said plurality of clonally amplified sample nucleic acid molecules to determine an identify of a nucleotide of said clonally amplified sample nucleic acid molecule. The stochastic photo-switching may comprise use of dark states in an emissive fluorophore to randomly switch the fluorophores on and off. This may enable imaging individual fluorophores, which can then be localized to provide a super resolution image. In some cases, the stochastic photo-switching can comprise use of stimulated emission depletion (STED), stochastic optical reconstruction (STORM), or the like.

[0183] In some cases, the super resolution imaging may comprise imaging at a resolution of at most about 1,000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, 50, or less nanometers. In some cases, the resolution of the super resolution imaging may be controlled by the numerical aperture of the system doing the imaging. In some cases, the resolution of the optical system may be sub-pixel resolution. Sub-pixel resolution may be imaged at a resolution higher than the resolution achievable given the size of the pixels used in imaging (e.g., by computer processing the image, etc.).

Light sources

[0184] In some embodiments, the light source 4901 as shown in FIGS. 49A-53B is a solid-state light source. In some embodiments, the solid-state light source is a light emitting diode (LED). In some embodiments, the light source 4901 is configured to emit a plurality of wavelengths. In some embodiments, the light source comprises a plurality of light sources. In some embodiments, each light source of the plurality is configured to emit a different wavelength of

light. In some embodiments, the light source 4901 is configured to emit: a first wavelength of light at a first time; a second wavelength of light at a second time and a third wavelength of light at a third time. In some embodiments, the first wavelength of light at a first time, the second wavelength of light at the second time and the third wavelength of light at the third time are emitted in a sequence. In some embodiments, the plurality of light sources is configured to be delivered for timed pulse sequences in sequential colors. In some embodiments, the plurality of optical subsystems 5001 are configured to increase speed of detection. In some embodiments, the solid-state light source is not a laser. For some applications, the light source comprises a filter to narrow the spectrum of the light emitted by the light source. In some embodiments, the light source is referred to as the excitation source. In some embodiments, the light emitted by the light source is referred to as excitation light.

Light delivery components

[0185] In some embodiments, the optical system comprises a light delivery component. In some embodiments, the light delivery component is a waveguide. In some embodiments, the light delivery component is a light pipe 4702 as shown in FIG. 47. In some embodiments, the light delivery component is a fiber optic. In some embodiments, the light source delivers light to the flow cell by the light delivery component. In some embodiments, the light source delivers light to the flow cell by a light pipe. In some embodiments, the light delivery component is positioned between the light source 4901 and the flow cell 4905. In some embodiments, a second light delivery component is positioned between the flow cell and the image sensor.

Imaging channels

[0186] The optical system as described herein may be configured for imaging one or more fluorophores. In some embodiments, the optical system is configured to distinctly image two, three, or more different fluorophores. In certain aspects, the optical system comprises one or more imaging channels. In some embodiments, a first imaging channel of the one or more imaging channels is configured to image a first fluorophore of the one or more fluorophores. In some embodiments, a second imaging channel of the one or more imaging channels is configured to image a second fluorophore of the one or more fluorophores. In some embodiments, a third imaging channel of the one or more imaging channels is configured to image a third fluorophore of the one or more fluorophores. In some embodiments, an imaging channel comprises at least one of a light source 4901, a filter 4910, an imaging sensor 4912, or a combination thereof.

Heater

[0187] Typically, assays require heating. In some instances, the flow cell 4905 further comprises a heater. In some embodiments, the heater is integrated with the flow cell. In some embodiments, the heater is integrated with a multiple surface imaging flow cell 4905. In some embodiments, the heater is integrated with the capillary flow cell 5201. In some embodiments, the integrated heater is a transparent heater block integrated heater. In some embodiments, the heater is an IR heater. In some embodiments, the transparent heater conforms to the surface of the flow cell. In some embodiments, a transparent heater conforms to and fully surrounds a flow cell with a non-rectangular cross section. In some embodiments, a transparent heater conforms to and fully surrounds a flow cell with a round cross section. In some embodiments, a transparent heater conforms to and fully surrounds a capillary flow cell 5201. In some embodiments, the transparent heater is transparent in all image channels of the one or more image channels of the optical system.

Aberration correction

[0188] In some embodiments, aberration correction methods may be applied to allow for imaging through air bubbles that may appear within the flow cell. In some embodiments, non-flat flow cell surfaces enable right angle or off-axis illumination. In some embodiments, the optical system described herein may comprise magnetic positioning of various elements. In some embodiments, the optical system may be configured to image flow cells with round edges.

Integrated field flatteners

[0189] Typically, the area of illumination and/or FOV of a standard fluorescence microscope imaging system is limited to the size of the single lens system and/or single imaging sensor present. Typically, the ability of a system to systematically capture brightness across the FOV may be referred to as field uniformity of the system. Non-uniformity of brightness and resolution across the FOV is, in some cases, observed from the center to the edge of the FOV. In some instances, illumination non-uniformity is caused by non-uniform field curvature effects of a lens the system, usually these are single lens systems. Systems, devices and methods designed to improve field uniformity are sometimes referred to as field flatteners or field flattening, respectively. The optical system described herein can comprise a field flattener. In some instances, the field flattener comprises a plurality of optical subsystems 5001 designed to provide overlapping coverage of the ‘active area of the flow cell surface. Where one image of an individual optical subsystem system 4914 of the plurality 5001 begins to become non-uniform (e.g., increased blurring, loss of intensity at corners and edges) the optimal imaging volume 4915 of a second optical subsystem 4914 may overlap. In some instances, the optimal imaging volume 4915 of a first optical subsystem overlaps with a second optical subsystem and a third optical subsystem.

[0190] In some embodiments, the surface 5101 of the flow cell comprising sample sites 4902 is not flat as shown in FIG. 52. In certain aspects, each optical subsystem 4914 of the plurality 5001 is positioned to match the contour of the active area of the flow cell as shown in FIG. 52

Optical systems with super-resolution

[0191] For imaging very small sample site features present in high surface densities, such as nucleic acid polonies (e.g., spots comprising amplified target nucleic acids) super resolution imaging techniques as described herein may be used. In some embodiments, stochastic photo-switching techniques as described herein may be used to improve image resolution. Alternatively, structured illumination techniques as described herein may be used to improve image resolution in the optical system. In some cases, the super resolution imaging technique can comprise structured illumination.

[0192] In some instances, improvements in imaging performance, *e.g.*, for multiple-side (flow cell) imaging applications comprising the use of thick flow cell walls (*e.g.*, wall (or coverslip) thickness > 700 μm) and fluid channels (*e.g.*, fluid channel height or thickness of 50 – 200 μm) may be achieved using novel objective lens designs that correct for optical aberration introduced by imaging surfaces on the opposite side of thick coverslips and/or fluid channels from the objective.

[0193] In some instances, improvements in imaging performance, *e.g.*, for multiple-side (flow cell) imaging applications comprising the use of thick flow cell walls (*e.g.*, wall (or coverslip) thickness > 700 μm) and fluid channels (*e.g.*, fluid channel height or thickness of 50 – 200 μm) may be achieved even when using commercially-available, off-the-shelf objectives by using a novel tube lens design that, unlike the tube lens in a conventional microscope that simply forms an image at the intermediate image plane, corrects for the optical aberrations induced by the thick flow cell walls and/or intervening fluid layer in combination with the objective.

[0194] In some instances, improvements in imaging performance, *e.g.*, for multichannel (*e.g.*, two-color or four-color) imaging applications, may be achieved by using multiple tube lenses, one for each imaging channel, where each tube lens design has been optimized for the specific wavelength range used in that imaging channel.

[0195] In some instances, improvements in imaging performance, *e.g.*, for multiple-side (flow cell) imaging applications, may be achieved by using an electro-optical phase plate in combination with an objective lens to compensate for the optical aberrations induced by the layer of fluid separating the upper (near) and lower (far) interior surfaces of a flow cell. In some instances, this design approach may also compensate for vibrations introduced by, *e.g.*, a

motion-actuated compensator that is moved in or out of the optical path depending on which surface of the flow cell is being imaged.

[0196] Various multichannel fluorescence imaging module designs are disclosed that may include illumination and imaging optical paths comprising folded optical paths (*e.g.*, comprising one or more beam splitters or beam combiners, such as dichroic beam splitters or combiners) that direct an excitation light beam to an objective lens, and direct emission light transmitted through the objective lens to a plurality of detection channels. Some particularly advantageous features of the fluorescence imaging modules described herein include specification of dichroic filter incidence angles that result in sharper and/or more uniform transitions between passband and stopband wavelength regions of the dichroic filters. Such filters may be included within the folded optics and may comprise dichroic beam splitters or combiners. Further advantageous features of the disclosed imaging optics designs may include the position and orientation of one or more excitation light sources and one or more detection optical paths with respect to the objective lens and to a dichroic filter that receives the excitation beam. The excitation beam may also be linearly-polarized and the orientation of the linear polarization may be such that s-polarized light is incident on the dichroic reflective surface of the dichroic filter. Such features may potentially improve excitation beam filtering and/or reduce wavefront error introduced into the emission light beam due to surface deformation of dichroic filters. The fluorescence imaging modules described herein may or may not include any of these features and may or may not include any of these advantages.

[0197] Also described herein are devices and systems configured to analyze large numbers of different nucleic acid sequences by imaging, *e.g.*, arrays of immobilized nucleic acid molecules or amplified nucleic acid clusters formed on flow cell surfaces. The devices and systems described herein can also be useful in, *e.g.*, performing sequencing for comparative genomics, tracking gene expression, performing micro RNA sequence analysis, epigenomics, aptamer and phage display library characterization, and for performing other sequencing applications. The devices and systems disclosed herein comprise various combinations of optical, mechanical, fluidic, thermal, electrical, and computing devices/aspects. The advantages conferred by the disclosed flow cell devices, cartridges, and systems include, but are not limited to: (i) reduced device and system manufacturing complexity and cost, (ii) significantly lower consumable costs (*e.g.*, as compared to those for currently available nucleic acid sequencing systems), (iii) compatibility with typical flow cell surface functionalization methods, (iv) flexible flow control when combined with microfluidic components, *e.g.*, syringe pumps and diaphragm valves, *etc.*, and (v) flexible system throughput.

[0198] Disclosed herein are capillary flow-cell devices and capillary flow cell cartridges that are constructed from off-the-shelf, disposable, single lumen (*e.g.*, single fluid flow channel) or multi-lumen capillaries that may also comprise fluidic adaptors, cartridge chassis, one or more integrated fluid flow control components, or any combination thereof. Also disclosed herein are capillary flow cell-based systems that may comprise one or more capillary flow cell devices (or microfluidic chips), one or more capillary flow cell cartridges (or microfluidic cartridges), fluid flow controller modules, temperature control modules, imaging modules, or any combination thereof.

[0199] The design features of some disclosed capillary flow cell devices, cartridges, and systems include, but are not limited to, (i) unitary flow channel construction, (ii) sealed, reliable, and repetitive switching between reagent flows that can be implemented with a simple load/unload mechanism such that fluidic interfaces between the system and capillaries are reliably sealed, thereby facilitating capillary replacement and system reuse, and enabling precise control of reaction conditions such as reagent concentration, pH, and temperature, (iii) replaceable single fluid flow channel devices or capillary flow cell cartridges comprising multiple flow channels that can be used interchangeably to provide flexible system throughput, and (iv) compatibility with a wide variety of detection methods such as fluorescence imaging.

[0200] Although the disclosed capillary flow cell devices and systems, capillary flow cell cartridges, capillary flow cell-based systems, microfluidic devices and cartridges, and microfluidic chip-based systems, are described primarily in the context of their use for nucleic acid sequencing applications, various aspects of the disclosed devices and systems may be applied not only to nucleic acid sequencing but also to any other type of chemical analysis, biochemical analysis, nucleic acid analysis, cell analysis, or tissue analysis application. It shall be understood that different aspects of the disclosed methods, devices, and systems can be appreciated individually, collectively, or in combination with each other. Although discussed herein primarily in the context of fluorescence imaging (including, *e.g.*, fluorescence microscopy imaging, fluorescence confocal imaging, two-photon fluorescence, and the like), it will be understood by those of skill in the art that many of the disclosed optical design approaches and features are applicable to other imaging modes, *e.g.*, bright-field imaging, dark-field imaging, phase contrast imaging, and the like.

Fluorescence imaging viewed as an information pipeline

[0201] A useful abstraction of the role that fluorescence imaging systems plays in typical genomic assay techniques (including nucleic acid sequencing applications) is as an information pipeline, where the photon signal enters at one end of the pipeline, *e.g.*, the objective lens used

for imaging, and location specific information regarding the fluorescence signal emerges at the other end of the pipeline, *e.g.*, at the position of the image sensor. When more information is pumped through this pipeline, some content, inevitably, will be lost during this transfer process and never recovered. An example of this case is when too many labeled molecules (or clonally-amplified clusters of molecules) are present within a small region of a substrate surface to be clearly resolved in the image; at the position of the image sensor, it becomes difficult to differentiate photon signals arising from adjacent clusters of molecules, thus increasing the probability of attributing the signal to the wrong cluster and leading to detection errors. In some cases, the clusters are polonies.

Design of optical imaging modules

[0202] The goal of designing an optical imaging module is thus to maximize the flow of information content through this detection pipeline and to minimize detection errors. Several key design elements need to be addressed in the design process, including:

[0203] 1) Matching the physical feature density on the substrate surface to be imaged with the overall image quality of the optical imaging system and the pixel sampling frequency of the image sensor used. A mismatch of these parameters may result in loss of information or sometimes even the generation of false information, *e.g.*, spatial aliasing may arise when pixel sampling frequency is lower than twice the optical resolution limit.

[0204] 2) Matching the size of the area to be imaged with the overall image quality of the optical imaging system and focus quality across the entire field-of-view.

[0205] 3) Matching the optical collection efficiency, modulation transfer function, and image sensor performance characteristics of the optical system design with the fluorescence photon flux expected for the input excitation photon flux, dye efficiency (related to dye extinction coefficient and fluorescence quantum yield), while accounting for background signal and system noise characteristics.

[0206] 4) Maximizing the separation of spectral content to reduce cross talk between fluorescence imaging channels.

[0207] 5) Effective synchronization of image acquisition steps with repositioning of the sample or optics between image capture of different fields-of-view to minimize the down time (or maximize the duty cycle) of the imaging system and thus maximize the overall throughput of the image capture process.

[0208] This disclosure describes a systematic way to address each of the design elements outlined above and to create component level specifications for the imaging system.

Improved optical resolution and image quality to improve or maximize information transfer and throughput

[0209] One non-limiting design practice may be to start with the optical resolution required to distinguish two adjacent features as specified in terms of a number, *X*, of line pairs per mm (lp/mm) and translate it to a corresponding numerical aperture (NA) requirement. The numerical aperture requirement can then be used to assess the resulting impact on modulation transfer function and image contrast.

[0210] The standard modulation transfer function (MTF) describes the spatial frequency response for image contrast (modulation) transferred through an optical system; image contrast decreases as a function of spatial frequency and increases with increasing NA. This function limits the contrast/modulation that can be achieved for a given NA. Furthermore, wave front error can negatively impact the MTF, thus making it desirable to improve or optimize the optical system design using the true system MTF instead of that predicted by diffraction-limited optics. Note that, as used herein, MTF will refer to the total system MTF (including the complete optical path from coverslip to image sensor) although design practice may primarily consider the MTF of the objective lens.

[0211] In genomic testing applications, where the target to be imaged is an array of high density "spots" on a surface (either randomly distributed or patterned), one can determine the minimum modulation transfer value required by downstream analysis to resolve two adjacent spots and discriminate between four possible states (*e.g.*, ON-OFF, ON-ON, OFF-ON and OFF-OFF). For example, assume that the spots are small enough to be approximated as point sources of light. Assuming that the detection task is to determine if the two adjacent spots separated by a distance, d, are ON or OFF (in other words, bright or dark), and that the contrast-to-noise ratio (CNR) for the fluorescence signals arising from the spots at the sample plane (or object plane) is C_{sample}, then under ideal conditions the CNR of the readout signal for the two adjacent spots at the image sensor plane, C_{image}, can be closely approximated as $C_{image} = C_{sample} * MTF(1/d)$, where MTF(1/d) is the MTF value at spatial frequency = (1/d).

[0212] In a typical design, the value of C may be at least 4 so that a simple threshold method can be used to avoid misclassification of fluorescence signals. Assuming a Gaussian distribution of fluorescence signal intensities around a mean value, at $C_{image} > 4$, the expected error in correctly classifying fluorescence signals (*e.g.*, as being ON or OFF) is <0.035%. The use of proprietary high CNR sequencing and surface chemistry, such as that described in U.S. Patent Application No. 16/363,842, allows one to achieve sample plane CNR (C_{sample}) values for clusters of clonally-amplified, labeled oligonucleotide molecules tethered to a substrate surface

of greater than 12 (or even much higher) when measured for a sparse field (*e.g.*, at a low surface density of clusters or spots) where the MTF has a value of close to 100%. Assuming a sample plane CNR value of $C_{sample} > 12$ and targeting a classification error rate of <0.1% (thus, $C_{image} > 4$), in some implementations the minimum value for M(1/d) can be determined as M(1/d) = 4/12 ~ 33%. Thus, a modulation transfer function threshold of at least 33% may be used to retain the information content of the transferred image.

[0213] Design practice can relate the minimum separation distance of two features or spots, *d*, to the optical resolution requirement (specified as noted above in terms of *X* (lp/mm)) as *d* = (1mm)/*X*, *e.g.*, *d* is the minimum separation distance between two features or spots which can be fully resolved by the optical system. In some designs disclosed herein, where the objective of the design analysis is to increase or maximize relevant information transfer, this design criterion can be relaxed to $d = (1mm)/X/A$, where $2 > A > 1$. For the same optical resolution of *X* lp/mm, the value of *d*, the minimum resolvable spot separation distance at the sample plane, is reduced, thereby enabling the use of higher feature densities.

[0214] Design practice determines the minimum spatial sampling frequency at the sample plane using the Nyquist criteria, where spatial sampling frequency $S \geq 2*X$ (and where *X* is the optical resolution of the imaging system specified in terms of *X* lp/mm as noted above). When the system spatial sampling frequency is close to the Nyquist criteria, as is often the case, imaging system resolution of greater than *S* results in aliasing as the higher frequency information resolved by the optical system cannot be sufficiently sampled by the image sensor.

[0215] In the some of the designs disclosed herein, an oversampling scheme based on the relationship $S = B*Y$ (where $B \geq 2$ and *Y* is the true optical system MTF limit) may be used to further improve the information transfer capacity of the imaging system. As indicated above, *X* (lp/mm) corresponds to a practical, non-zero (>33%) minimum modulation transfer value, whereas *Y* (lp/mm) is the limit of optical resolution so modulation at Y(lp/mm) is 0. Thus, in the disclosed designs, *Y* (lp/mm) may advantageously be significantly greater than *X*. For values of $B \geq 2$, the disclosed designs are oversampling for the sample object frequency *X*, *e.g.*, $S \geq B*Y > 2*X$.

[0216] The above relationship can be used to determine the system magnification and may provide an upper bound for image sensor pixel size. The choice of image sensor pixel size is matched to the system optical quality as well to the spatial sampling frequency required to reduce aliasing. The lower bound of image sensor pixel size can be determined based on photon throughput, as relative noise contributions increase with smaller pixels.

[0217] Other design approaches are, however, also possible. For example, reducing the NA to less than 0.6 (*e.g.*, 0.5 or less,) may provide increased depth of field. Such increased depth of

field may enable multiple surface imagining wherein two surfaces at different depths can be imaged at the same time with or without refocusing. As discussed above, reducing NA may reduce optical resolution. In some implementations, use of higher excitation beam power, *e.g.*, 1 Watt or higher, may be employed to produce strong signal. An inherently high contrast sample (*e.g.*, comprising a sample surface that exhibits strong foreground signal and dramatically reduced background signal, may also be used to facilitate acquisition of high contrast-to-noise ratio (CNR) images, *e.g.*, having CNR values of > 20, that provide for improved signal discrimination for base-calling in nucleic acid sequencing applications, *etc.* In some optical system designs disclosed herein, sample support structures such as flow cells having hydrophilic surfaces are used to reduce background noise.

[0218] In various implementations, a large field-of-view (FOV) is provided by the disclosed optical systems. For example, a FOV of greater than 2 or 3 mm may be provided with some optical imaging systems comprising, *e.g.*, an objective lens and a tube lens. In some cases, the optical imaging system provides a reduced magnification, for example, a magnification of less than 10x. Such reduced magnification may in some implementations facilitate large FOV designs. Despite a reduced magnification, the optical resolution of such systems can still be sufficient as detector arrays having small pixel size or pitch may be used. In some implementations, image sensors comprising a pixel size that is smaller than twice the optical resolution provided by the optical imaging system (*e.g.*, objective and tube lens) may be used to satisfy the Nyquist theorem.

[0219] Still other designs are also possible. In some optical designs configured to provide for multiple surface imaging where two surfaces at different depths can be imaged at the same time, the optical imaging system (*e.g.*, the objective lens and/or tube lens) is configured to reduce optical aberration for imaging said two surfaces (*e.g.*, two planes) at those two respective depths more than at other locations (*e.g.*, other planes) at other depths. Additionally, the optical imaging system may be configured to reduce aberration for imaging said two surfaces (*e.g.*, two planes) at those two respective depths through a transmissive layer on said sample support structure (such as a layer of glass (*e.g.*, a cover slip) and through a solution (*e.g.*, an aqueous solution) comprising the sample or in contact with a sample on at least one of said two surfaces.

Multichannel fluorescence imaging modules and systems

[0220] In some instances, the imaging modules or systems disclosed herein may comprise fluorescence imaging modules or systems. In some instances, the fluorescence imaging systems disclosed herein may comprise a single fluorescence excitation light source (for providing excitation light at a single wavelength or within a single excitation wavelength range) and an

optical path configured to deliver the excitation light to a sample (*e.g.*, fluorescently-tagged nucleic acid molecules or clusters thereof disposed on a substrate surface). In some instances, the fluorescence imaging systems disclosed herein may comprise a single fluorescence emission imaging and detection channel, *e.g.*, an optical path configured to collect fluorescence emitted by the sample and deliver an image of the sample (*e.g.*, an image of a substrate surface on which fluorescently-tagged nucleic acid molecules or clusters thereof are disposed) to an image sensor or other photodetection device. In some instances, the fluorescence imaging systems may comprise two, three, four, or more than four fluorescence excitation light sources and/or optical paths configured to deliver excitation light at two, three, four, or more than four excitation wavelengths (or within two, three, four, or more than four excitation wavelength ranges). In some instances, the fluorescence imaging systems disclosed herein may comprise two, three, four, or more than four fluorescence emission imaging and detection channels configured to collect fluorescence emitted by the sample at two, three, four, or more than four emission wavelengths (or within two, three, four, or more than four emission wavelength ranges and deliver an image of the sample (*e.g.*, an image of a substrate surface on which fluorescently-tagged nucleic acid molecules or clusters thereof are disposed) to two, three, four, or more than four image sensors or other photodetection devices.

Multiple surface imaging

[0221] In some instances, the imaging systems disclosed herein, including fluorescence imaging systems, may be configured to acquire high-resolution images of a single sample support structure or substrate surface. In some instances, the imaging systems disclosed herein, including fluorescence imaging systems, may be configured to acquire high-resolution images of two or more sample support structures or substrate surfaces, *e.g.*, two, three, or more surfaces of a flow cell. The multiple surfaces of a sample support structure or a flow cell device can be axially displaced from each other, along the axial or z direction. The multiple surfaces of a sample support structure or a flow cell device can be interior surfaces facing fluidic channel(s) disclosed herein. The fluidic channels or capillaries of the sample support structure or flow cell can be axially displaced from each other, along the axial or z direction.

[0222] In some instances, the high-resolution images provided by the disclosed imaging systems may be used to monitor reactions occurring on the two or more surfaces of the flow cell (*e.g.*, nucleic acid hybridization, amplification, and/or sequencing reactions) as various reagents flow through the flow cell or around a flow cell substrate. FIG. 1A and FIG. 1B provide schematic illustrations of a multiple surface support structures. FIGS. 64A-64F provide schematic illustrations of a quad surface support structure as a flow cell.

[0223] FIG. 1A shows a multiple surface support structure such as a flow cell that includes an internal flow channel through which an analyte or reagent can be flowed. The flow channel may be formed between first and second, top and bottom, and/or front and back layers such as first and second, top and bottom, and/or front and back plates as shown. One or more of the plates may include a glass plate, such as a coverslip, or the like. In some implementations, the layer comprises borosilicate glass, quartz, or plastic. Interior surfaces of these top and bottom layers provide walls of the flow channel that assist in confining the flow of analyte or reagent through the flow channel of the flow cell. In some designs, these interior surfaces are planar. Similarly, the top and bottom layers may be planar. In some designs, at least one additional layer (not shown) is disposed between the top and bottom layers. This additional layer may have one or more pathways cut therein that assist in defining one or more flow channels and controlling the flow of the analyte or reagent within the flow channel. Additional discussion of sample support structures, *e.g.*, flow cells, can be found below.

[0224] FIG. 1A schematically illustrates a plurality of fluorescing sample sites on the first and second, top and bottom, and/or front and back interior surfaces of the flow cell. In some implementations, reactions may occur at these sites to bind sample such that fluorescence is emitted from these sites (note that FIG. 1A is schematic and not drawn to scale; for example, the size and spacing of the fluorescing sample sites may be smaller than shown).

[0225] FIG. 1B shows another multiple surface support structure having two surfaces containing fluorescing sample sites to be imaged. The sample support structure comprises a substrate having first and second, top and bottom, and/or front and back exterior surfaces. In some designs, these exterior surfaces are planar. In various implementations, the analyte or reagent is flowed across these first and second exterior surfaces. FIG. 1B schematically illustrates a plurality of fluorescing sample sites on the first and second, top and bottom, and/or front and back exterior surfaces of the sample support structure. In some implementations, reactions may occur at these sites to bind sample such that fluorescence is emitted from these sites (note that FIG. 1B is schematic and not drawn to scale; for example, the size and spacing of the fluorescing sample sites may be smaller than shown). Support structures with one or more surfaces, e.g., in FIGS. 64A-64E, can have similar sample site distributions as shown in FIGS. 1A or 1B on each of the surfaces.

[0226] In some instances, the fluorescence imaging modules and systems described herein may be configured to image such fluorescing sample sites on each of the multiple surfaces at different distances from the objective lens. In some designs, only one of the multiple surfaces is in focus at a time. Accordingly, in such designs, one of the surfaces is imaged at a first time, and the other surface is imaged at a second time. The focus of the fluorescence imaging module

may be changed after imaging one of the surfaces in order to image a next surface with comparable optical resolution, as the images of the multiple surfaces are not simultaneously in focus. In some designs, an optical compensation element may be introduced into the optical path between the sample support structure and the image sensor in order to image one of the surfaces. The depth of field in such fluorescence imaging configurations may not be sufficiently large to include two or more surfaces of the multiple surfaces. In some implementations of the fluorescence imaging modules described herein, two or more surfaces may be imaged at the same time, *e.g.*, simultaneously. For example, the fluorescence imaging module may have a depth of field that is sufficiently large to include two or more surfaces. In some instances, this increased depth of field may be provided by, for example, reducing the numerical aperture of the objective lens (or microscope objective) as will be discussed in more detail below.

[0227] As shown in FIGS. 1A and 1B, the imaging optics (*e.g.*, an objective lens) may be positioned at a suitable distance (*e.g.*, a distance corresponding to the working distance) from the surfaces to form in-focus images of the surfaces on an image sensor of a detection channel. The first surface, e.g., 6418 in FIG. 64C may be between the objective lens and the second surface, e.g., 6419. For example, as illustrated in FIGS. 1A and 1B, the objective lens is disposed above the multiple surfaces. the first surface is disposed above the second surface. As illustrated in FIG. 64C, the second surface 6419 is above the third surface 6420, and the third surface is above the fourth surface 6421. The multiple surfaces can be at different depths. The surfaces are at different distances from any one or more of the fluorescence imaging module, the illumination and imaging module, imaging optics, or the objective lens. The multiple surfaces are separated from each other along the z direction. The surfaces can be planar surfaces and are separated from each other along a direction normal to the planar surfaces. In some embodiments, the objective lens has an optical axis and the surfaces are separated from each other along the direction of the optical axis. Similarly, the separation between the surfaces may correspond to the axial distance such as along the optical path of the excitation beam and/or along an optical axis through the fluorescence imaging module and/or the objective lens. Accordingly, these surfaces may be separated by a distance from each other in the axial (Z) direction, which may be along the direction of the central axis of the excitation beam and/or the optical axis of the objective lens and/or the fluorescence imaging module. This separation may correspond, for example, to a flow channel within a flow cell in some implementations between the first and second surfaces or between the third and fourth surfaces. This separation may correspond, for example, to an interposer substrate, within a flow cell in some implementations between the second and third surface.

[0228] In various designs, the objective lens (possibly in combination with another optical component, *e.g.*, a tube lens) have a depth of field and/or depth of focus that is at least as large as the axial separation (in the Z direction) between two adjacent surfaces of the multiple surfaces. In some embodiments, the depth of filed and/or depth of focus that is at least as large as the axial separation (in the z direction) between the first and the last (e.g., the fourth) surfaces along the optical path from the objective lens. The objective lens, alone or in combination with the additional optical component, may thus simultaneously form in-focus images of at least two adjacent surfaces on an image sensor of one or more detection channels where these images have comparable optical resolution. In some implementations, the imaging module may or may not need to be re-focused to capture images of at least two adjacent surfaces with comparable optical resolution. In some implementations, compensation optics need not be moved into or out of an optical path of the imaging module to form in-focus images of the surfaces. Similarly, in some implementations, one or more optical elements (*e.g.*, lens elements) in the imaging module (*e.g.*, the objective lens and/or a tube lens) need not be moved, for example, in the axial direction along the first and/or second optical paths (*e.g.*, along the optical axis of the imaging optics) to form in-focus images of one of the surfaces, e.g., the first surface, in comparison to the location of said one or more optical element when used to form in-focus images of another one of the surfaces, e.g., the second, third, or fourth surface. In some implementations, however, the imaging module includes an autofocus system configured to provide at least two adjacent surfaces in focus at the same time. In various implementations, the sample is in focus to sufficiently resolve the sample sites, which are closely spaced together in lateral directions (*e.g.*, the X and Y directions). Accordingly, in various implementations, no optical element enters an optical path between the sample support structure (*e.g.*, between a translation stage that supports the sample support structure) and an image sensor (or photodetector array) in the at least one detection channel in order to form in-focus images of fluorescing sample sites on one surface of the sample support structure and on two other surfaces of the sample support structure. In various implementations, no optical compensation is used to form an in-focus image of fluorescing sample sites on one surface, e.g., the first surface, of the sample support structure on the image sensor or photodetector array that is not identical to optical compensation used to form an in-focus image of fluorescing sample sites on another surface, e.g., the second, third, or fourth surface, of the sample support structure on the image sensor or photodetector array. Additionally, in certain implementations, no optical element in an optical path between the sample support structure (*e.g.*, between a translation stage that supports the sample support structure) and an image sensor in the at least one detection channel is adjusted differently to form an in-focus image of fluorescing sample sites on one surface, e.g., the first surface of the

sample support structure than to form an in-focus image of fluorescing sample sites on another surface, e.g., the second, third, or fourth surface of the sample support structure. Similarly, in some various implementations, no optical element in an optical path between the sample support structure (*e.g.*, between a translation stage that supports the sample support structure) and an image sensor in the at least one detection channel is moved a different amount or a different direction to form an in-focus image of fluorescing sample sites on one surface, e.g., the first surface, of the sample support structure on the image sensor than to form an in-focus image of fluorescing sample sites on another surface, e.g., the second, third, fourth surface of the sample support structure on the image sensor. Any combination of the features herein can be possible. For example, in some implementations, in-focus images of the first interior surface and the second interior surface of the flow cell can be obtained without moving an optical compensator into or out of an optical path between the flow cell and the at least one image sensor and without moving one or more optical elements of the imaging system (*e.g.*, the objective and/or tube lens) along the optical path (*e.g.*, optical axis) therebetween. For example, in-focus images of the first interior surface and the second, third interior surfaces of the flow cell can be obtained without moving one or more optical elements of the tube lens into or out of the optical path, or without moving one or more optical elements of the tube lens along the optical path (*e.g.*, optical axis) therebetween.

[0229] Any one or more of the fluorescence imaging module, the illumination optical path, the imaging optical path, the objective lens, or the tube lens may be designed to reduce or minimize optical aberration at multiple locations such as the planes corresponding to the multiple surfaces on a flow cell or other sample support structure, for example, where fluorescing sample sites are located. Any one or more of the fluorescence imaging module, the illumination optical path, the imaging optical path, the objective lens, or the tube lens may be designed to reduce or minimize optical aberration at the selected locations or planes relative to other locations or planes, such as the surfaces containing fluorescing sample sites on a flow cell. For example, any one or more of the fluorescence imaging module, the illumination optical path, the imaging optical path, the objective lens, or the tube lens may be designed to reduce or minimize optical aberration at two depths or planes located at different distances from the objective lens as compared to the aberrations associated with other depths or planes at other distances from the objective lens. For example, optical aberration may be less for imaging the surfaces than elsewhere in a region ranging from about 1 to about 10 mm from the objective lens. Additionally, any one or more of the fluorescence imaging module, the illumination optical path, the imaging optical path, the objective lens, or the tube lens may, in some instances, be configured to compensate for optical aberration induced by transmission of emission light through one or more portions of the sample

support structure such as a layer that includes one of the surfaces on which sample adheres as well as possibly a solution that is in contact with the sample. This layer (*e.g.*, a coverslip or the wall of a flow cell) may comprise, *e.g.*, glass, quartz, plastic, or other transparent material having a refractive index and that introduces optical aberration.

[0230] Accordingly, the imaging performance may be substantially the same when imaging the multiple surfaces, e.g., three or four surfaces. For example, the optical transfer functions (OTF) and/or modulation transfer functions (MTF) may be substantially the same for imaging of the multiple surfaces. Either or both of these transfer functions may, for example, be within 20%, within 15%, within 10%, within 5%, within 2.5%, or within 1% of each other, or within any range formed by any of these values at one or more specified spatial frequencies or when averaged over a range of spatial frequencies. Accordingly, an imaging performance metric may be substantially the same for imaging each surface of the multiple surfaces of the flow cell without moving an optical compensator into or out of an optical path between the flow cell and the at least one image sensor, and without moving one or more optical elements of the imaging system (*e.g.*, the objective and/or tube lens) along the optical path (*e.g.*, optical axis) therebetween. For example, an imaging performance metric may be substantially the same for imaging the first, second, third, and fourth surfaces of the flow cell without moving one or more optical elements of the tube lens into or out of the optical path or without moving one or more optical elements of the tube lens along the optical path therebetween. In some embodiments, the optical path is an optical axis. Additional discussion of MTF is included below and in U.S. Provisional Application No. 62/962,723 filed January 17, 2020, which is incorporated herein by reference in its entirety.

[0231] It will be understood by those of skill in the art that the disclosed imaging modules or systems may, in some instances, be stand-alone optical systems designed for imaging a sample or substrate surface. In some instances, they may comprise one or more processors or computers. In some instances, they may comprise one or more software packages that provide instrument control functionality and/or image processing functionality. In some instances, in addition to optical components such as light sources (*e.g.*, solid-state lasers, dye lasers, diode lasers, arc lamps, tungsten-halogen lamps, *etc.*), lenses, prisms, mirrors, dichroic reflectors, beam splitters, optical filters, optical bandpass filters, light guides, optical fibers, apertures, and image sensors (*e.g.*, complementary metal oxide semiconductor (CMOS) image sensors and cameras, charge-coupled device (CCD) image sensors and cameras, *etc.*), they may also include mechanical and/or optomechanical components, such as X-Y translation stages, X-Y-Z translation stages, piezoelectic focusing mechanisms, electro-optical phase plates, and the like. In some instances, they may function as modules, components, sub-assemblies, or sub-systems

of larger systems designed for, *e.g.*, genomics applications (*e.g.*, genetic testing and/or nucleic acid sequencing applications). For example, in some instances, they may function as modules, components, sub-assemblies, or sub-systems of larger systems that further comprise light-tight and/or other environmental control housings, temperature control modules, flow cells and cartridges, fluidics control modules, fluid dispensing robotics, cartridge- and/or microplate-handling (pick-and-place) robotics, one or more processors or computers, one or more local and/or cloud-based software packages (*e.g.*, instrument / system control software packages, image processing software packages, data analysis software packages), data storage modules, data communication modules (*e.g.*, Bluetooth, WiFi, intranet, or internet communication hardware and associated software), display modules, *etc.*, or any combination thereof. These additional components of larger systems, *e.g.*, systems designed for genomics applications, will be discussed in more detail below.

[0232] FIGS. 2A and 2B illustrate a non-limiting example of an illumination and imaging module 100 for multi-channel fluorescence imaging. The illumination and imaging module 100 includes an objective lens 110, an illumination source 115, a plurality of detection channels 120, and a first dichroic filter 130, which may comprise a dichroic reflector or beam splitter. An autofocus system, which may include an autofocus laser 102, for example, which projects a spot the size of which is monitored to determine when the imaging system is in-focus may be included in some designs. Some or all components of the illumination and imaging module 100 may be coupled to a baseplate 105.

[0233] The illumination or light source 115 may include any suitable light source configured to produce light of at least a desired excitation wavelength (discussed in more detail below). The light source may be a broadband source that emits light within one or more excitation wavelength ranges (or bands). The light source may be a narrowband source that emits light within one or more narrower wavelength ranges. In some instances, the light source may produce a single isolated wavelength (or line) corresponding to the desired excitation wavelength, or multiple isolated wavelengths (or lines). In some instances, the lines may have some very narrow bandwidth. Example light sources that may be suitable for use in the illumination source 115 include, but are not limited to, an incandescent filament, xenon arc lamp, mercury-vapor lamp, a light-emitting diode, a laser source such as a laser diode or a solid-state laser, or other types of light sources. As discussed below, in some designs, the light source may comprise a polarized light source such as a linearly polarized light source. In some implementations, the orientation of the light source is such that s-polarized light is incident on one or more surfaces of one or more optical components such as the dichroic reflective surface of one or more dichroic filters.

[0234] The illumination source 115 may further include one or more additional optical components such as lenses, filters, optical fibers, or any other suitable transmissive or reflective optics as appropriate to output an excitation light beam having suitable characteristics toward a first dichroic filter 130. For example, beam shaping optics may be included, for example, to receive light from a light emitter in the light source and produce a beam and/or provide a desired beam characteristic. Such optics may, for example, comprise a collimating lens configured to reduce the divergence of light and/or increase collimation and/or to collimate the light.

[0235] In some implementations, multiple light sources are included in the illumination and imaging module 100. In some such implementations, different light sources may produce light having different spectral characteristics, for example, to excite different fluorescence dyes. In some implementations, light produced by the different light sources may be directed to coincide and form an aggregate excitation light beam. This composite excitation light beam may be composed of excitation light beams from each of the light sources. The composite excitation light beam will have more optical power than the individual beams that overlap to form the composite beam. For example, in some implementations that include two light sources that produce two excitation light beams, the composite excitation light beam formed from the two individual excitation light beams may have optical power that is the sum of the optical power of the individual beams. Similarly, in some implementations, three, four, five or more light sources may be included, and these light sources may each output excitation light beams that together form a composite beam that has an optical power that is the sum of the optical power of the individual beams.

[0236] In some implementations, the light source 115 outputs a sufficiently large amount of light to produce sufficiently strong fluorescence emission. Stronger fluorescence emission can increase the signal-to-noise ratio (SNR) and the contrast-to-noise ratio (CNR) of images acquired by the fluorescence imaging module. In some implementations, the output of the light source and/or an excitation light beam derived therefrom (including a composite excitation light beam) may range in power from about 0.5 watts (W) to about 5.0 W, or more (as will be discussed in more detail below).

[0237] Referring again to FIGS. 2A and 2B, the first dichroic filter 130 is disposed with respect to the light source to receive light therefrom. The first dichroic filter may comprise a dichroic mirror, dichroic reflector, dichroic beam splitter, or dichroic beam combiner configured to transmit light in a first spectral region (or wavelength range) and reflect light having a second spectral region (or wavelength range). The first spectral region may include one or more spectral bands, *e.g.*, one or more spectral bands in the ultraviolet and blue wavelength ranges. Similarly, a second spectral region may include one or more spectral bands, *e.g.*, one or more

spectral bands extending from the green to red and infrared wavelengths. Other spectral regions or wavelength ranges are also possible.

[0238] In some implementations, the first dichroic filter may be configured to transmit light from the light source to a sample support structure such as to a microscope slide, a capillary, a flow cell, a microfluidic chip, or other substrate or support structure. The sample support structure supports and positions the sample, *e.g.*, a composition comprising a fluorescently-labeled nucleic acid molecule or complement thereof, with respect to the illumination and imaging module 100. Accordingly, a first optical path extends from the light source to the sample via the first dichroic filter. In various implementations, the sample support structure includes at least one surface on which the sample is disposed or to which the sample binds. In some instances, the sample may be disposed within or bound to different localized regions or sites on the at least one surface of the sample support structure.

[0239] In some instances, the support structure may include two, three, four, or even more surfaces located at different distances from objective lens 110 (*e.g.*, at different positions or depths along the optical axis of objective lens 110 or an axial direction) on which the sample is disposed. As discussed below, for example, the flow cell may comprise a fluid channel formed at least in part by first and second (*e.g.*, upper and lower) interior surfaces, and the sample may be disposed at localized sites on the first interior surface, the second interior surface, or both interior surfaces. The first and second surface may be separated by the region corresponding to the fluid channel through which a solution flows, and thus be at different distances or depth with respect to objective lens 110 of the illumination and imaging module 100. The flow cell may comprise a second fluid channel, axially displaced from a first channel, that is formed at least in part by the third and fourth (*e.g.*, upper and lower) interior surfaces, and the sample may be disposed at localized sites on the third interior surface, the fourth interior surface, or both. The third and fourth surface may be separated by the region corresponding to the second fluid channel through which a solution flows, and thus be at different distances or depth with respect to objective lens 110 of the illumination and imaging module 100. The first and the second fluidic channel may be separated axially by an interposer substrate disposed in between.

[0240] The objective lens 110 may be included in the first optical path between the first dichroic filter and the sample. This objective lens may be configured, for example, to have a focal length, working distance, and/or be positioned to focus light from the light source(s) onto the sample, *e.g.*, onto a surface of the microscope slide, capillary, flow cell, microfluidic chip, or other substrate or support structure. Similarly, the objective lens 110 may be configured to have suitable focal length, working distance, and/or be positioned to collect light reflected, scattered,

or emitted from the sample (*e.g.*, fluorescence emission) and to form an image of the sample (*e.g.*, a fluorescence image).

[0241] In some implementations, objective lens 110 may comprise a microscope objective such as an off-the-shelf objective. In some implementations, objective lens 110 may comprise a custom objective. An example of a custom objective lens and/or custom objective - tube lens combination is described below and in U.S. Provisional Application No. 62/962,723 filed on January 17, 2020, which is incorporated herein by reference in its entirety. The objective lens 110 may be designed to reduce or minimize optical aberration at two, three, four, or more locations. For example, such locations can include planes corresponding to the multiple surfaces of a flow cell. The objective lens 110 may be designed to reduce the optical aberration at the selected locations or planes, *e.g.*, the first and second surfaces of a multiple surface flow cell, or the first, second, third, and fourth surfaces of a quad surface flow cell, relative to other locations or planes in the optical path. For example, the objective lens 110 may be designed to reduce the optical aberration at two, three, or four depths or planes located at different distances from the objective lens as compared to the optical aberrations associated with other depths or planes at other distances from the objective. For example, in some instances, optical aberration may be less for imaging the surfaces of a flow cell than that exhibited elsewhere in a region spanning from 1 to 10 mm from the front surface of the objective lens. Additionally, a custom objective lens 110 may in some instances be configured to compensate for optical aberration induced by transmission of fluorescence emission light through one or more portions of the sample support structure, such as a layer that includes one or more of the flow cell surfaces on which a sample is disposed, or a layer comprising a solution filling the fluid channel of a flow cell. These layers may comprise, *e.g.*, glass, quartz, plastic, or other transparent material having a refractive index, and which may introduce optical aberration.

[0242] In some implementations, objective lens 110 may have a numerical aperture (NA) of 0.6 or more (as discussed in more detail below). Such a numerical aperture may provide for reduced depth of focus and/or depth of field, improved background discrimination, and increased imaging resolution.

[0243] In some implementations, objective lens 110 may have a numerical aperture (NA) of 0.6 or less (as discussed in more detail below). Such a numerical aperture may provide for increased depth of focus and/or depth of field. Such increased depth of focus and/or depth of field may increase the ability to image planes separated by a distance such as the first and second surfaces, the second and third surface, or the third and fourth surfaces. In some implementations, objective lens 110 may have a numerical aperture (NA) of 0.5 or less, e.g., 0.4. Such a numerical aperture may provide for lower optical aberration that needs to be

compensated by the optical system than higher NA values. Such a numerical aperture may provide for the capability of focusing and imaging additional image planes, e.g., the planes of the third or fourth surfaces of the flow cell, with minimal changes to the optical system design (e.g., with no requirement for adding or removing a compensator from the optical path from the objective lens to the flow cell being imaged) that is configured to image one or multiple surfaces of the flow cell.

[0244] As discussed above, a flow cell may comprise, for example, first and second layers comprising first and second interior surfaces respectively that are separated by a fluid channel through which an analyte or reagent can flow. The flow cell may also include third and fourth layers comprising third and fourth interior surfaces respectively that are separated by a second fluid channel through which an analyte or reagent can flow. In some implementations, the objective lens 110 and/or illumination and imaging module 100 may be configured to provide a depth of field and/or depth of focus sufficiently large to image at least two adjacent surfaces of the flow cell, either sequentially by re-focusing the imaging module between imaging the at least two surfaces, or simultaneously by ensuring a sufficiently large depth of field and/or depth of focus, with comparable optical resolution. In some instances, the depth of field and/or depth of focus may be at least as large or larger than the distance separating the two adjacent surfaces of the flow cell to be imaged. In some instances, the two adjacent surfaces, *e.g.*, the first and second interior surfaces of a dual surface flow cell or the third and fourth surfaces of a quad surface flow cell, may be separated, for example, by a distance ranging from about 10 μm to about 700 μm, or more (as will be discussed in more detail below). In some instances, the depth of field and/or depth of focus may thus range from about 10 μm to about 700 μm, or more (as will be discussed in more detail below).

[0245] In some designs, compensation optics (*e.g.*, an "optical compensator" or "compensator") may be moved into or out of an optical path in the imaging module, for example, an optical path by which light collected by the objective lens 110 is delivered to an image sensor, to enable the imaging module to image the surfaces of the flow cell. The imaging module may be configured, for example, to image one surface, e.g., the first surface, when a first compensation optics is included in the optical path between the objective lens and an image sensor or photodetector array configured to capture an image of the first surface. The imaging module may be configured, for example, to image another surface, e.g., the second surface when a second compensation optics is included in the optical path between the objective lens and an image sensor or photodetector array configured to capture an image of the second surface. In such a design, the imaging module may be configured to image yet another surface, e.g., the third surface, when the first and the second compensation optics is removed from or not included in

the optical path between the objective lens 110 and the image sensor or photodetector array configured to capture an image of the third surface. The need for an optical compensator may be more pronounced when using an objective lens 110 with a high numerical aperture (NA) value, *e.g.*, for numerical aperture values of at least 0.6, least 0.65, at least 0.7, at least 0.75, at least 0.8, at least 0.85, at least 0.9, at least 0.95, at least 1.0, or higher. In some implementations, the optical compensation optics (*e.g.*, an optical compensator or compensator) comprises a refractive optical element such as a lens, a plate of optically-transparent material such as glass, a plate of optically-transparent material such as glass, or in the case of polarized light beams, a quarter-wave plate or half-wave plate, *etc*. Other configurations may be employed to enable the surfaces to be imaged at different times. For example, one or more lenses or optical elements may be configured to be translated in and out of, or along, an optical path between the objective lens 110 and the image sensor.

[0246] In certain designs, the objective lens 110 is configured to be adjusted to change the NA of the optical system. In some embodiments, the objective lens 110 can be adjusted so that the NA of the optical system can be adjusted within the range of 0.25 to 0.6. In some embodiments, the objective lens 110 can be adjusted so that the NA of the optical system can be adjusted within the range of 0.35 to 0.55. In some embodiments, the objective lens 110 can be adjusted so that the NA of the optical system can be adjusted within the range of 0.4 to 0.5. In some embodiments, the NA of the optical system can be adjusted by changing the objective lens 110. In some embodiments, the NA of the optical system can be adjusted by only changing the objective lens 110 without moving any optical compensator in, out of, or along the optical path from the objective lens to the sample. In some embodiments, the NA of the optical system can be adjusted by changing an optical element within the objective lens 110, without removing an existing objective lens and adding a new objective lens.

[0247] In some embodiments, the objective lens 110 can include an aperture stop, and adjustment of the aperture stop's size can result in change of the NA of the optical system. In some embodiments, changing the aperture stop's size does not involve translating the objective lens or any other optical elements in, out of, or along an optical path between the objective lens and the image sensor. As a non-limiting example, changing the aperture stop's size comprises rotating an optical element or part of the objective lens about an axial axis or a longitudinal axis of the objective lens. As another non-limiting example, changing the aperture stop's size comprises moving an optical element or part of the objective lens orthogonal to an axial axis or a longitudinal axis of the objective lens.

[0248] In some embodiments, the NA of the optical system can be adjusted without changing the aperture's stop's size. Instead, the objective lens and/or the tube lens can be redesigned from

an optical system with a first NA, e.g., NA of 0.5, to make the NA of the optical system to a predetermined different value, e.g., 0.4. The redesign can involve changing one or more characteristics of the objective lens and/or the tube lens including but is not limited to: a diameter, a size, a magnification, a length, a cover thickness, a working distance, and a lens design that functions to correct aberration.

[0249] In some embodiments, the NA of the optical system can be adjusted, from the NA used in imaging traditional one or dual surface flow cells (e.g., NA of 0.5 to NA of 0.4), to image multiple surface flow cell with three or more axially-displaced surfaces with predetermined image quality. In some embodiments, the NA of the optical system can remain unaltered from the NA used in imaging traditional one or dual surface flow cells (e.g., NA of 0.4), to image multiple surface flow cell with three or more axially-displaced surface with predetermined image quality.

[0250] The optical systems herein can allow the adjustment of the objective lens to change the NA (e.g., between 0.5 and 0.4 or in the range from 0.4 to 0.5). The optical system also can allow usage of the same NA, e.g., NA= 0.4 to image flow cell devices with a total thickness that is within a matching range of the NA, e.g., a range from about 220 um to about 360 um. As such, the optical systems herein can provide flexibility and compatibility to image both (1) traditional flow cells with one or dual surfaces and (2) the multiple surface flow cells herein (e.g., with three, four, or even more axially displaced surfaces) with sufficient image quality, e.g., a CNR of at least 5, 10, 15, or 20.

[0251] In certain designs, however, the objective lens 110 is configured to provide sufficiently large depth of focus and/or depth of field to enable the surfaces to be imaged with comparable optical resolution without such compensation optics moving into and/or out of an optical path in the imaging module, such as an optical path between the objective lens and the image sensor or photodetector array. In various designs, the objective lens 110 is configured to provide sufficiently large depth of focus and/or depth of field to enable the surfaces to be imaged with comparable optical resolution without optics being moved, such as one or more lenses or other optical components being translated along an optical path in the imaging module, such as an optical path between the objective lens and the image sensor or photodetector array. Examples of such objective lenses will be described in more detail below.

[0252] In some implementations, the objective lens (or microscope objective) 110 may be configured to have reduced magnification. The objective lens 110 may be configured, for example, such that the fluorescence imaging module has a magnification of from less than 2x to less than 10x (as will be discussed in more detail below). Such reduced magnification may alter design constraints such that other design parameters can be achieved. For example, the

objective lens 110 may also be configured such that the fluorescence imaging module has a large field-of-view (FOV) ranging, for example, from about 1.0 mm to about 5.0 mm (*e.g.*, in diameter, width, length, or longest dimension) as will be discussed in more detail below.

[0253] In some implementations, the objective lens 110 may be configured to provide the fluorescence imaging module with a field-of-view as indicated above such that the FOV has diffraction-limited performance, *e.g.*, less than 0.15 waves of aberration over at least 60%, 70%, 80%, 90%, or 95% of the field, as will be discussed in more detail below.

[0254] In some implementations, the objective lens 110 may be configured to provide the fluorescence imaging module with a field-of-view as indicated above such that the FOV has diffraction-limited performance, *e.g.*, a Strehl ratio of greater than 0.8 over at least 60%, 70%, 80%, 90%, or 95% of the field, as will be discussed in more detail below.

[0255] Referring again to FIGS. 2A and 2B, the first dichroic beam splitter or beam combiner is disposed in the first optical path between the light source and the sample so as to illuminate the sample with one or more excitation beams. This first dichroic beam splitter or combiner is also in one or more second optical path(s) from the sample to the different optical channels used to detect the fluorescence emission. Accordingly, the first dichroic filter 130 couples the first optical path of the excitation beam emitted by the illumination source 115 and second optical path of the emission light emitted by a sample specimen to the various optical channels where the light is directed to respective image sensors or photodetector arrays for capturing images of the sample.

[0256] In various implementations, the first dichroic filter 130, *e.g.*, first dichroic reflector or beam splitter or beam combiner, has a passband selected to transmit light from the illumination source 115 only within a specified wavelength band or possibly a plurality of wavelength bands that include the desired excitation wavelength or wavelengths. For example, the first dichroic beam splitter 130 includes a reflective surface comprising a dichroic reflector that has spectral transmissivity response that is, *e.g.*, configured to transmit light having at least some of the wavelengths output by the light source that form part of the excitation beam. The spectral transmissivity response may be configured not to transmit (*e.g.*, instead to reflect) light of one or more other wavelengths, for example, of one or more other fluorescence emission wavelengths. In some implementations, the spectral transmissivity response may also be configured not to transmit (*e.g.*, instead to reflect) light of one or more other wavelengths output by the light source. Accordingly, the first dichroic filter 130 may be utilized to select which wavelength or wavelengths of light output by the light source reach the sample. Conversely, the dichroic reflector in the first dichroic beam splitter 130 has a spectral reflectivity response that reflects light having one or more wavelengths corresponding to the desired fluorescence emission from

the sample and possible reflects light having one or more wavelengths output from the light source that is not intended to reach the sample. Accordingly, in some implementations, the dichroic reflector has a spectral transmissivity that includes one or more pass bands to transmit the light to be incident on the sample and one or more stop bands that reflects light outside the pass bands, for example, light at one or more emission wavelengths and possibly one or more wavelengths output by the light source that are not intended to reach the sample. Likewise, in some implementations the dichroic reflector has a spectral reflectivity that includes one or more spectral regions configured to reflect one or more emission wavelengths and possible one or more wavelengths output by the light source that are not intended to reach the sample and includes one or more regions that transmit light outside these reflection regions. The dichroic reflector included in the first dichroic filter 130 may comprise a reflective filter such as an interference filter (*e.g.*, a quarter-wave stack) configured to provide the appropriate spectral transmission and reflection distributions. FIGS. 2A and 2B also show a dichroic filter 105, which may comprise for example a dichroic beam splitter or beam combiner, which may be used to direct the autofocus laser 102 though the objective and to the sample support structure.

[0257] In some embodiments, the dichroic filter 105, 130, 530 can include one or more spectral passband(s) that increase intensity thus SNR of the light signal(s) being passed by the filter. As such, the dichroic filter(s) herein can provide increased uniformity of frequency response than traditional dichroic filter(s) with narrower spectral passbands.

[0258] Although the imaging module 100 shown in FIGS. 2A and 2B and discussed above is configured such that the excitation beam is transmitted by the first dichroic filter 130 to the objective lens 110, in some designs the illumination source 115 may be disposed with respect to the first dichroic filter 130 and/or the first dichroic filter is configured (*e.g.*, oriented) such that the excitation beam is reflected by the first dichroic filter 130 to the objective lens 110. Similarly, in some such designs, the first dichroic filter 130 is configured to transmit fluorescence emission from the sample and possibly transmit light having one or more wavelengths output from the light source that is not intended to reach the sample. As will be discussed below, a design where the fluorescence emission is transmitted instead of reflected may potentially reduce wavefront error in the detected emission and/or possibly have other advantages. In either case, in various implementations the first dichroic reflector 130 is disposed in the second optical path so as to receive fluorescence emission from the sample, at least some of which continues on to the detection channels 120.

[0259] FIGS. 3A and 3B illustrate the optical paths within the multi-channel fluorescence imaging module of FIGS. 2A and 2B. In the example shown in FIG. 2A and FIG. 3A, the detection channels 120 are disposed to receive fluorescence emission from a sample specimen

that is transmitted by the objective lens 110 and reflected by the first dichroic filter 130. As referred to above and described more below, in some designs the detection channels 120 may be disposed to receive the portion of the emission light that is transmitted, rather than reflected, by the first dichroic filter. In either case, the detection channels 120 may include optics for receiving at least a portion of the emission light. For example, the detection channels 120 may include one or more lenses, such as tube lenses, and may include one or more image sensors or detectors such as photodetector arrays (*e.g.*, CCD or CMOS sensor arrays) for imaging or otherwise producing a signal based on the received light. The tube lenses may, for example, comprise one or more lens elements configured to form an image of the sample onto the sensor or photodetector array to capture an image thereof. Additional discussion of detection channels is included below and in U.S. Provisional Application No. 62/962,723, filed January 17, 2020, which is incorporated herein by reference in its entirety. In some instances, improved optical resolution may be achieved using an image sensor having relatively high sensitivity, small pixels, and high pixel count, in conjunction with a suitable sampling scheme, which may include oversampling or undersampling.

[0260] FIGS. 3A and 3B are ray tracing diagrams illustrating optical paths of the illumination and imaging module 100 of FIGS. 2A and 2B. FIG. 3A corresponds to a top view of the illumination and imaging module 100. FIG. 3B corresponds to a side view of the illumination and imaging module 100. The illumination and imaging module 100 illustrated in these figures includes four detection channels 120. However, it will be understood that the disclosed illumination and imaging modules may equally be implemented in systems including more or fewer than four detection channels 120. For example, the multi-channel systems disclosed herein may be implemented with as few as one detection channel 120, or as many as two detection channels 120, three detection channels 120, four detection channels 120, five detection channels 120, six detection channels 120, seven detection channels 120, eight detection channels 120, or more than eight detection channels 120, without departing from the spirit or scope of the present disclosure.

[0261] The non-limiting example of imaging module 100 illustrated in FIGS. 3A and 3B includes four detection channels 120, a first dichroic filter 130 that reflects a beam 150 of emission light, a second dichroic filter (*e.g.*, a dichroic beam splitter) 135 that splits the beam 150 into a transmitted portion and a reflected portion, and two channel-specific dichroic filters (*e.g.*, dichroic beam splitters) 140 that further split the transmitted and reflected portions of the beam 150 among individual detection channels 120. The dichroic reflecting surface in the dichroic beam splitters 135 and 140 for splitting the beam 150 among detection channels are shown disposed at 45 degrees relative to a central beam axis of the beam 150 or an optical axis

of the imaging module. However, as discussed below, an angle smaller than 45 degrees may be employed and may offer advantages such as sharper transitions from pass band to stop band.

[0262] The different detection channels 120 includes imaging devices 124, which may include an image sensor or photodetector array (*e.g.*, a CCD or CMOS detector array). The different detection channels 120 further include optics 126 such as lenses (*e.g.*, one or more tube lenses each comprising one or more lens elements) disposed to focus the portion of the emission light entering the detection channel 120 at a focal plane coincident with a plane of the photodetector array 124. The optics 126 (*e.g.*, a tube lens) combined with the objective lens 110 are configured to form an image of the sample onto the photodetector array 124 to capture an image of the sample, for example, an image of a surface on the flow cell or other sample support structure after the sample has bound to that surface. Accordingly, such an image of the sample may comprise a plurality of fluorescent emitting spots or regions across a spatial extent of the sample support structure where the sample is emitting fluorescence light. The objective lens 110 together with the optics 126 (*e.g.*, tube lens) may provide a field-of-view (FOV) that includes a portion of the sample or the entire sample. Similarly, the photodetector array 124 of the different detection channels 120 may be configured to capture images of a full field-of-view (FOV) provided by the objective lens and the tube lens, or a portion thereof. In some implementations, the photodetector array 124 of some or all detection channels 120 can detect the emission light emitted by a sample disposed on the sample support structure, *e.g.*, a surface of the flow cell, or a portion thereof and record electronic data representing an image thereof. In some implementations, the photodetector array 124 of some or all detection channels 120 can detect features in the emission light emitted by a specimen without capturing and/or storing an image of the sample disposed on the flow cell surface and/or of the full field-of-view (FOV) provided by the objective lens and optics 126 and/or 122 (*e.g.*, elements of a tube lens). In some implementations, the FOV of the disclosed imaging modules (*e.g.*, that provided by the combination of objective lens 110 and optics 126 and/or 122) may range, for example, between about 1 mm and 5 mm (*e.g.*, in diameter, width, length, or longest dimension) as will be discussed below. The FOV may be selected, for example, to provide a balance between magnification and resolution of the imaging module and/or based on one or more characteristics of the image sensors and/or objective lenses. For example, a relatively smaller FOV may be provided in conjunction with a smaller and faster imaging sensor to achieve high throughput.

[0263] Referring again to FIGS. 3A and 3B, in some implementations, the optics 126 in the detection channel (*e.g.*, the tube lens) may be configured to reduce optical aberration in images acquired using optics 126 in combination with objective lens 110. In some implementations comprising multiple detection channels for imaging at different emission wavelengths, the optics

126 (*e.g.*, the tube lens) for different detection channels have different designs to reduce aberration for the respective emission wavelengths at which that particular channel is configured to image. In some implementations, the optics 126 (*e.g.*, the tube lens) may be configured to reduce aberrations when imaging a specific surface (*e.g.*, a plane, object plane, *etc.*) on the sample support structure comprising fluorescing sample sites disposed thereon as compared to other locations (*e.g.*, other planes in object space). In some implementations, the optics 126 (*e.g.*, the tube lens) may be configured to reduce aberrations when imaging the multiple surfaces (*e.g.*, first and second planes, first and second object planes, *etc.*) on a sample support structure (*e.g.*, a dual or quad surface flow cell) having fluorescing sample sites disposed thereon as compared to other locations (*e.g.*, other planes in object space). For example, the optics 126 in the detection channel (*e.g.*, tube lens) may be designed to reduce the aberration at two, three, or more depths or planes located at different distances from the objective lens as compared to the aberrations associated with other depths or planes at other distances from the objective. For example, optical aberration may be less for imaging the multiple surfaces than elsewhere in a region from about 1 to about 10 mm from the objective lens. Additionally, custom optic 126 in the detection channel (*e.g.*, a tube lens) may in some embodiments be configured to compensate for aberration induced by transmission of emission light through one or more portions of the sample support structure such as a layer that includes one of the surfaces on which the sample is disposed as well as possibly a solution adjacent to and in contact with the surface on which the sample is disposed. The layer comprising one of the surfaces on which the sample is disposed may comprise, *e.g.*, glass, quartz, plastic, or other transparent material having a refractive index, and which introduces optical aberration. Custom optic 126 in the detection channel (*e.g.*, the tube lens), for example, may in some implementations be configured to compensate for optical aberration induced by a sample support structure, *e.g.*, a coverslip or flow cell wall, or other sample support structure components, as well as possibly a solution adjacent to and in contact with the surface on which the sample is disposed.

[0264] In some implementations, the optics 126 in the detection channel (*e.g.*, a tube lens) are configured to have reduced magnification. The optics 126 in the detection channel (*e.g.*, a tube lens) may be configured, for example, such that the fluorescence imaging module has a magnification of less than, for example, 10x, as will be discussed further below. Such reduced magnification may alter design constraints such that other design parameters can be achieved. For example, the optics 126 (*e.g.*, a tube lens) may also be configured such that the fluorescence imaging module has a large field-of-view (FOV), for example, of at least 1.0 mm or larger (*e.g.*, in diameter, width, length, or longest dimension), as will be discussed further below.

[0265] In some implementations, the optics 126 (*e.g.*, a tube lens) may be configured to provide the fluorescence imaging module with a field-of-view as indicated above such that the FOV has less than 0.15 waves of aberration over at least 60%, 70%, 80%, 90%, or 95% of the field, as will be discussed further below.

[0266] Referring again to FIGS. 3A and 3B, in various implementations, a sample is located at or near a focal position 112 of the objective lens 110. As described above with reference to FIGS. 2A and 2B, a light source such as a laser source provides an excitation beam to the sample to induce fluorescence. At least a portion of fluorescence emission is collected by the objective lens 110 as emission light. The objective lens 110 transmits the emission light toward the first dichroic filter 130, which reflects some or all of the emission light as the beam 150 incident upon the second dichroic filter 135 and to the different detection channels, each comprising optics 126 that form an image of the sample (*e.g.*, a plurality of fluorescing sample sites on a surface of a sample support structure) onto a photodetector array 124.

[0267] As discussed above, in some implementations, the sample support structure comprises a flow cell such as a flow cell having multiple surfaces (*e.g.*, two or more interior surfaces) containing sample sites that emit fluorescent emission. These surfaces may be separated by a distance from each other in the longitudinal (Z) direction along the direction of the central axis of the excitation beam and/or the optical axis of the objective lens. This separation may correspond, for example, to one or more flow channels within the flow cell. Analytes or reagents may be flowed through the flow channel(s) and contact the surfaces of the flow cell, which may thereby be contacted with a binding composition such that fluorescence emission is radiated from a plurality of sites on the surfaces. The imaging optics (*e.g.*, objective lens 110) may be positioned at a suitable distance (*e.g.*, a distance corresponding to the working distance) from the sample to form in-focus images of the sample on one or more detector arrays 124. As discussed above, in various designs, the objective lens 110 (possibly in combination with the optics 126) may have a depth of field and/or depth of focus that is at least as large as the longitudinal separation between two adjacent surfaces or any two surfaces of the multiple surfaces. The objective lens 110 and the optics 126 (of each detection channel) can thus simultaneously form images of multiple surfaces on the photodetector array 124, and these images of the surfaces are in focus and have comparable optical resolution (or may be brought into focus with only minor refocusing of the objects to acquire images that have comparable optical resolution). In various implementations, compensation optics need not be moved into or out of an optical path of the imaging module (*e.g.*, into or out of the first and/or second optical paths) to form in-focus images of the surfaces that are of comparable optical resolution. Similarly, in various implementations, one or more optical elements (*e.g.*, lens elements) in the

imaging module (*e.g.*, the objective lens 110 or optics 126) need not be moved, for example, in the longitudinal direction along the first and/or second optical paths to form in-focus images of the first surface in comparison to the location of said one or more optical elements when used to form in-focus images of the second surface. In some implementations, the imaging module includes an autofocus system configured to quickly and sequentially refocus the imaging module on one or more of the multiple surfaces such that the images have comparable optical resolution. In some implementations, objective lens 110 and/or optics 126 are configured such that at least two of the multiple surfaces, e.g., two adjacent surfaces, are in focus simultaneously with comparable optical resolution without moving an optical compensator into or out of the first and/or second optical path, and without moving one or more lens elements (*e.g.*, objective lens 110 and/or optics 126 (such as a tube lens) longitudinally along the first and/or second optics path. In some implementations, images of the surfaces, acquired either sequentially (*e.g.*, with refocusing between surfaces) or simultaneously (*e.g.*, without refocusing between surfaces) using the novel objective lens and/or tube lens designs disclosed herein, may be further processed using a suitable image processing algorithm to enhance the effective optical resolution of the images such that the images have comparable optical resolution. In various implementations, the sample plane is sufficiently in focus to resolve sample sites on the flow cell surfaces, the sample sites being closely spaced in lateral directions (*e.g.*, in the X and Y directions).

[0268] As discussed above, the dichroic filters may comprise interference filters that selectively transmit and reflect light of different wavelengths based on the principle of thin-film interference, using layers of optical coatings having different refractive indices and particular thickness. Accordingly, the spectral response (*e.g.*, transmission and/or reflection spectra) of the dichroic filters implemented within multi-channel fluorescence imaging modules may be at least partially dependent upon the angle of incidence, or range of angles of incidence, at which the light of the excitation and/or emission beams are incident upon the dichroic filters. Such effects may be especially significant with respect to the dichroic filters of the detection optical path (*e.g.*, the dichroic filters 135 and 140 of FIGS. 3A and 3B).

[0269] FIG. 4 is a graph illustrating a relationship between dichroic filter performance and beam angle of incidence (AOI). Specifically, the graph of FIG. 4 illustrates the effect of angle of incidence on the transition width or spectral span of a dichroic filter, which corresponds to the range of wavelengths where the spectral response (*e.g.*, transmission spectrum and/or reflection spectrum) transitions between the passband and stopband regions of a dichroic filter. Thus, a transmission edge (or reflection edge) having a relatively small spectral span (*e.g.*, a small delta λ value in the graph of FIG. 4) corresponds to a sharper transition between passband and

stopband regions or the transmission and reflection regions (or conversely between reflection and transmission regions), while a transmission edge (or reflection edge) having a relatively large spectral span (*e.g.*, a large delta_ λ value in the graph of FIG. 4) corresponds to a less sharp transition between passband and stopband regions. In various implementations, sharper transitions between passband and stopband regions are generally desirable. Moreover, it may also be desirable to have increased consistency or a relatively consistent transition width across all or most of the field-of-view and/or beam area.

[0270] Fluorescence imaging modules, in which the dichroic mirrors are disposed at 45 degrees relative to a central beam axis of the emission light or the optical axis of the optical paths (*e.g.*, of the objective lens and/or tube lens), accordingly can have a transition width of roughly 50 nm for an example dichroic filter, as shown in FIG. 4. Because the emission light beam is not collimated and has some degree of divergence, fluorescence imaging modules may have a range of angles of incidence of approximately 5 degrees between opposing sides of the beam. Thus, as shown in FIG. 4, different portions of the beam of emission light may be incident upon a channel splitting dichroic filter at various angles of incidence between 40 degrees and 50 degrees. This range of relatively large angles of incidence corresponds to a range of transition widths between about 40 nm and about 62 nm. This range of relatively large angles of incidence thereby leads to an increase in transition width of the dichroic filter in the imaging module. Performance of multi-channel fluorescence imaging modules may thus be improved by providing smaller angles of incidence across the full beam, thereby making the transmission edge sharper and allowing better discrimination between different fluorescence emission bands.

[0271] FIG. 5 is a graph illustrating a relationship between beam footprint size (DBS) and beam angle of incidence (DBS angle) on a dichroic filter. In some instances, a smaller beam footprint may be desirable. For example, a small beam footprint allows smaller dichroic filters to be used to split a beam into different wavelength ranges. The use of smaller dichroic filters in turn reduces manufacturing costs and improves the ease of manufacturing suitably flat dichroic filters. As shown in FIG. 5, any angle of incidence greater than 0 degrees (*e.g.*, perpendicular to the surface of the dichroic filter) results in an elliptical beam footprint having an area larger than the cross-sectional area of the beam. An angle of incidence of 45 degrees results in a large beam footprint on the dichroic reflector that is greater than 1.4 times the cross-sectional area of the beam when incident at zero degrees.

[0272] FIGS. 6A and 6B schematically illustrate a non-limiting example configuration of dichroic filters and detection channels in a multi-channel fluorescence imaging module where the dichroic mirrors are disposed at an angle of less than 45 degrees relative to a central beam axis of the emission light or the optical axis of the optical paths (*e.g.*, of the objective lens and/or

tube lens). FIG. 6A depicts an imaging module 500 including a plurality of detection channels 520a, 520b, 520c, 520d. FIG. 6B is a detailed view of the portion of the imaging module 500 within the circle 5B as shown in FIG. 6A. As will be described in greater detail, the configuration illustrated in FIGS. 6A and 6B includes a number of aspects that may result in significant improvements over conventional multi-channel fluorescence imaging module designs. In some instances, fluorescence imaging modules and systems of the present disclosure may, however, may be implemented with one or a subset of the features described with respect to FIGS. 6A and 6B without departing from the spirit or scope of the present disclosure.

[0273] The imaging module 500 depicted in FIG. 6A includes an objective lens 510 and four detection channels 520a, 520b, 520c, and 520d disposed to receive and/or image emission light transmitted by the objective lens 510. A first dichroic filter 530 is provided to couple the excitation and detection optical paths. In contrast to the design shown in FIGS. 2A and 2B, as well as in FIGS. 3A and 3B, the first dichroic filter 530 (*e.g.*, a dichroic beam splitter or combiner) is configured to reflect light from the light source to the objective lens 510 and sample, and transmit fluorescence emission from the sample to the detection channels 520a, 520b, 520c, and 520d. A second dichroic filter 535 splits a beam of emission light among at least two detection channels 520a, 520b by transmitting a first portion 550a and reflecting a second portion 550b. Additional dichroic filters 540a, 540b are provided to further split the emission light. Dichroic filter 540a transmits at least a portion of the first portion 550a of the emission light and reflects a portion 550c to a third detection channel 520c. Dichroic filter 540b transmits at least a portion of the second portion 550b of the emission light and reflects a portion 550d to a fourth detection channel 520d. Although the imaging module 500 is depicted with four detection channels, in various embodiments the imaging module 500 may include more or fewer detection channels, with a correspondingly larger or smaller number of dichroic filters as appropriate to provide a portion of the emission light to each detection channel. For example, in some embodiments, the features of the imaging module 500 may be implemented with similar advantageous effects in an imaging module including only two detection channels 520a, 520b, and omitting additional dichroic filters 540a, 540b. In some implementations, only one detection channel may be included. Alternatively, three or more detection channels may be employed.

[0274] The detection channels 520a, 520b, 520c, 520d illustrated in FIG. 6A may include some or all of the same or similar components to those of the detection channels 120 illustrated in FIGS. 2A - 3B. For example, different detection channel 520a, 520b, 520c, 520d may include one or more image sensors or photodetectors arrays, and may include transmissive and/or

reflective optics such as one or more lenses (*e.g.*, tube lenses) that focus the light received by the detection channel onto its respective image sensor or photodetector array.

[0275] The objective lens 510 is disposed to receive emission light emitted by fluorescence from a specimen. In particular, the first dichroic filter 530 is disposed to receive the emission light collected and transmitted by the objective lens 510. As discussed above and shown in FIG. 6A, in some designs, an illumination source (*e.g.*, the illumination source 115 of FIGS. 2A and 2B) such as a laser source or the like is disposed to provide an excitation beam which is incident on the first dichroic filter 530 such that the first dichroic filter 530 reflects the excitation beam into the same objective lens 510 that transmits the emission light, for example, in an epifluorescence configuration. In some other designs, the illumination source may be directed to the specimen by other optical components along a different optical path that does not include the same objective lens 510. In such configurations, the first dichroic filter 530 may be omitted.

[0276] Similarly, as discussed above and shown in FIG. 6A, the detection optics (*e.g.*, including the detection channels 520a, 520b, 520c, 520d and any optical components such as dichroic filters 535, 540a, 540b along the optical path between the objective lens 510 and the detection channels 520a, 520b, 520c, 520d) may be disposed on the transmission path of the first dichroic filter 530, rather than on the reflected path of the first dichroic filter 530. In one example implementations, the objective lens 510 and detection optics are disposed such that the objective lens 510 transmits the beam 550 of emission light directly toward the second dichroic filter 535. The wavefront quality of the emission light may be degraded somewhat by the presence of the first dichroic filter 530 along the path of the beam 550 of emission light (*e.g.*, by imparting some wavefront error to the beam 550). However, the wavefront error introduced by a beam transmitted through a dichroic reflector of a dichroic beam splitter is generally significantly smaller than the wavefront error of a beam reflected from the dichroic reflecting surface of a dichroic beam splitter (*e.g.*, an order of magnitude smaller). Thus, the wavefront quality and subsequent imaging quality of the emission light in a multi-channel fluorescence imaging module may be substantially improved by placing the detection optics along the transmitted beam path of the first dichroic filter 530 rather than along the reflected beam path.

[0277] Still referring to FIG. 6A, within the detection optics of the imaging module 500, dichroic filters 535, 540a, and 540b are provided to split the beam 550 of emission light among the detection channels 520a, 520b, 520c, 520d. For example, the dichroic filters 535, 540a, and 540b split the beam 550 on the basis of wavelength, such that a first wavelength or wavelength band of the emission light can be received by the first detection channel 520a, a second wavelength or wavelength band of the emission light can be received by the second detection channel 520b, a third wavelength or wavelength band of the emission light can be received by

the third detection channel 520c, and a fourth wavelength or wavelength band of the emission light can be received by the fourth detection channel 520d. In some implementations, multiple separated wavelengths or wavelength bands can be received by the detection channel.

[0278] In contrast to the multi-channel fluorescence imaging module design shown in FIGS. 2A and 2B, as well as FIGS. 3A and 3B, the imaging module 500 has dichroic filters 535, 540a, and 540b disposed at angles of incidence of less than 45 degrees with respect to the central beam axis of the incident beams. As shown in FIG. 6B, the different beams 550, 550a, 550b have respective central beam axes 552, 552a, 552b. In various implementations, the central beam axes 552, 552a, 552b is at the center of a cross-section of the beam orthogonal to the propagation direction of the beam. These central beam axes 552, 552a, 552b may correspond to the optical axis of the objective lens and/or the optics within the separate channels, for example, the optical axes of the respective tube lenses. Additional rays 554, 554a, 554b of each beam 550, 550a, 550b are illustrated in FIG. 6B to indicate the diameter of each beam 550, 550a, 550b. Beam diameter may be defined, for example, as a full width at half maximum diameter, a D4σ (*e.g.*, 4 times σ, where σ is the standard deviation of the horizontal or vertical marginal distribution of the beam respectively) or second-moment width, or any other suitable definition of beam diameter.

[0279] The central beam axis 552 of the beam 550 of emission light may serve as a reference point for defining the angle of incidence of the beam 550 on the second dichroic filter 535. Accordingly, the "angle of incidence" (AOI) of a beam 550 may be the angle between the central beam axis 552 of the incident beam 550 and a line N normal to the surface the beam is incident on, for example, the dichroic reflective surface. When the beam 550 of emission light is incident upon the dichroic reflective surface of the second dichroic filter 535 at an angle of incidence AOI, the second dichroic filter 535 transmits a first portion 550a of the emission light (*e.g.*, the portion having wavelengths within the passband region of the second dichroic filter 535) and reflects a second portion 550b of the emission light (*e.g.*, the portion having wavelengths within the stopband region of the second dichroic filter 535). The first portion 550a and the second portion 550b may each be similarly described in terms of a central beam axis 552a, 552b. As referred to above, the optical axis may alternatively or additionally be used.

[0280] In the example configuration of FIGS. 6A and 6B, the second dichroic filter 535 is disposed such that the central beam axis 552 of the beam 550 is incident at an angle of incidence of 30 degrees. Similarly, the additional dichroic filters 540a, 540b are disposed such that the central beam axes 552a, 552b of the first and second portions 550a, 550b of the beam 550 are also incident at angles of incidence of 30 degrees. However, in various implementations these angles of incidence may be other angles smaller than 45 degrees. In some instances, for

example, the angles of incidence may range between about 20 degrees and about 45 degrees, as will be discussed further below. Moreover, the angles of incidence on each of the dichroic filters 535, 540a, 540b need not necessarily be the same. In some embodiments, some or all of the dichroic filters 535, 540a, 540b may be disposed such that their incident beams 550, 550a, 550b have different angles of incidence. As described above, the angle of incidence may be with respect to the optical axis of the optics within the imaging module, for example, the objective lens and/or the optics in the detection channels (*e.g.*, the tube lenses) and the dichroic reflective surface in the respective dichroic beam splitter. The same ranges and values for the angle of incidence apply to the case when the optical axis is used to specify the AOI.

[0281] The beams 550, 550a, 550b of emission light in a fluorescence imaging module system are typically diverging beams. As noted above, the beams of emission light can have a beam divergence large enough that regions of the beam within the beam diameter are incident upon the dichroic filters at angles of incidence that differ by up to 5 degrees or more relative to the angle of incidence of the central beam axis and/or optical axis of the optics. In some designs, the objective lens 510 may be configured, for example, to have an f-number or numerical aperture selected to produce a smaller beam diameter for a given field-of-view of the microscope. In one example, the f-number or numerical aperture of the objective lens 510 may be selected such that the full diameter of the beams 550, 550a, 550b are incident upon dichroic filters 535, 540a, 540b at angles of incidence within, for example, 1 degree, 1.5 degrees, 2 degrees, 2.5 degrees, 3 degrees, 3.5 degrees, 4 degrees, 4.5 degrees, or 5 degrees of the angle of incidence of the central beam axes 552, 552a, 552b.

[0282] In some implementations, the focal length of the objective lens that is suitable for producing such a narrow beam diameter may be longer than those typically employed in fluorescence microscopes or imaging systems. For example, in some implementations, the focal length of the objective lens may range between 20 mm and 40 mm, as will be discussed further below. In one example, an objective lens 510 having a focal length of 36 mm may produce a beam 550 characterized by a divergence small enough that light across the full diameter of the beam 550 is incident upon the second dichroic filter 535 at angles within 2.5 degrees of the angle of incidence of the central beam axis.

[0283] FIG. 7 and FIG. 8 provide graphs illustrating improved dichroic filter performance due to aspects of the imaging module configuration of FIGS. 6A and 6B (or any of the imaging module configurations disclosed herein). The graph in FIG. 7 is similar to that of FIG. 4 and illustrates the effect of angle of incidence on the transition width (*e.g.*, the spectral span of the transmission edge) of a dichroic filter. FIG. 7 shows an example where the orientation of a dichroic filter (*e.g.*, dichroic filters 535, 540a, and 540b) and the dichroic reflective surface therein is such that

its incident beam has an angle of incidence of 30 degrees, rather than 45 degrees. FIG. 7 shows how this reduced angle of incidence significantly improves the sharpness and the uniformity of the transition width across the full beam diameter. For example, while an angle of incidence of 45 degrees at the central beam axis results in a range of transition widths between about 40 nm and about 62 nm, an angle of incidence of 30 degrees at the central beam axis results in a range of transition widths between about 16 nm and about 30 nm. In this example, the average transition width is reduced from about 51 nm to about 23 nm, indicating a sharper transition between passband and stopband. Moreover, the variation in transition widths across the beam diameter is reduced by nearly 40% from a 22 nm range to a 14 nm range, indicating a more uniform sharpness of the transition over the area of the beam.

[0284] FIG. 8 illustrates additional advantages that may be realized by selecting the appropriate f-number or numerical aperture for the objective lens to reduce beam divergence in any of the imaging module configurations disclosed herein. In some implementations, a longer focal length is used. In the example of FIG. 8, the objective lens 510 has a focal length of 36 mm, which with the appropriate numerical aperture (*e.g.*, less than 5), reduces the range of angles of incidence within the beam 550 from 30 degrees ± 5 degrees to 30 degrees ± 2.5 degrees. With this design, the range of transition widths may be reduced to between about 19 nm and about 26 nm. When compared to the improved system of FIG. 7, although the average transition width is substantially the same (*e.g.*, a spectral span of roughly 23 nm), the variation in transition widths across the beam diameter is further reduced to a 7 nm range, representing a reduction of nearly 70% relative to the range of transition widths illustrated in FIG. 4.

[0285] Referring again to FIG. 5, the reduction in angle of incidence from 45 degrees to 30 degrees at the central beam axis is further advantageous because it reduces the beam spot size on the dichroic filter. As shown in FIG. 5, an angle of incidence of 45 degrees results in a beam footprint on the dichroic filter having an area greater than 1.4 times the cross-sectional area of the beam. However, an angle of incidence of 30 degrees results in a beam footprint on the dichroic filter having an area only about 1.15 times the cross-sectional area of the beam. Thus, reducing the angle of incidence at the dichroic filters 535, 540a, 540b from 45 degrees to 30 degrees results in a reduction of about 18% in the area of the beam footprint on the dichroic filters 535, 540a, 540b. This reduction in beam footprint area allows smaller dichroic filters to be used.

[0286] Referring now jointly to FIGS. 9A-B, the reduction in angle of incidence from 45 degrees to 30 degrees may also provide improved performance with regard to surface deformation caused by the dichroic filters in any of the imaging module configurations disclosed herein, as indicated by improvements in the modulation transfer function. In general, the

amount of surface deformation increases with larger area optical elements. If a larger area on the dichroic filter is employed, a larger amount of surface deformation is encountered, thereby introducing more wavefront error into the beam. FIG. 9A illustrates the effect of folding angle on image quality degradation induced by the addition of 1 wave of peak-to-valley (PV) spherical power to the last mirror. FIG. 9B illustrates the effect of folding angle on image quality degradation induced by the addition of 0.1 wave of PV spherical power to the last mirror. As shown in FIGS. 9A and 9B, the reduction in angle of incidence to 30 degrees significantly reduces the effect of surface deformation to achieve close to diffraction-limited performance of the detection optics.

[0287] In some implementations of the disclosed imaging modules, the polarization state of the excitation beam may be utilized to further improve the performance of the multi-channel fluorescence imaging modules disclosed herein. Referring back to FIGS. 2A, 2B, and 6A, for example, some implementations of the multi-channel fluorescence imaging modules disclosed herein have an epifluorescence configuration in which a first dichroic filter 130 or 530 merges the optical paths of the excitation beam and the beam of emission light such that both the excitation and emission light are transmitted through the objective lens 110, 510. As discussed above, the illumination source 115 may include a light source such as a laser or other source which provides the light that forms the excitation beam. In some designs, the light source comprises a linearly polarized light source and the excitation beam may be linearly polarized. In some designs, polarization optics are included to polarize the light and/or rotate the polarization of the light. For example, a polarizer such as a linear polarizer may be included in an optical path of the excitation beam to polarize the excitation beam. Retarders such as half wave retarders or a plurality of quarter wave retarders or retarders having other amounts of retardance may be included to rotate the linear polarization in some designs.

[0288] The linearly polarized excitation beam, when it is incident upon any dichroic filter or other planar interface, may be p-polarized (*e.g.*, having an electric field component parallel to the plane of incidence), s-polarized (*e.g.*, having an electric field component normal to the plane of incidence), or may have a combination of p-polarization and s-polarization states within the beam. The p- or s-polarization state of the excitation beam may be selected and/or changed by selecting the orientation of the illumination source 115 and/or one or more components thereof with respect to the first dichroic filter 130, 530 and/or with respect to any other surfaces with which the excitation beam will interact. In some implementations where the light source outputs linearly polarized light, the light source can be configured to provide s-polarized light. For example, the light source may comprise an emitter such as a solid-state laser or a laser diode that may be rotated about its optical axis or the central axis of the beam to orient the linearly

polarized light output therefrom. Alternatively, or in addition, retarders may be employed to rotate the linear polarization about the optical axis or the central axis of the beam. As discussed above, in some implementations, for example when the light source does not output polarized light, a polarizer disposed in the optical path of the excitation beam can polarize the excitation beam. In some designs, for example, a linear polarizer is disposed in the optical path of the excitation beam. This polarizer may be rotated to provide the proper orientation of the linear polarization to provide s-polarized light.

[0289] In some designs, the linear polarization is rotated about the optical axis or the central axis of the beam such that s-polarization is incident on the dichroic reflector of the dichroic beam splitter. When s-polarized light is incident on the dichroic reflector of the dichroic beam splitter the transition between the pass band and the stop band is sharper as opposed to when p-polarized light is incident on the dichroic reflector of the dichroic beam splitter.

[0290] As shown in FIGS. 10A and 10B, use of the p- or s-polarization state of the excitation beam may significantly affect the narrowband performance of any excitation filters such as the first dichroic filter 130, 530. FIG. 10A illustrates a transmission spectrum between 610 nm and 670 nm for an example bandpass dichroic filter at angles of incidence of 40 degrees and 45 degrees, where the incident beam is linearly polarized and is p-polarized with respect to the plane of the dichroic filter. As shown in FIG. 10B, changing the orientation of the light source with respect to the dichroic filter, such that the incident beam is s-polarized with respect to the plane of the dichroic filter, results in a substantially sharper edge between the passband and the stopband of the dichroic filter. Thus, the illumination and imaging modules 100, 500 disclosed herein may advantageously have an illumination source 115 oriented relative to the first dichroic filter 130, 530 such that the excitation beam is s-polarized with respect to the plane of the first dichroic filter 130, 530. As discussed above, in some implementations, a polarizer such as a linear polarizer may be used to polarize the excitation beam. This polarizer may be rotated to provide an orientation of the linearly polarized light corresponding to s-polarized light. Also as discussed above, in some implementations, other approaches to rotating the linearly polarized light may be used. For example, optical retarders such as half wave retarders or multiple quarter wave retarders may be used to rotate the polarization direction. Other arrangements are also possible.

[0291] As discussed elsewhere herein, reducing the numerical aperture (NA) of the fluorescence imaging module and/or of the objective lens may increase the depth of field to enable the comparable imaging of multiple surfaces, e.g., 3, 4, or more surfaces. FIGS. 11A-16B, show how the MTF is more similar at first and second surfaces separated by 1 mm of glass for lower numerical apertures than for larger numerical apertures.

[0292] FIGS. 11A and 11B show the MTF at first (FIG. 11A) and second (FIG. 11B) surfaces for an NA of 0.3.

[0293] FIGS. 12A and 12B show the MTF at first (FIG. 12A) and second (FIG. 12B) surfaces for an NA of 0.4.

[0294] FIGS. 13A and 13B show the MTF at first (FIG. 13A) and second (FIG. 13B) surfaces for an NA of 0.5.

[0295] FIGS. 14A and 14B show the MTF at first (FIG. 14A) and second (FIG. 14B) surfaces for an NA of 0.6.

[0296] FIGS. 15A and 15B show the MTF at first (FIG. 15A) and second (FIG. 15B) surfaces for an NA of 0.7.

[0297] FIGS. 16A and 16B show the MTF at first (FIG. 16A) and second (FIG. 16B) surfaces for an NA of 0.8. The first and second surfaces in each of these figures correspond to, *e.g.*, the top and bottom surfaces of a flow cell.

[0298] FIGS. 17A-B provide plots of the calculated Strehl ratio (*e.g.*, the ratio of peak light intensity focused or collected by the optical system versus that focused or collected by an ideal optical system and point light source) for imaging a second flow cell surface through a first flow cell surface. FIG. 17A shows a plot of the Strehl ratios for imaging a second flow cell surface through a first flow cell surface as a function of the thickness of the intervening fluid layer (fluid channel height) for different objective lens and/or optical system numerical apertures. In some embodiments, the Strehl ratio decreases with increasing separation between two adjacent surfaces, e.g., the first and second surfaces. One of the surfaces can thus have deteriorated image quality with increasing separation between the two surfaces. The decrease in surface imaging performance with increased separation distance between the two adjacent surfaces is reduced for imaging systems having smaller numerical apertures, e.g., NA of 0.5 or 0.4 as compared to those having larger numerical apertures. FIG. 17B shows a plot of the Strehl ratio as a function of numerical aperture for imaging a second flow cell surface through a first flow cell surface and an intervening layer of water having a thickness of 0.1 mm. The loss of imaging performance at higher numerical apertures may be attributed to the increased optical aberration induced by the fluid for the second surface imaging. With increasing NA, the increased optical aberration introduced by the fluid for the second, third, or fourth surface imaging can degrade the image quality significantly. In general, however, reducing the numerical aperture of the optical system reduces the achievable resolution. This loss of image quality can be at least partially offset by providing an increased sample plane (or object plane) contrast-to-noise ratio, for example, by using chemistries for nucleic acid sequencing applications that enhance the fluorescence emission for labeled nucleic acid clusters and/or that

reduce background fluorescence emission. In some instances, for example, sample support structures comprising hydrophilic substrate materials and/or hydrophilic coatings may be employed. In some cases, such hydrophilic substrates and/or hydrophilic coatings may reduce background noise. Additional discussion of sample support structures, hydrophilic surfaces and coatings, and methods for enhancing contrast-to-noise ratios, *e.g.*, for nucleic acid sequencing applications, can be found below.

[0299] In some implementations, any one or more of the fluorescence imaging system, the illumination and imaging module 100, the imaging optics (*e.g.*, optics 126), the objective lens, and/or the tube lens is configured to have reduced magnification, such as a magnification of less than 10x, as will be discussed further below. Such reduced magnification may adjust design constraints such that other design parameters can be achieved. For example, any one or more of the fluorescence microscope, illumination and imaging module 100, the imaging optics (*e.g.*, optics 126), the objective lens or the tube lens may also be configured such that the fluorescence imaging module has a large field-of-view (FOV), for example, a field-of-view of at least 3.0 mm or larger (*e.g.*, in diameter, width, height, or longest dimension), as will be discussed further below. Any one or more of the fluorescence imaging system, the illumination and imaging module 100, the imaging optics (*e.g.*, optics 126), the objective lens and/or the tube lens may be configured to provide the fluorescence microscope with such a field-of-view such that the FOV has less than, *e.g.*, 0.1 waves of aberration over at least 80% of field. Similarly, any one or more of the fluorescence imaging system, illumination and imaging module 100, the imaging optics (*e.g.*, optics 126), the objective lens and/or the tube lens may be configured such that the fluorescence imaging module has such a FOV and is diffraction limited or is diffraction limited over such an FOV.

[0300] As discussed above, in various implementations, a large field-of-view (FOV) is provided by the disclosed optical systems. In some implementations, obtaining an increased FOV is facilitated in part by the use of larger image sensors or photodetector arrays. The photodetector array, for example, may have an active area with a diagonal of at least 15 mm or larger, as will be discussed further below. As discussed above, in some implementations the disclosed optical imaging systems provide a reduced magnification, for example, of less than 10x which may facilitate large FOV designs. Despite the reduced magnification, the optical resolution of the imaging module may still be sufficient as detector arrays having small pixel size or pitch may be used. The pixel size and/or pitch may, for example, be about 5 μm or less, as will be discussed in more detail below. In some implementations, the pixel size is smaller than twice the optical resolution provided by the optical imaging system (*e.g.*, objective and tube lens) to satisfy the Nyquist theorem. Accordingly, the pixel dimension and/or pitch for the image sensor(s) may be

such that a spatial sampling frequency for the imaging module is at least twice an optical resolution of the imaging module. For example, the spatial sampling frequency for the photodetector array may be is at least 2 times, at least 2.5 times, at least 3 times, at least 4 times, or at least 5 times the optical resolution of the fluorescence imaging module (*e.g.*, the illumination and imaging module, the objective and tube lens, the object lens and optics 126 in the detection channel, the imaging optics between the sample support structure or stage configured to support the sample support stage and the photodetector array) or any spatial sampling frequency in a range between any of these values.

[0301] Although a wide range of features are discussed herein with respect to fluorescence imaging modules, any of the features and designs described herein may be applied to other types of optical imaging systems including without limitation bright-field and dark-field imaging, and may apply to luminescence or phosphorescence imaging.

Dual wavelength excitation / four channel imaging systems

[0302] FIG. 18 illustrates a dual excitation wavelength / four channel imaging system for dual-side or quad-side imaging applications that includes an objective and tube lens combination that is scanned in a direction perpendicular to the optical axis to provide for large area imaging, *e.g.*, by tiling several images to create a composite image having a total field-of-view (FOV) that is much larger than that for each individual image. The system comprises two excitation light sources, *e.g.*, lasers or laser diodes, operating at different wavelengths and an autofocus laser. The two excitation light beams and autofocus laser beam are combined using a series of mirrors and/or dichroic reflectors and delivered to the surfaces of the flow cell through the objective. Fluorescence that is emitted by labeled oligonucleotides (or other biomolecules) tethered to one of the flow cell surfaces is collected by the objective, transmitted through the tube lens, and directed to one of four imaging sensors according to the wavelength of the emitted light by a series of intermediate dichroic reflectors. Autofocus laser light that has been reflected from the flow cell surface is collected by the objective, transmitted through the tube lens, and directed to an autofocus sensor by a series of intermediate dichroic reflectors. The system allows accurate focus to be maintained (*e.g.*, by adjusting the relative distance between the flow cell surface and the objective using a precision linear actuator, translation stage, or microscope turret-mounted focus adjustment mechanism, to reduce or minimize the reflected light spot size on the autofocus image sensor) while the objective/tube lens combination is scanned in a direction perpendicular to the optical axis of the objective. Dual wavelength excitation used in combination with four channel (*e.g.*, four wavelength) imaging capability provides for high-throughput imaging of the multiple surfaces of the flow cell.

Multiplexed optical read-heads

[0303] In some instances, miniaturized versions of any of the imaging modules described herein may be assembled to create a multiplexed read-head that may be translated in one or more directions horizontally relative to a sample surface, *e.g.*, an interior surface of a flow cell, to image several sections of the surface simultaneously. A non-limiting example of a multiplexed read-head has recently been described in U.S. Published Patent Application No. 2020/0139375 Al.

[0304] In some instances, for example, a miniaturized imaging module may comprise a "microfluorometer" comprising an illumination or excitation light source such as an LED or laser diode (or the tip of an optical fiber connected to an external light source), one or more lenses for collimating or focusing the illumination or excitation light, one or more dichroic reflectors, one or more optical filters, one or more mirrors, beam-splitters, prisms, apertures, *etc.*, one or more objectives, one or more custom tube lenses for enabling multiple surface imaging with minimal focus adjustment as described elsewhere herein, one or more image sensors, or any combination thereof, as described elsewhere herein. In some instances, a miniaturized imaging module (*e.g.*, a "microfluorometer") may further comprise an autofocus mechanism, a microprocessor, power and data transfer connectors, a light-tight housing, *etc.* The resulting miniaturized imaging module may thus comprise an integrated imaging package or unit having a small form factor. In some instances, the shortest dimension (*e.g.*, width or diameter) of the miniaturized imaging module may be less than 5 cm, less than 4.5 cm, less than 4 cm, less than 3.5 cm, less than 3 cm, less than 2.5 cm, less than 2 cm, less than 1.8 cm, less than 1.6 cm, less than 1.4 cm, less than 1.2 cm, less than 1 cm, less than 0.8 cm, or less than 0.6 cm. In some instances, the longest dimension (*e.g.*, height or length) of the miniaturized imaging module may be less than 16 cm, less than 14 cm, less than 12 cm, less than 10 cm, less than 9 cm, less than 8 cm, less than 7 cm, less than 5 cm, less than 5 cm, less than 4.5 cm, less than 4 cm, less than 3.5 cm, less than 3 cm, less than 2.5 cm, less than 2 cm, less than 1.8 cm, less than 1.6 cm, less than 1.4 cm, less than 1.2 cm, or less than 1 cm. In some instances, one or more individual miniaturized imaging modules within the multiplexed read-head may comprise an autofocus mechanism.

[0305] In some instances, multiplexed read-heads as described herein may comprise an assembly of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 miniaturized imaging modules or microfluorometers held in fixed position relative to each other. In some instances, the optical design specifications and performance properties of the individual miniaturized imaging

modules or microfluorometers, e.g., for numerical aperture, field-of-view, depth-of-field, image resolution, *etc.*, may be the same as described elsewhere herein for other versions of the disclosed imaging modules. In some instances, the plurality of individual miniaturized imaging modules may be arranged in a linear arrangement comprising one, two, three, four, or more than four rows and/or columns. In some instances, the plurality of individual miniaturized imaging modules may be arranged in, *e.g.*, a hexagonal close pack arrangement. In some instances, the plurality of individual miniaturized imaging modules may be arranged in a circular or spiral arrangement, a randomly distributed arrangement, or in any other arrangement known to those of skill in the art.

[0306] FIGS. 43A-B provide non-limiting schematic illustrations of a multiplexed read-head as disclosed herein. FIG. 43A shows a side view of a multiplexed read-head in which two rows of individual microfluorometers (seen from the end on) having common optical design specifications, *e.g.*, numerical aperture, field-of-view, working distance, *etc.*, are configured to image a common surface, *e.g.*, a first interior surface of a flow cell. FIG. 43B shows a top view of the same multiplexed read-head illustrating the overlapping imaging paths acquired by individual microfluorometers of the multiplexed read-head as the read-head is translated relative to the flow cell (or *vice versa*). In some instances, the individual fields-of-view for the individual microfluorometers may overlap, as indicated in FIG. 43B. In some instances, they may not overlap. In some instances, the multiplexed-read head may be designed such that it aligns with and images predetermined features, *e.g.*, individual fluid channels, within a flow cell.

[0307] FIGS. 44A-B provide non-limiting schematic illustrations of a multiplexed read-head where a first subset of the plurality of individual miniaturized imaging modules is configured to image a first sample plane, *e.g.*, a first interior surface of a flow cell, and a second subset of the plurality is configured to simultaneously or sequentially image a second sample plane, *e.g.*, a second interior surface of a flow cell. FIG. 44A shows a side view of the multiplexed read-head in which the first subset of individual microfluorimeters is configured to image, *e.g.*, the first or upper interior surface of a flow cell, and the second subset is configured to image a second surface, *e.g.*, the second or lower interior surface of a flow cell. FIG. 44B shows a top view of the multiplexed read-head of FIG. 44A illustrating the imaging paths acquired by individual microfluorimeters of the multiplexed read-head. Again, in some instances, the individual fields-of-view for the individual microfluorometers in a given subset may overlap. In some instances, they may not overlap. In some instances, the multiplexed-read head may be designed such that the individual miniaturized imaging modules of the first and second subsets align with and image predetermined features, *e.g.*, individual fluid channels, within a flow cell.

[0308] In some embodiments, the multiplexed read-head can further include a third subset of the plurality of individual miniaturized imaging modules is configured to image a third sample plane, *e.g.*, a third interior surface of a flow cell, and a fourth subset of the plurality is configured to simultaneously or sequentially image a fourth sample plane, *e.g.*, a fourth interior surface of a flow cell.

Improved or optimized objective and/or tube lens for use with thicker coverslips

[0309] Existing design practice includes the design of objective lenses and/or use of commonly available off-the-shelf microscope objectives to optimize image quality when images are acquired through thin (*e.g.*, < 200 μm thick) microscope coverslips. When used to image on both sides of a fluidic channel or flow cell, the extra height of the gap between the two surfaces (*e.g.*, the height of the fluid channel; typically, about 50 μm to 200 μm) introduces optical aberration in images captured for the non-optimal side of the fluidic channel, thereby causing reduction in optical resolution. This is primarily because the additional gap height is significant compared to the optimal coverslip thickness (typical fluid channel or gap heights of 50 – 200 μm vs. coverslip thicknesses of < 200 μm). Another common design practice is to utilize an additional "compensator" lens in the optical path when imaging is to be performed on the non-optimal side of the fluid channel or flow cell. This "compensator" lens and the mechanism required to move it in or out of the optical path so that all the surfaces of the flow cell may be imaged. The compensator can further increase system complexity and imaging system down time, and potentially degrades image quality due to vibration, or motion *etc.*

[0310] In the present disclosure, the imaging system is designed for compatibility with flow cell consumables that comprise a thicker coverslip or flow cell wall (thickness ≥ 700 μm). The objective lens design may be improved or optimized for a coverslip that is equal to the true cover slip thickness plus half of the effective gap thickness (*e.g.*, 700 μm + ½ * fluid channel (gap) height). This design can significantly reduce the effect of gap height on image quality for the multiple surfaces of the fluid channel and balances the optical quality for images of the surfaces, as the gap height is small relative to the total coverslip thickness and thus its impact on optical quality is reduced.

[0311] Additional advantages of using a thicker coverslip include improved control of thickness tolerance error during manufacturing, and a reduced likelihood that the coverslip undergoes deformation due to thermal and mounting-induced stress. Coverslip thickness error and deformation can adversely impact imaging quality for all surfaces of a flow cell.

[0312] To further improve the imaging quality for sequencing applications, our optical system design places a strong emphasis on improving or optimizing MTF (*e.g.*, through improving or

optimizing the objective lens and/or tube lens design) in the mid- to high-spatial frequency range that is most suitable for imaging and resolving small spots or clusters.

Improved or optimized tube lens design for use in combination with commercially available, off-the-shelf objectives

[0313] For low-cost sequencer design, the use of a commercially available, off-the-shelf objective lens may be preferred due to its relatively low price. However, as noted above, low-cost, off-the-shelf objectives are mostly optimized for use with thin coverslips of about 170 μm in thickness. In some instances, the disclosed optical systems may utilize a tube lens design that compensates for a thicker flow cell coverslip while enabling high image quality for the surfaces of a flow cell in multiple-surface imaging applications. In some instances, the tube lens designs disclosed herein enable high quality imaging for the multiple surfaces of a flow cell without moving an optical compensator into or out of the optical path between the flow cell and an image sensor, without moving one or more optical elements or components of the tube lens along the optical path, and without moving one or more optical elements or components of the tube lens into and/or out of the optical path.

[0314] FIG. 19 provides an optical ray tracing diagram for a low light objective lens design that has been improved or optimized for imaging a surface on the opposite side of a 0.17 mm thick coverslip. The plot of modulation transfer function for this objective, shown in FIG. 20, indicates near-diffraction limited imaging performance when used with the designed-for 0.17 mm thick coverslip.

[0315] FIG. 21 provides a plot of the modulation transfer function for the same objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface on the opposite side of a 0.3 mm thick coverslip. The relatively minor deviations of MTF value over the spatial frequency range of about 100 to about 800 lines/mm (or cycles/mm) indicates that the image quality obtained even when using a 0.3 mm thick coverslip is still reasonable.

[0316] FIG. 22 provides a plot of the modulation transfer function for the same objective lens illustrated in FIG. 19 as a function of spatial frequency when used to image a surface that is separated from that on the opposite side of a 0.3 mm thick coverslip by a 0.1 mm thick layer of aqueous fluid (*e.g.*, under the kind of conditions encountered for multiple-side imaging of a flow cell when imaging the far surface). As can be seen in the plot of FIG. 22, imaging performance is degraded, as indicated by the deviations of the MTF curves from those for an ideal, diffraction-limited case over the spatial frequency range of about 50 lp/mm to about 900 lp/mm.

[0317] FIG. 23 and FIG. 24 provide plots of the modulation transfer function as a function of spatial frequency for the upper (or near) interior surface (FIG. 23) and lower (or far) interior

surface (FIG. 24) of a flow cell when imaged using the objective lens illustrated in FIG. 19 through a 1.0 mm thick coverslip, and when the upper and lower interior surfaces are separated by a 0.1 mm thick layer of aqueous fluid. As can be seen, imaging performance is significantly degraded for both surfaces.

[0318] FIG. 25 provides a ray tracing diagram for a tube lens design which, if used in conjunction with the objective lens illustrated in FIG. 19, provides for improved multiple-side imaging through a 1 mm thick coverslip. The optical design 700 comprising a compound objective (lens elements 702, 703, 704, 705, 706, 707, 708, 709, and 710) and a tube lens (lens elements 711, 712, 713, and 714) is improved or optimized for use with flow cells comprising a thick coverslip (or wall), *e.g.*, greater than 700 μm thick, and a fluid channel thickness of at least 50 μm, and transfers the image of an interior surface from the flow cell 701 to the image sensor 715 with dramatically improved optical image quality and higher CNR.

[0319] In some instances, the tube lens (or tube lens assembly) may comprise at least two optical lens elements, at least three optical lens elements, at least four optical lens elements, at least five optical lens elements, at least six optical lens elements, at least seven optical lens elements, at least eight optical lens elements, at least nine optical lens elements, at least ten optical lens elements, or more, where the number of optical lens elements, the surface geometry of each element, and the order in which they are placed in the assembly is improved or optimized to correct for optical aberrations induced by the thick wall of the flow cell, and in some instances, allows one to use a commercially-available, off-the-shelf objective while still maintaining high-quality, multiple-side imaging capability.

[0320] In some instances, as illustrated in FIG. 25, the tube lens assembly may comprise, in order, a first asymmetric convex-convex lens 711, a second convex-plano lens 712, a third asymmetric concave-concave lens 713, and a fourth asymmetric convex-concave lens 714.

[0321] FIG. 26 and FIG. 27 provide plots of the modulation transfer function as a function of spatial frequency for the upper (or near) interior surface (FIG. 26) and lower (or far) interior surface (FIG. 27) of a flow cell when imaged using the objective lens (corrected for a 0.17 mm coverslip) and tube lens combination illustrated in FIG. 25 through a 1.0 mm thick coverslip, and when the upper and lower interior surfaces are separated by a 0.1 mm thick layer of aqueous fluid. As can be seen, the imaging performance achieved is nearly that expected for a diffraction-limited optical design.

[0322] FIG. 28 provides ray tracing diagrams for tube lens design (left) of the present disclosure that has been improved or optimized to provide high-quality, multiple-side imaging performance. Because the tube lens is no longer infinity-corrected, an appropriately designed

null lens (right) may be used in combination with the tube lens to compensate for the non-infinity-corrected tube lens for manufacturing and testing purposes.

Imaging channel-specific tube lens adaptation or optimization

[0323] In imaging system design, it is possible to improve or optimize both the objective lens and the tube lens in the same wavelength region for all imaging channels. Typically, the same objective lens is shared by all imaging channels (see, for example, FIG. 18), and each imaging channel either uses the same tube lens or has a tube lens that shares the same design.

[0324] In some instances, the imaging systems disclosed herein may further comprise a tube lens for each imaging channel where the tube lens has been independently improved or optimized for the specific imaging channel to improve image quality, *e.g.*, to reduce or minimize distortion and field curvature, and improve depth-of-field (DOF) performance for each channel. Because the wavelength range (or bandpass) for each specific imaging channel is much narrower than the combined wavelength range for all channels, the wavelength- or channel-specific adaptation or optimization of the tube lens used in the disclosed systems results in significant improvements in imaging quality and performance. This channel-specific adaptation or optimization results in improved image quality for the multiple surfaces of the flow cell in multiple-side imaging applications.

Multiple surface imaging w/o fluid present in flow cell

[0325] For optimal imaging performance of the multiple surfaces of a flow cell, a motion-actuated compensator is typically required to correct for optical aberrations induced by the fluid in the flow cell (typically comprising a fluid layer thickness of about 50 – 200 μm). In some instances of the disclosed optical system designs, the first interior surface of the flow cell may be imaged with fluid present in the flow cell. Once the sequencing chemistry cycle has been completed, the fluid may be extracted from the flow cell for imaging of the surfaces below the first surface. Similarly, the fluid may be extracted from the flow cell from the first and second surfaces for imaging of the third surface. Thus, in some instances, even without the use of a compensator, the image quality for the lower surfaces can be maintained.

Compensation for optical aberration and/or vibration using electro-optical phase plates

[0326] In some instances, image quality may be improved without requiring the removal of the fluid from the flow cell by using an electro-optical phase plate (or other corrective lens) in combination with the objective to cancel the optical aberrations induced by the presence of the fluid. In some instances, the use of an electro-optical phase plate (or lens) may be used to remove the effects of vibration arising from the mechanical motion of a motion-actuated

compensator and may provide faster image acquisition times and sequencing cycle times for genomic sequencing applications.

Improved contrast-to-noise ratio (CNR), field-of-view (FOV), spectral separation, and timing design to increase or maximize information transfer and throughput

[0327] Another way to increase or maximize information transfer in imaging systems designed for genomics applications is to increase the size of the field-of-view (FOV) and reduce the time required to image a specific FOV. With typical large NA optical imaging systems, it may be common to acquire images for fields-of-view that are on the order of $1mm^2$ in area, where in the presently disclosed imaging system designs large FOV objectives with long working distances are specified to enable imaging of areas of 2 mm^2 or larger.

[0328] In some cases, the disclosed imaging systems are designed for use in combination with proprietary low-binding substrate surfaces and DNA amplification processes that reduce fluorescence background arising from a variety of confounding signals including, but are not limited to, nonspecific adsorption of fluorescent dyes to substrate surfaces, nonspecific nucleic acid amplification products (*e.g.*, nucleic acid amplification products that arise the substrate surface in areas between the spots or features corresponding to clonally-amplified clusters of nucleic acid molecules (*e.g.*, specifically amplified colonies), nonspecific nucleic acid amplification products that may arise within the amplified colonies, phased and pre-phased nucleic acid strands, *etc.* The use of low-binding substrate surfaces and DNA amplification processes that reduce fluorescence background in combination with the disclosed optical imaging systems may significantly cut down on the time required to image each FOV.

[0329] The presently disclosed system designs may further reduce the required imaging time through imaging sequence improvement or optimization where multiple channels of fluorescence images are acquired simultaneously or with overlapping timing, and where spectral separation of the fluorescence signals is designed to reduce cross-talks between fluorescence detection channels and between the excitation light and the fluorescence signal(s).

[0330] The presently disclosed system designs may further reduce the required imaging time through improvement or optimization of scanning motion sequence. In the typical approach, an X-Y translation stage is used to move the target FOV into position underneath the objective, an autofocus step is performed where optimal focal position is determined and the objective is moved in the Z direction to the determined focal position, and an image is acquired. A sequence of fluorescence images is acquired by cycling through a series of target FOV positions. From an information transfer duty cycle perspective, information is only transferred during the fluorescence image acquisition portion of the cycle. In the presently disclosed imaging system

designs, a single-step motion in which all axes (X-Y-Z) are repositioned simultaneously is performed, and the autofocus step is used to check focal position error. The additional Z motion is only commanded if the focal position error (*e.g.*, the difference between the focal plane position and the sample plane position) exceeds a certain limit (*e.g.*, a specified error threshold). Coupled with high speed X-Y motion, this approach increases the duty cycle of the system, and thus increases the imaging throughput per unit time.

[0331] In some embodiments, the system run time, e.g., to complete a sequencing analysis run, comprises imaging time and motion time of relative motion of the target FOV to the objective.

[0332] In some embodiments, the imaging time for scanning the target FOV can be doubled when multiple surface instead of single surfaces are imaged, and the imaging time can double again when quad surface instead of multiple surfaces are imaged. The motion time of moving the target FOV relative to the objective along the x, y, and/or z directions is increased when quad surface instead of multiple surface are imaged, but the increase is much less than doubled because motion time in x and y directions are not increased but only motion time in z direction is increased. Thus, the optical system herein can increase the duty cycle of the system and increase the imaging throughput per unit time. For example, the information is only transferred during the fluorescence image acquisition portion of the cycle, and the information is doubled by imaging quad surface in comparison to dual surface of the flow cell while the run time is much less than doubled because only the motion time in z direction is increased. The throughput of the system per unit time for imaging quad surface flow cell can be more than doubled when compared to the system throughput for imaging dual surface flow cells.

[0333] Furthermore, by matching the optical collection efficiency, modulation transfer function, and image sensor performance characteristics of the design with the fluorescence photon flux expected for the input excitation photon flux, dye efficiency (related to dye extinction coefficient and fluorescence quantum yield), while accounting for background signal and system noise characteristics, the time required to acquire high quality (high contrast-to-noise ratio (CNR) images) may be reduced or minimized.

[0334] The combination of efficient image acquisition and improved or optimized translation stage step and settle times leads to fast imaging times (*e.g.*, the overall time required per field-of-view) and higher throughput imaging system performance.

[0335] Along with the large FOV and fast image acquisition duty cycle, the disclosed designs may comprise also specifying image plane flatness, chromatic focus performance between fluorescence detection channels, sensor flatness, image distortion, and focus quality specifications.

[0336] Chromatic focus performance is further improved by individually aligning the image sensors for different fluorescence detection channels such that the best focal plane for each detection channel overlaps. The design goal is to ensure that images across more than 90 percent of the field-of-view are acquired within ± 100nm (or less) relative to the best focal plane for each channel, thus increasing or maximizing the transfer of individual spot intensity signals. In some instances, the disclosed designs further ensure that images across 99 percent of the field-of-view are acquired within ± 150nm (or less) relative to the best focal plane for each channel, and that images across more the entire field-of-view are acquired within ± 200nm (or less) relative to the best focal plane for each imaging channel.

Illumination optical path design

[0337] Another factor for improving signal-to-noise ratio (SNR), contrast-to-noise ratio (CNR), and/or increasing throughput is to increase illumination power density to the sample. In some instances, the disclosed imaging systems may comprise an illumination path design that utilizes a high-power laser or laser diode coupled with a liquid light guide. The liquid light guide removes optical speckle that is intrinsic to coherent light sources such as lasers and laser diodes. Furthermore, the coupling optics are designed in such a way as to underfill the entrance aperture of the liquid light guide. The underfilling of the liquid light guide entrance aperture reduces the effective numerical aperture of the illumination beam entering the objective lens, and thus improves light delivery efficiency through the objective onto the sample plane. With this design innovation, one can achieve illumination power densities up to 3x that for conventional designs over a large field-of-view (FOV).

[0338] By utilizing the angle-dependent discrimination of s- and p- polarization, in some instances, the illumination beam polarization may be orientated to reduce the amount of back-scattered and back-reflected illumination light that reaches the imaging sensors.

Illumination systems

[0339] In some instances, the disclosed imaging modules and systems may comprise a structured illumination optical design to increase the effective spatial resolution of the imaging system and thus enable the use of higher surface densities of clonally-amplified target nucleic acid sequences (clusters) on flow cell surfaces for improved sequencing throughput. Structured illumination microscopy (SIM) utilizes spatially structured (*e.g.*, periodic) patterns of light for illumination of the sample plane and relies on the generation of interference patterns known as Moiré fringes. Several images are acquired under slightly different illumination conditions, *e.g.*, by shifting and/or rotating the pattern of the structured illumination, to create the Moiré fringes.

Mathematical deconvolution of the resulting interference signal allows reconstruction of a super-resolution image having up to about a two-fold improvement in spatial resolution over that achieved using diffraction-limited imaging optics [Lutz (2011), "Biological Imaging by Superresolution Light Microscopy", Comprehensive Biotechnology (Second Ed.), vol. 1, pages 579-589, Elsevier; Feiner-Gracia, et al. (2018), "15 - Advanced Optical Microscopy Techniques for the Investigation of Cell-Nanoparticle Interactions", Smart Nanoparticles for Biomedicine: Micro and Nano Technologies, pages 219-236, Elsevier; Nylk, et al. (2019), "Light-Sheet Fluorescence Microscopy With Structured Light", Neurophotonics and Biomedical Spectroscopy, pages 477-501, Elsevier]. An example of structured illumination microscopy imaging systems has recently been described in Hong, U.S. Patent Application Publication No. 2020/0218052.

[0340] FIG. 41 provides a non-limiting schematic illustration of an imaging system 4100 comprising a branched structured illumination optical design as disclosed herein. The first branch (or arm) of the illumination optical path of system 4100 comprises, *e.g.*, a light source (light emitter) 4110A, an optical collimator 4120A to collimate light emitted by light source 4110A, a diffraction grating 4130A in a first orientation with respect to the optical axis, a rotating window 4140A, and a lens 4150A. The second branch of the illumination optical path of system 4100 comprises, *e.g.*, a light source 4110B, an optical collimator 4120B to collimate light emitted by light source 4110B, a diffraction grating 4130B in a second orientation with respect to the optical axis, a rotating window 4140B, and a lens 4150B. The diffraction gratings 4130A and 4130B enable projection of patterns of light fringes on the sample plane.

[0341] In some instances, the light sources 4110A and 4110B may be incoherent light sources (*e.g.*, comprising one or more light emitting diodes (LEDs)) or coherent light sources (*e.g.*, comprising one or more lasers or laser diodes). In some instances, the light sources 4110A and 4110B may comprise an optical fiber coupled to, *e.g.*, an LED, laser, or laser diode that outputs a light beam that is then collimated by the respective collimator lenses 4120A and 4120B. In some instances, light sources 4110A and 4110B may output light of the same wavelength. In some instances, light sources 4110A and 4110B may output light of different wavelengths. Either of light sources 4110A and 4110B may be configured to output light of any wavelength and/or wavelength range described elsewhere herein. During imaging, light sources 4110A and 4110B may be switched on or off using, for example, a high-speed shutter (not shown) positioned in the optical path or by pulsing the light sources at a predetermined frequency.

[0342] In the example shown in FIG. 41, the first illumination arm of system 4100 includes a fixed vertical grating 4130A used to project a grating pattern (*e.g.*, a vertical light fringe pattern) in a first orientation onto the sample plane, *e.g.*, a first interior surface 4188 of a flow cell 4187,

and the second illumination arm includes a fixed horizontal grating 4130B to project a grating pattern (*e.g.*, a horizontal light fringe pattern) in a second orientation onto the sample plane 4188. Advantageously, the diffraction gratings of imaging system 4100 do not need to be mechanically rotated or translated during imaging in this non-limiting example, which may provide improved imaging speed, system reliability, and system repeatability. In some instances, diffraction gratings 4130A and/or 4130B may be rotatable about their respective optical axes such that the angle between the light fringe patterns projected on the sample plane is adjustable.

[0343] As illustrated in FIG. 41, in some instances, diffraction gratings 4130A and 4130B may be transmissive diffraction gratings that comprise a plurality of diffracting elements (*e.g.*, parallel slits or grooves) formed in a glass substrate or other suitable surface. In some instances, the gratings may be implemented as phase gratings that provide a periodic variation of the refractive index of the grating material. In some instances, the groove or feature spacing may be chosen to diffract light at suitable angles and/or be tuned to the minimum resolvable feature size of the imaged samples for operation of imaging system 4100. In other instances, the diffraction gratings may be reflective diffraction gratings.

[0344] In the example illustrated in FIG. 41, the orientations of the vertical and horizontal light fringe patterns are offset by about 90 degrees. In other instances, other orientations of the diffraction gratings may be used to create an offset of about 90 degrees. For example, the diffraction gratings may be oriented such that they project light fringe patterns that are offset ±45 degrees from the x or y axes of sample plane (*e.g.*, first interior flow cell surface) 4188. The configuration of imaging system 4100 illustrated in FIG. 41 may be particularly advantageous in the case of a sample support surface (*e.g.*, an interior surface 4188 of a flow cell 4187) comprising regularly patterned features laid out on a rectangular grid, as enhancement of image resolution using the structured illumination approach can be achieved using only two perpendicular grating orientations (*e.g.*, the vertical grating orientation and horizontal grating orientation). The flow cell 4187 is not limited to having only two interior surfaces as shown in FIG. 41. In some embodiments, the flow cell 4187 can have one or more surfaces as shown in FIGS. 64A-64F.

[0345] Diffraction gratings 4130A and 4130B, in the example of system 4100, may be configured to diffract the input illumination light beams into a series of intensity maxima due to constructive interference according to the relationship:

$$m = \text{order number} = d \sin(\theta)/\lambda$$

[0346] where d = the distance between slits or grooves in the diffraction grating, θ = the angle of incidence of the illumination light relative to a normal to the surface of the diffraction grating, λ = the wavelength of the illumination light, and m = an integer value corresponding to an intensity maxima of the diffracted light, *e.g.*, m = 0, ±1, ±2, *etc.* In some instances, a specific order of the diffracted illumination light, *e.g.*, the first order (m = ±1) light may be projected on the sample plane, *e.g.*, interior flow cell surface 4188. In some instances, for example, vertical grating 4130A may diffract a collimated light beam into first order diffracted beams (±1 orders) which are focused onto the sample plane in a first orientation, and horizontal grating 4130B may diffract a collimated light beam into first order diffracted beams which are focused onto the sample plane in a second orientation. In some instances, the zeroth order beam and/or all other higher order beams (*e.g.*, m = ±2 or higher) may be blocked, *e.g.*, filtered out of the illumination pattern projected onto the sample plane 4188, using, for example, a beam blocking element (not shown) such as an order filter that may be inserted into the optical paths following the diffraction gratings.

[0347] Each branch of the illumination system in the example of 4100 includes an optical phase modulator or phase shifter 4140A and 4140B to phase shift the diffracted light transmitted or reflected by each of the diffraction gratings 4130A and 4130B. During structured imaging, the optical phase of each diffracted beam may be shifted by some fraction (*e.g.*, ½, ½, ¼, *etc.*) of the pitch (X) of each fringe of the structured pattern. In the example of FIG. 41, phase modulators 4140A and 4140B may be implemented, *e.g.*, as rotating optical phase plates actuated by rotatory actuators or other actuator mechanisms to rotate and modulate the optical path-length of each diffracted beam. For example, optical phase plate 4140A may be rotated about the vertical axis to shift the image projected by vertical grating 4130A on sample plane 4188 left or right, and optical phase plate 4140B may rotate about the horizontal axis to shift the image projected by horizontal grating 4130B on sample plane 4188 in the perpendicular direction.

[0348] In other implementations, other types of phase modulators that change the optical path length of the diffracted light (*e.g.*, optical wedges mounted on linear translation stages, *etc.*) may be used. Additionally, although optical phase modulators 4140A and 4140B are illustrated as being placed after diffraction gratings 4130A and 4130B, in other implementations they may be placed at other positions in the illumination optical path. In some instances, a single optical phase modulator may be operated in two different directions to produce different light fringe patterns, or the position of a single optical phase modulator may be adjusted using a single motion to simultaneously adjust the path lengths of both arms of the illumination optical path.

[0349] In the example illustrated in FIG. 41, optical component 4160 may be used to combine light from the two illumination optical paths. Optical component 4160 may comprise, for

example, a partially-silvered mirror, a dichroic mirror (depending on the wavelengths of light output by light sources 4110A and 4110B), a mirror comprising a pattern of holes or a patterned reflective coating such that light from the two arms of the illumination system are combined in a lossless or nearly lossless manner (*e.g.*, without significant loss of optical power other than a small amount of absorption by the reflective coating), a polarizing beam splitter (in the case that light sources 4110A and 4110B are configured to produce polarized light), and the like. Optical component 4160 may be located such that the desired diffracted orders of light reflected or transmitted by each of the diffraction gratings are spatially resolved, and the unwanted orders of light are blocked. In some instances, optical component 4160 may pass the first order light output by the first illumination light path and reflect the first order light output by the second illumination light path. In some instances, the structured illumination pattern on the sample surface 4188 may be switched from a vertical orientation (*e.g.*, using diffraction grating 4130A) to a horizontal orientation (*e.g.*, using diffraction grating 4130B) by turning each light source on or off, or by opening and closing an optical shutter in the optical path for the light source. In other instances, the structured illumination pattern may be switched by using an optical switch to change the illumination optical path used to illuminate the sample plane.

[0350] Referring again to FIG. 41, a lens 4170, a semi-reflective mirror or dichroic mirror 4180, and an objective 4185 may be used to focus the structured illumination light onto sample surface 4188 (*e.g.*, the first interior surface of a flow cell 4187). Light that is emitted by, reflected by, or scattered by the sample surface 4188 is then collected by objective 4185, transmitted through mirror 4180, and imaged by image sensor or camera 4195. As noted, mirror 4180 may be a dichroic mirror to reflect structured illumination light received from each branch of the illumination optical path into objective 4185 for projection onto sample plane 4188, and to pass through light emitted by the sample plane 4188 (*e.g.*, fluorescent light, which is emitted at different wavelengths than the excitation light) for imaging onto image sensor 4195.

[0351] In some instances, system 4100 may optionally comprise a custom tube lens 4190 as described elsewhere herein such that the focus of the imaging system may be shifted from the first interior surface 4188 to the second interior surface 4189 of the flow cell 4187, or to a third interior surface or a fourth interior surface (not shown), with minimal adjustment. In some instances, lens 4170 may comprise a custom tube lens as described elsewhere herein such that the focus of the illumination optical path may be shifted from the first interior surface 4188 to the second interior surface 4189 of the flow cell 4187, to a third surface or a fourth surface, with minimal adjustment. In some instances, lens 4170 may be implemented to articulate along the optical axis to adjust the focus of the structured illumination pattern on the sample plane. In some instances, system 4100 may comprise an autofocus mechanism (not shown) to adjust focus

of the illumination light and/or the focus of the image at the plane of image sensor 4195. In some instances, the system 4100 illustrated in FIG. 41 may provide a high optical efficiency due to the absence of a polarizer in the optical path. The use of unpolarized light may or may not have a significant impact on illumination pattern contrast depending on the numerical aperture of objective 4185.

[0352] For the sake of simplicity, some optical components of imaging system 4100 may have been omitted from FIG. 41 and the foregoing discussion. Although system 4100 is illustrated in this non-limiting example as a single channel detection system, in other instances, it may be implemented as a multi-channel detection system (*e.g.*, using two different image sensors and appropriate optics as well as light sources that emit at two different wavelengths). Furthermore, although the illumination optical path of system 4100 is illustrated in this non-limiting example as comprising two branches, in some instances it may be implemented as comprising, *e.g.*, three branches, four branches, or more than four branches, each of which comprises a diffraction grating at a fixed or adjustable relative orientation to each other.

[0353] In some instances, alternative illumination path optical designs may be used to create structured illumination. For example, in some instances, a single large, rotating optical phase modulator may be positioned after optical component 4160 and used in place of optical phase modulators 4140A and 4140B to modulate the phases of both diffracted beams output by the vertical and horizontal diffraction gratings 4130A and 4130B. In some instances, instead of being parallel with respect to the optical axis of one of the diffraction gratings, the axis of rotation for the single rotating optical compensator may be offset by 45 degrees (or another angular offset) from the optical axis of each of the vertical and horizontal diffraction gratings to allow for phase shifting along both illumination directions. In some instances, the single rotating optical phase modulator may be replaced by, *e.g.*, a wedged optical component rotating about the nominal beam axis.

[0354] In another alternative illumination optical path design, diffraction gratings 4130A and 4130B may be mounted on respective linear motion stages so that they may be translated to change the optical path length (and thus the phase) of light reflected or transmitted by diffraction gratings 4130A and 4130B. The axis of motion of the linear motion stages may be perpendicular or otherwise offset from the orientation of their respective diffraction grating to provide translation of the diffraction grating's fringe pattern along sample plane 4188. Suitable translation stages may comprise, *e.g.*, crossed roller bearing stages, a linear motor, a high-accuracy linear encoder, and/or other linear actuator technologies to provide precise linear translation of the diffraction gratings.

[0355] FIG. 42 provides a non-limiting example of a workflow for acquiring and processing images using structured illumination to enhance the spatial resolution of the imaging system. In some instances, the workflow illustrated in FIG. 42 may be performed to image an entire sample plane (*e.g.*, an interior surface of a flow cell by image tiling) or to image a single area of a larger sample plane. The vertical 4130A and horizontal 4130B diffraction gratings of the system 4100 illustrated in FIG. 41 may be used to project illumination light fringe patterns onto the sample plane that have different known orientations and/or different known phase shifts. For example, the imaging system 4100 may use vertical grating 4130A and horizontal grating 4130B to generate the horizontal and vertical illumination patterns respectively, while optical phase modulators 4140A and 4140B may be set to three different positions to produce the three phase shifts shown for each orientation.

[0356] During operation, a first illumination condition (*e.g.*, a specific orientation of the diffraction grating and phase shift setting) may be used to project a grating light fringe pattern on the sample plane, *e.g.*, flow cell surface. Following capture of an image using the first illumination condition, one or more additional images acquired using one or more phase shifted illumination patterns (*e.g.*, 1, 2, 3, 4, 5, 6, or more than 6 additional images acquired using 1, 2, 3, 4, 5, 6, or more than 6 phase shifted illumination patterns) may be acquired. If the imaging system comprises a second branch of the illumination optical path, the image acquisition process may be repeated using a second illumination condition as a starting point (*e.g.*, a second specific orientation of the diffraction grating and phase shift setting), and the image acquisition process may be repeated. In some instances, images may be acquired for at least three different orientations of the diffraction grating (*e.g.*, spaced apart by 60 degrees relative to each other) using at least 5 different phase shifted light fringe patterns. If no more images are to be acquired using different orientations of the diffraction grating or phase shifted illumination light fringe patterns, an image reconstruction algorithm may be used to process the acquired images and produce a reconstructed super-resolution image. In some instances, images may be acquired for at least 1, 2, 3, 4, 5, 6, or more than 6 different orientations of the diffraction grating using at least 1, 2, 3, 4, 5, 6, or more than 6 different phase-shifted light fringe patterns at each orientation.

[0357] A potential disadvantage of acquiring multiple images for use in reconstructing single, super- resolution images is the time required to adjust the orientation and/or relative phase shift of the projected light fringe patterns and the exposure time required for acquiring each image, as well as the downstream image processing. Therefore, optical designs that minimize the time required to change diffraction grating orientation and relative phase, along with highly efficient image reconstruction algorithms, are to be preferred. In some instances, fewer images may be

required to reconstruct super-resolution images of, *e.g.*, flow cell surfaces comprising discrete, fluorescently labeled clusters of amplified target nucleic acid sequences tethered to the low-nonspecific binding surfaces described elsewhere herein than may ordinarily be required for reconstructing higher resolution images of conventional samples, *e.g.*, stained tissue samples.

[0358] Referring again to FIG. 42, the afore-mentioned cycle may be repeated for different areas of a given flow cell surface, *e.g.*, in the case that the images will be tiled to create a higher resolution image of the entire flow cell surface. In some instances, the afore-mentioned cycle may be repeated after adjusting the focus of the imaging system if, *e.g.*, a second, third, or fourth flow cell surface is to be imaged.

Other super-resolution imaging techniques

[0359] In some instances, the disclosed imaging systems may comprise the use of an alternative super-resolution imaging technique, *e.g.*, photoactivation localization microscopy (PALM), fluorescence photoactivation localization microscopy (FPALM), and/or stochastic optical reconstruction microscopy (STORM) [see, for example, Lutz, *et al.* (2011), "Biological Imaging by Superresolution Light Microscopy", Comprehensive Biotechnology (Second Ed.), vol. 1, pages 579-589, Elsevier), which are based on statistical curve fitting of the intensity distribution observed in images of a single molecule's point spread function (PSF) to a Gaussian distribution function. The Gaussian distribution function is then used to define location of the molecule in the sample plane with much higher precision than allowed by the classical resolution limit. The same approach may be used to image, *e.g.*, small, dispersed subsets of fluorescently labeled molecules such as clonally amplified clusters of target nucleic acid sequences tethered to a low non-specific binding surface on a sample support or the interior surface of a flow cell.

[0360] The spatial accuracy or resolution achieved using these methods depends upon the number of photons collected from the molecule before it is photobleached and upon the background noise level [Lutz, *et al.* (2011), ibid]. In the case that background noise is negligible and collection of at least 10,000 photons per molecule is possible, position accuracies of 1–2 nm have been demonstrated. In some instances, *e.g.*, using the sequencing-by-avidity approach described elsewhere herein, nucleotide conjugates comprising a plurality of fluorescent labels (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 labels per conjugate) to ensure a high photon count, optionally used in combination with the low non-specific binding surfaces disclosed elsewhere herein to ensure very low background signals, may facilitate the use of these super-resolution imaging techniques for genetic testing and sequencing applications. Spatial accuracy or resolution decreases with decreasing numbers of photons collected, however, even in the case that only moderate numbers of photons are collected, position location accuracy or resolution of

20 nm is possible. In some cases, an improvement of 10-fold or better in lateral spatial resolution may be achieved. In some cases, an image resolution of better than 500 nm, 400 nm, 300 nm, 200 nm, 175 nm, 150 nm, 125 nm, 100 nm, 75 nm, 50 nm, 25 nm, or 10 nm may be achieved.

[0361] The second principle fundamental to this class of imaging is that small numbers of spatially separated fluorescent molecules within the sample are imaged at any given time.

[0362] In some instances, the ability to control fluorescence emission of small, dispersed subsets of fluorescent molecules in the sample plane is key to facilitating super-resolution imaging. In the case of fluorescence photoactivation localization microscopy (FPALM) and photoactivation localization microscopy (PALM), for example, the use of photoactivatable green fluorescent proteins (PA-GFP) as a label has allowed for controlled induction of fluorescent subsets in a sample using short pulses of 405 nm light to photo convert the PA-GFP from a dark, nonfluorescent state to a 488 nm excitable fluorescent state, thereby resulting in spatially separated subsets of fluorescent molecules that can be imaged [Lutz, *et al.* (2011), ibid]. In the case of stochastic optical reconstruction microscopy (STORM), the photo-switching properties of, for example, the cyanine dye pairs Cy5–Cy3 may be used in a similar fashion to enable the stochastic induction of Cy5 fluorescence from a small subset of the molecules in the sample at any given time, *e.g.*, small subsets of molecules that are spatially separated by at least several resolution units. In some instances, *e.g.*, when combined with the sequencing-by-avidity approach described elsewhere herein, nucleotide conjugates may comprise a photoactivatable green fluorescent protein (PA-GFP) or a subdomain or portion thereof. In some instances, the nucleotide conjugates may comprise a mixture of conjugates in which a first portion is labeled with, *e.g.*, Cy3 labels, and a second portion is labeled with, *e.g.*, Cy5 labels. In some instances, the nucleotide conjugates may comprise a mixture of, *e.g.*, Cy3 and Cy5 labels within the same conjugate.

[0363] The super-resolved image is reconstructed from the sum of the Gaussian fits from all molecules or features (*e.g.*, labeled nucleic acid clusters) imaged in a time stack of acquired images [Lutz, *et al.* (2011), ibid], where the intensity corresponds to the positional uncertainty of the location of each molecule or subset of molecules. Unique to this kind of data set is the ability to render the image with different localization precisions or resolutions. In some instances, an imaging module comprising a total internal reflectance fluorescence (TIRF) optical imaging design may be advantageous in implementing the use of these super-resolution imaging techniques as the evanescent wave used for excitation of fluorescence is restricted in the axial dimension to less than 200 nm from the sample support or flow cell surface and thus suppresses background fluorescence signal. In some instances, the imaging system may comprise a higher

numerical aperture objective than utilized in other imaging module designs disclosed herein. The use of higher numerical aperture objectives may facilitate implementation of evanescent wave excitation and highly efficient capture of photons from the fluorescent probes. In some instances, wide-field imaging using single-photon-sensitive EM-CCD cameras or other types of image sensors may enable simultaneous imaging of many molecules or subsets of molecules (*e.g.*, nucleic acid sequence clusters) per frame, thereby improving the throughput of image acquisition.

[0364] In some instances, the data acquisition time required to acquire enough images for adequate feature definition and resolution may be shortened by improvements in the sensitivity and speed of the imaging system, through the use of the sequencing-by-avidity reagents and low non-specific binding surfaced disclosed herein to increase signal while reducing or eliminating background, and the use of improved image reconstruction algorithms.

Assessing image quality

[0365] For any of the embodiments of the optical imaging designs disclosed herein, imaging performance or imaging quality may be assessed using any of a variety of performance metrics known to those of skill in the art. Examples include, but are not limited to, measurements of modulation transfer function (MTF) at one or more specified spatial frequencies, defocus, spherical aberration, chromatic aberration, coma, astigmatism, field curvature, image distortion, contrast-to-noise ratio (CNR), or any combination thereof.

[0366] In some instances, the disclosed optical designs for dual-side or quad-side surface imaging (*e.g.*, the disclosed objective lens designs, tube lens designs, the use of an electro-optical phase plate in combination with an objective, *etc.*, alone or in combination) may yield significant improvements for image quality for all interior surfaces of a flow cell, such that the difference in an imaging performance metric for imaging the multiple surfaces, e.g., the first, second, third, or fourth surface of the flow cell is less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% for any of the imaging performance metrics listed above, either individually or in combination.

[0367] In some instances, the disclosed optical designs for multiple-side imaging (*e.g.*, comprising the disclosed tube lens designs, the use of an electro-optical phase plate in combination with an objective, *etc.*) may yield significant improvements for image quality. In some embodiments, an image quality performance metric for multiple-side imaging provides for an at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, or at least 30% improvement for multiple-side imaging compared to dual-side imaging of a conventional system comprising, *e.g.*, an objective lens, a motion-

actuated compensator (that is moved out of or into the optical path when imaging the near surface, e.g., the first surface or far interior surfaces, e.g., the second surface, of a flow cell), and an image sensor for any of the imaging performance metrics listed above, either individually or in combination. In some instances, fluorescence imaging systems comprising one or more of the disclosed tube lens designs provides for an at least equivalent or better improvement in an imaging performance metric for multiple-side imaging compared to that for a conventional system comprising an objective lens, a motion-actuated compensator, and an image sensor. In some instances, fluorescence imaging systems comprising one or more of the disclosed tube lens designs provides for an at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% improvement in an imaging performance metric for multiple-side imaging compared to that for a conventional system comprising an objective lens, a motion-actuated compensator, and an image sensor.

Imaging module specifications

[0368] *Excitation light wavelength(s):* In any of the disclosed optical imaging module designs, or optical system designs, the light source(s) of the disclosed imaging modules may produce visible light, such as green light and/or red light. the light source(s) of the disclosed imaging modules may produce visible light, such as blue light. In some instances, the light source(s), alone or in combination with one or more optical components, *e.g.*, excitation optical filters and/or dichroic beam splitters, may produce excitation light at about 350 nm, 375 nm, 400 nm, 425 nm, 450 nm, 475 nm, 500 nm, 525 nm, 550 m, 575 nm, 600 nm, 625 nm, 650 nm, 675 nm, 700 nm, 725 nm, 750 nm, 775 nm, 800 nm, 825 nm, 850 nm, 875 nm, or 900 nm. Those of skill in the art will recognize that the excitation wavelength may have any value within this range, *e.g.*, about 620 nm. In some instances, the light source(s), alone or in combination with one or more optical components, *e.g.*, excitation optical filters and/or dichroic beam splitters, may produce excitation light with a single wave length in a range from 300 nm to 600 nm, from 400 to 500 nm, from 450 to 500 nm, from 420 to 520 nm, or from 350 to 850 nm. In some instances, the light source(s), alone or in combination with one or more optical components, *e.g.*, excitation optical filters and/or dichroic beam splitters, may produce excitation light with a single wave length in a range from 300 nm to 600 nm, from 400 to 500 nm, from 450 to 500 nm, from 420 to 520 nm, or from 350 to 850 nm, with a wavelength bandwidth of ± 2 nm, ± 5 nm, ± 10 nm, ± 20 nm, ± 40 nm, ± 80 nm, or greater. For example, the light source(s) may include a blue light with a wavelength of 460 nm, with a wavelength range of ± 5 nm.

[0369] *Excitation light bandwidths:* In any of the disclosed optical imaging module designs or optical system designs, the light source(s), alone or in combination with one or more optical

components, *e.g.*, excitation optical filters and/or dichroic beam splitters, may produce light at the specified excitation wavelength within a bandwidth of ± 2 nm, ± 5 nm, ± 10 nm, ± 20 nm, ± 40 nm, ± 80 nm, or greater. Those of skill in the art will recognize that the excitation bandwidths may have any value within this range, *e.g.*, about ± 18 nm.

[0370] *Light source power output:* In any of the disclosed optical imaging module designs, the output of the light source(s) and/or an excitation light beam derived therefrom (including a composite excitation light beam) may range in power from about 0.5 Watts to about 5.0 Watts, or more (as will be discussed in more detail below). In some instances, the output of the light source and/or the power of an excitation light beam derived therefrom may be at least 0.5 Watts, at least 0.6 Watts, at least 0.7 Watts, at least 0.8 Watts, at least 1 Watts, at least 1.1 Watts, at least 1.2 Watts, at least 1.3 Watts, at least 1.4 Watts, at least 1.5 Watts, at least 1.6 Watts, at least 1.8 Watts, at least 2.0 Watts, at least 2.2 Watts, at least 2.4 Watts, at least 2.6 Watts, at least 2.8 Watts, at least 3.0 Watts, at least 3.5 Watts, at least 4.0 Watts, at least 4.5 Watts, or at least 5.0 Watts. In some implementations, the output of the light source and/or the power of an excitation light beam derived therefrom (including a composite excitation light beam) may be at most 5.0 Watts, at most 4.5 Watts, at most 4.0 Watts, at most 3.5 Watts, at most 3.0 Watts, at most 2.8 Watts, at most 2.6 Watts, at most 2.4 Watts, at most 2.2 Watts, at most 2.0 Watts, at most 1.8 Watts, at most 1.6 Watts, at most 1.5 Watts, at most 1.4 Watts, at most 1.3 Watts, at most 1.2 Watts, at most 1.1 Watts, at most 1 Watts, at most 0.8 Watts, at most 0.7 Watts, at most 0.6 Watts, or at most 0.5 Watts. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the output of the light source and/or the power of an excitation light beam derived therefrom (including a composite excitation light beam) may range from about 0.8 Watts to about 2.4 Watts. Those of skill in the art will recognize that the output of the light source and/or the power of an excitation light beam derived therefrom (including a composite excitation light beam) may have any value within this range, *e.g.*, about 1.28 Watts.

[0371] *Light source output power and CNR:* In some implementations of the disclosed optical imaging module designs, the output power of the light source(s) and/or the power of excitation light beam(s) derived therefrom (including a composite excitation light beam) is sufficient, in combination with an appropriate sample, to provide for a contrast-to-noise ratio (CNR) in images acquired by the illumination and imaging module of at least 5, at least 10, at least 15, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 30, at least 35, at least 40, or at least 50 or more, or any CNR within any range formed by any of these values.

[0372] In some embodiment, the light source herein can include an illumination system that can illuminate a wide FOV, e.g., 60 mm^2, with uniformity in its illumination power, e.g., less than 10% variance or difference across the illuminated area. Exemplary illumination systems and corresponding illumination uniformity are disclosed in PCT Application PCT/US24/12802 and are incorporated herein by reference in its entirety.

[0373] *Fluorescence emission bands:* In some instances, the disclosed fluorescence optical imaging modules may be configured to detect fluorescence emission produced by any of a variety of fluorophores known to those of skill in the art. Examples of suitable fluorescence dyes for use in, *e.g.*, genotyping and nucleic acid sequencing applications (*e.g.*, by conjugation to nucleotides, oligonucleotides, or proteins) include, but are not limited to, fluorescein, rhodamine, coumarin, cyanine, and derivatives thereof, including the cyanine derivatives cyanine dye-3 (Cy3), cyanine dye-5 (Cy5), cyanine dye-7 (Cy7), *etc.*

[0374] *Fluorescence emission wavelengths:* In any of the disclosed optical imaging module designs or optical system designs, the detection channel or imaging channel of the disclosed optical systems may include one or more optical components, *e.g.*, emission optical filters and/or dichroic beam splitters, configured to collect emission light at about 350 nanometer (nm), 375 nm, 400 nm, 425 nm, 450 nm, 475 nm, 500 nm, 525 nm, 550 m, 575 nm, 600 nm, 625 nm, 650 nm, 675 nm, 700 nm, 725 nm, 750 nm, 775 nm, 800 nm, 825 nm, 850 nm, 875 nm, or 900 nm. In some embodiments, the emission light can be in a range from 500 nm to 750 nm, from 400 to 1200 nm, or from 450 to 850 nm. Those of skill in the art will recognize that the emission wavelength may have any value within this range, *e.g.*, about 825 nm.

[0375] *Fluorescence emission light bandwidths:* In any of the disclosed optical imaging module designs or optical system designs, the detection channel or imaging channel may comprise one or more optical components, *e.g.*, emission optical filters and/or dichroic beam splitters, configured to collect light at the specified emission wavelength within a bandwidth of ± 2 nm, ± 5 nm, ± 10 nm, ± 20 nm, ± 40 nm, ± 80 nm, or greater. Those of skill in the art will recognize that the excitation bandwidths may have any value within this range, *e.g.*, about ± 18 nm.

[0376] *Numerical aperture:* In some instances, the numerical aperture of the objective lens and/or optical imaging module (*e.g.*, comprising an objective lens and/or tube lens) in any of the disclosed optical system designs may range from about 0.1 to about 1.4. In some instances, the numerical aperture may be at least 0.1, at least 0.2, at least 0.3, at least 0.4, at least 0.5, at least 0.6, at least 0.7, at least 0.8, at least 0.9, at least 1.0, at least 1.1, at least 1.2, at least 1.3, or at least 1.4. In some instances, the numerical aperture may be at most 1.4, at most 1.3, at most 1.2, at most 1.1, at most 1.0, at most 0.9, at most 0.8, at most 0.7, at most 0.6, at most 0.5, at most

0.4, at most 0.3, at most 0.2, or at most 0.1. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the numerical aperture may range from about 0.1 to about 0.6. Those of skill in the art will recognize that the numerical aperture may have any value within this range, *e.g.*, about 0.55.

[0377] *Optical resolution:* In some instances, depending on the numerical aperture of the objective lens and/or optical system (*e.g.*, comprising an objective lens and/or tube lens), the minimum resolvable spot (or feature) separation distance at the sample plane achieved by any of the disclosed optical system designs may range from about 0.5 μm to about 2 μm. In some instances, the minimum resolvable spot separation distance at the sample plane may be at least 0.5 μm, at least 0.6 μm, at least 0.7 μm, at least 0.8 μm, at least 0.9 μm, at least 1.0 μm, at least 1.2 μm, at least 1.4 μm, at least 1.6 μm, at least 1.8 μm, or at least 1.0 μm. In some instances, the minimum resolvable spot separation distance may be at most 2.0 μm, at most 1.8 μm, at most 1.6 μm, at most 1.4 μm, at most 1.2 μm, at most 1.0 μm, at most 0.9 μm, at most 0.8 μm, at most 0.7 μm, at most 0.6 μm, or at most 0.5 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the minimum resolvable spot separation distance may range from about 0.8 μm to about 1.6 μm. Those of skill in the art will recognize that the minimum resolvable spot separation distance may have any value within this range, *e.g.*, about 0.95 μm.

[0378] In some instances, the use of the novel illumination system and other designs of the optical system disclosed herein, in any of the optical modules or systems disclosed herein, may confer comparable optical resolution for the multiple surfaces (*e.g.* the first, second, third, and/or fourth interior surfaces of a flow cell) with or without the need to refocus between acquiring the images of the surfaces. In some instances, the optical resolution of the images thus obtained of the surfaces may be within 20%, 18%, 16%, 14%, 12%, 10%, 8%, 6%, 4%, 2%, or 1% of each other, or within any value within this range.

[0379] *Magnification:* In some instances, the magnification of the objective lens and/or tube lens, and/or optical system (*e.g.*, comprising an objective lens and/or tube lens) in any of the disclosed optical configurations may range from about 2x to about 20x. In some instances, the optical system magnification may be at least 2x, at least 3x, at least 4x, at least 5x, at least 6x, at least 7x, at least 8x, at least 9x, at least 10x, at least 15x, or at least 20x. In some instances, the optical system magnification may be at most 20x, at most 15x, at most 10x, at most 9x, at most 8x, at most 7x, at most 6x, at most 5x, at most 4x, at most 3x, or at most 2x. Any of the lower and upper values described in this paragraph may be combined to form a range included within

the present disclosure, for example, in some instances the optical system magnification may range from about 3x to about 10x. Those of skill in the art will recognize that the optical system magnification may have any value within this range, *e.g.*, about 7.5x.

[0380] *Objective lens focal length:* In some implementations of the disclosed optical designs, the focal length of the objective lens may range between 20 mm and 40 mm. In some instances, the focal length of the objective lens may be at least 20 mm, at least 25 mm, at least 30 mm, at least 35 mm, or at least 40 mm. In some instances, the focal length of the objective lens may be at most 40 mm, at most 35 mm, at most 30 mm, at most 25 mm, or at most 20 mm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the focal length of the objective lens may range from 25 mm to 35 mm. Those of skill in the art will recognize that the focal length of the objective lens may have any value within the range of values specified above, *e.g.*, about 37 mm.

[0381] *Objective lens working distance:* In some implementations of the disclosed optical designs, the working distance of the objective lens may range between about 100 μm and 30 mm. In some instances, the working distance may be at least 100 μm, at least 200 μm, at least 300 μm, at least 400 μm, at least 500 μm, at least 600 μm, at least 700 μm, at least 800 μm, at least 900 μm, at least 1 mm, at least 2 mm, at least 4 mm, at least 6 mm, at least 8 mm, at least 10 mm, at least 15 mm, at least 20 mm, at least 25 mm, or at least 30 mm. In some instances, the working distance may be at most 30 mm, at most 25 mm, at most 20 mm, at most 15 mm, at most 10 mm, at most 8 mm, at most 6 mm, at most 4 mm, at most 2 mm, at most 1 mm, at most 900 μm, at most 800 μm, at most 700 μm, at most 600 μm, at most 500 μm, at most 400 μm, at most 300 μm, at most 200 μm, at most 100 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the working distance of the objective lens may range from 500 μm to 2 mm. Those of skill in the art will recognize that the working distance of the objective lens may have any value within the range of values specified above, *e.g.*, about 1.25 mm.

[0382] *Objectives optimized for imaging through thick coverslips:* In some instances of the disclosed optical designs, the design of the objective lens may be improved or optimized for a different coverslip of flow cell thickness. For example, in some instances the objective lens may be designed for optimal optical performance for a coverslip that is from about 200 μm to about 1,000 μm thick. In some instances, the objective lens may be designed for optimal performance with a coverslip that is at least 200 μm, at least 300 μm, at least 400 μm, at least 500 μm, at least 600 μm, at least 700 μm, at least 800 μm, at least 900 μm, or at least 1,000 μm thick. In some

instances, the objective lens may be designed for optimal performance with a coverslip that is at most 1,000 μm, at most 900 μm, at most 800 μm, at most 700 μm, at most 600 μm, at most 500 μm, at most 400 μm, at most 300 μm, or at most 200 μm thick. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the objective lens may be designed for optimal optical performance for a coverslip that may range from about 300 μm to about 900 μm. Those of skill in the art will recognize that the objective lens may be designed for optimal optical performance for a coverslip that may have any value within this range, *e.g.*, about 725 μm.

[0383] *Depth of field and depth of focus:* In some instances, the depth of field and/or depth of focus for any of the disclosed imaging module (*e.g.*, comprising an objective lens and/or tube lens) designs may range from about 10 μm to about 800 μm, or more. In some instances, the depth of field and/or depth of focus may be at least 10 μm, at least 20 μm, at least 30 μm, at least 40 μm, at least 50 μm, at least 75 μm, at least 100 μm, at least 125 μm, at least 150 μm, at least 175 μm, at least 200 μm, at least 250 μm, at least 300 μm, at least 300 μm, at least 400 μm, at least 500 μm, at least 600 μm, at least 700 μm, or at least 800 μm, or more. In some instances, the depth of field and/or depth of focus be at most 800 μm, at most 700 μm, at most 600 μm, at most 500 μm, at most 400 μm, at most 300 μm, at most 250 μm, at most 200 μm, at most 175 μm, at most 150 μm, at most 125 μm, at most 100 μm, at most 75 μm, at most 50 μm, at most 40 μm, at most 30 μm, at most 20 μm, at most 10 μm, or less. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the depth of field and/or depth of focus may range from about 100 μm to about 175 μm. Those of skill in the art will recognize that the depth of field and/or depth of focus may have any value within the range of values specified above, *e.g.*, about 132 μm.

[0384] *Field-of-view (FOV):* In some implementations, the FOV of any of the disclosed imaging module designs or illumination system designs (*e.g.*, that provided by a combination of objective lens and detection channel optics (such as a tube lens)) may range, for example, between about 1 mm and 5 mm (*e.g.*, in diameter, width, length, or longest dimension). In some instances, the FOV may be at least 1.0 mm, at least 1.5 mm, at least 2.0 mm, at least 2.5 mm, at least 3.0 mm, at least 3.5 mm, at least 4.0 mm, at least 4.5 mm, or at least 5.0 mm (*e.g.*, in diameter, width, length, or longest dimension). In some instances, the FOV may be at most 5.0 mm, at most 4.5 mm, at most 4.0 mm, at most 3.5 mm, at most 3.0 mm, at most 2.5 mm, at most 2.0 mm, at most 1.5 mm, or at most 1.0 mm (*e.g.*, in diameter, width, length, or longest dimension). Any of the lower and upper values described in this paragraph may be combined to

form a range included within the present disclosure, for example, in some instances the FOV may range from about 1.5 mm to about 3.5 mm (*e.g.*, in diameter, width, length, or longest dimension). Those of skill in the art will recognize that the FOV may have any value within the range of values specified above, *e.g.*, about 3.2 mm (*e.g.*, in diameter, width, length, or longest dimension).

[0385] In some instances of the disclosed optical system designs, the area of the field-of-view may range from about 2 mm^2 to about 5 mm^2. In some embodiments, the area of the field-of-view may range from 1 mm^2 to 10 mm^2. In some embodiments, the area of the field-of-view may range from 1 mm^2 to 200 mm^2. In some embodiments, the area of the field-of-view may range from 4 mm^2 to 80 mm^2. In some instances, the field-of-view may be at least 2 mm^2, at least 3 mm^2, at least 4 mm^2, or at least 5 mm^2 in area. In some instances, the field-of-view may be at least 5 mm^2, at least 10 mm^2, at least 20 mm^2, or at least 50 mm^2 in area. In some instances, the field-of-view may be at most 5 mm^2, at most 4 mm^2, at most 3 mm^2, or at most 2 mm^2 in area. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the field-of-view may range from about 5 mm^2 to about 10 mm^2 in area. Those of skill in the art will recognize that the area of the field-of-view may have any value within this range, *e.g.*, 2.75 mm^2.

[0386] *Optimization of objective lens and/or tube lens MTF:* In some instances, the design of the objective lens and/or at least one tube lens in the disclosed imaging modules and systems is configured to optimize the modulation transfer function in the mid to high spatial frequency range. For example, in some instances, the design of the objective lens and/or at least one tube lens in the disclosed imaging modules and systems is configured to optimize the modulation transfer function in the spatial frequency range from 500 cycles per mm to 900 cycles per mm, from 700 cycles per mm to 1100 cycles per mm, from 800 cycles per mm to 1200 cycles per mm, or from 600 cycles per mm to 1000 cycles per mm in the sample plane.

[0387] *Optical aberration and diffraction-limited imaging performance:* In some implementations of any of the optical imaging module designs disclosed herein, the objective lens and/or tube lens may be configured to provide the imaging module with a field-of-view as indicated above such that the FOV has less than 0.15 waves of aberration over at least 60%, 70%, 80%, 90%, or 95% of the field. In some implementations, the objective lens and/or tube lens may be configured to provide the imaging module with a field-of-view as indicated above such that the FOV has less than 0.1 waves of aberration over at least 60%, 70%, 80%, 90%, or 95% of the field. In some implementations, the objective lens and/or tube lens may be

configured to provide the imaging module with a field-of-view as indicated above such that the FOV has less than 0.075 waves of aberration over at least 60%, 70%, 80%, 90%, or 95% of the field. In some implementations, the objective lens and/or tube lens may be configured to provide the imaging module with a field-of-view as indicated above such that the FOV is diffraction-limited over at least 60%, 70%, 80%, 90%, or 95% of the field.

[0388] *Angle of incidence of light beams on dichroic reflectors, beam splitter, and beam combiners:* In some instances of the disclosed optical designs, the angles of incidence for a light beam incident on a dichroic reflector, beam splitter, or beam combiner may range between about 20 degrees and about 45 degrees. In some instances, the angles of incidence may be at least 20 degrees, at least 25 degrees, at least 30 degrees, at least 35 degrees, at least 40 degrees, or at least 45 degrees. In some instances, the angles of incidence may be at most 45 degrees, at most 40 degrees, at most 35 degrees, at most 30 degrees, at most 25 degrees, or at most 20 degrees. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the angles of incidence may range from about 25 degrees to about 40 degrees. Those of skill in the art will recognize that the angles of incidence may have any value within the range of values specified above, *e.g.*, about 43 degrees.

[0389] *Image sensor (photodetector array) size:* In some embodiments, the disclosed optical systems may comprise image sensor(s) having an active area that is identical to the FOVs disclosed herein. In some embodiments, the disclosed optical systems may comprise image sensor(s) having an active area that is no less than the area of the FOV being acquired using the image sensor.

[0390] In some instances, the disclosed optical systems may comprise image sensor(s) having an active area with a diagonal ranging from about 10 mm to about 30 mm, or larger. In some instances, the image sensors may have an active area with a diagonal of at least 10 mm, at least 12 mm, at least 14 mm, at least 16 mm, at least 18 mm, at least 20 mm, at least 22 mm, at least 24 mm, at least 26 mm, at least 28 mm, or at least 30 mm. In some instances, the image sensors may have an active area with a diagonal of at most 30 mm, at most 28 mm, at most 26 mm, at most 24 mm, at most 22 mm, at most 20 mm, at most 18 mm, at most 16 mm, at most 14 mm, at most 12 mm, or at most 10 mm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the image sensor(s) may have an active area with a diagonal ranging from about 12 mm to about 24 mm. Those of skill in the art will recognize that the image sensor(s) may have

an active area with a diagonal having any value within the range of values specified above, *e.g.*, about 28.5 mm.

[0391] *Image sensor pixel size and pitch:* In some instances, the pixel size and/or pitch selected for the image sensor(s) used in the disclosed optical system designs may range in at least one dimension from about 1 μm to about 10 μm. In some instances, the pixel size and/or pitch may be at least 1 μm, at least 2 μm, at least 3 μm, at least 4 μm, at least 5 μm, at least 6 μm, at least 7 μm, at least 8 μm, at least 9 μm, or at least 10 μm. In some instances, the pixel size and/or pitch may be at most 10 μm, at most 9 μm, at most 8 μm, at most 7 μm, at most 6 μm, at most 5 μm, at most 4 μm, at most 3 μm, at most 2 μm, or at most 1 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the pixel size and/or pitch may range from about 3 μm to about 9 μm. Those of skill in the art will recognize that the pixel size and/or pitch may have any value within this range, *e.g.*, about 1.4 μm. In some embodiments, the pixel may include a width, length, diagonal of less than 3 um, 2 um, 1 um, 0.8 um, 0.6 um, 0.5 um, 0.4 um, 0.3 um, or 0.2 um.

[0392] *Oversampling:* In some instances of the disclosed optical designs, a spatial oversampling scheme is utilized wherein the spatial sampling frequency is at least 2x, 2.5x, 3x, 3.5x, 4x, 4.5x, 5x, 6x, 7x, 8x, 9x, or 10x the optical resolution X (lp/mm).

[0393] *Maximum translation stage velocity:* In some instances of the disclosed optical imaging modules, the maximum translation stage velocity on any one axis may range from about 1 mm/sec to about 5 mm/sec. In some instances, the maximum translation stage velocity may be at least 1 mm/sec, at least 2 mm/sec, at least 3 mm/sec, at least 4 mm/sec, or at least 5 mm/sec. In some instances, the maximum translation stage velocity may be at most 5 mm/sec, at most 4 mm/sec, at most 3 mm/sec, at most 2 mm/sec, or at most 1 mm/sec. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the maximum translation stage velocity may range from about 2 mm/sec to about 4 mm/sec. Those of skill in the art will recognize that the maximum translation stage velocity may have any value within this range, *e.g.*, about 2.6 mm/sec.

[0394] *Maximum translation stage acceleration:* In some instances of the disclosed optical imaging modules, the maximum acceleration on any one axis of motion may range from about 2 mm/sec^2 to about 10 mm/sec^2. In some instances, the maximum acceleration may be at least 2 mm/sec^2, at least 3 mm/sec^2, at least 4 mm/sec^2, at least 5 mm/sec^2, at least 6 mm/sec^2, at least 7 mm/sec^2, at least 8 mm/sec^2, at least 9 mm/sec^2, or at least 10 mm/sec^2. In some instances, the

maximum acceleration may be at most 10 mm/sec^2, at most 9 mm/sec^2, at most 8 mm/sec^2, at most 7 mm/sec^2, at most 6 mm/sec^2, at most 5 mm/sec^2, at most 4 mm/sec^2, at most 3 mm/sec^2, or at most 2 mm/sec^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the maximum acceleration may range from about 2 mm/sec^2 to about 8 mm/sec^2. Those of skill in the art will recognize that the maximum acceleration may have any value within this range, *e.g.*, about 3.7 mm/sec^2.

[0395] *Translation stage positioning repeatability:* In some instances of the disclosed optical imaging modules, the repeatability of positioning for any one axis may range from about 0.1 μm to about 2 μm. In some instances, the repeatability of positioning may be at least 0.1 μm, at least 0.2 μm, at least 0.3 μm, at least 0.4 μm, at least 0.5 μm, at least 0.6 μm, at least 0.7 μm, at least 0.8 μm, at least 0.9 μm, at least 1.0 μm, at least 1.2 μm, at least 1.4 μm, at least 1.6 μm, at least 1.8 μm, or at least 2.0 μm. In some instances, the repeatability of positioning may be at most 2.0 μm, at most 1.8 μm, at most 1.6 μm, at most 1.4 μm, at most 1.2 μm, at most 1.0 μm, at most 0.9 μm, at most 0.8 μm, at most 0.7 μm, at most 0.6 μm, at most 0.5 μm, at most 0.4 μm, at most 0.3 μm, at most 0.2 μm, or at most 0.1 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the repeatability of positioning may range from about 0.3 μm to about 1.2 μm. Those of skill in the art will recognize that the repeatability of positioning may have any value within this range, *e.g.*, about 0.47 μm.

[0396] *FOV repositioning time:* In some instances of the disclosed optical imaging modules, the maximum time required to reposition the sample plane (field-of-view) relative to the optics, or *vice versa*, may range from about 0.1 sec to about 0.5 sec. In some instances, the maximum repositioning time (*e.g.*, the scan stage step and settle time) may be at least 0.1 sec, at least 0.2 sec, at least 0.3 sec, at least 0.4 sec, or at least 0.5 sec. In some instances, the maximum repositioning time may be at most 0.5 sec, at most 0.4 sec, at most 0.3 sec, at most 0.2 sec, or at most 0.1 sec. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the maximum repositioning time may range from about 0.2 sec to about 0.4 sec. Those of skill in the art will recognize that the maximum repositioning time may have any value within this range, *e.g.*, about 0.45 sec.

[0397] In some embodiments, the maximum time required to reposition the sample plane (FOV or target FOV) relative to the optics or vice versa may include motion in the x, y, and/or z

directions. The reposition time in z direction, from surface to surface, of a distance, can be smaller than the reposition time in x or y direction, of the same distance.

[0398] In some embodiments, the total reposition time for imaging four axially displaced surfaces is less than double of the total reposition time for imaging existing dual surface flow cells because only the reposition time in z direction is doubled but the reposition time in x and y direction remain unaltered.

[0399] *Error threshold for autofocus correction:* In some instances of the disclosed optical imaging modules, the specified error threshold for triggering an autofocus correction may range from about 50 nm to about 200 nm. In some instances, the error threshold may be at least 50 nm, at least 75 nm, at least 100 nm, at least 125 nm, at least 150 nm, at least 175 nm, or at least 200 nm. In some instances, the error threshold may be at most 200 nm, at most 175 nm, at most 150 nm, at most 125 nm, at most 100 nm, at most 75 nm, or at most 50 nm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the error threshold may range from about 75 nm to about 150 nm. Those of skill in the art will recognize that the error threshold may have any value within this range, *e.g.*, about 105 nm.

[0400] *Image acquisition time:* In some instances of the disclosed optical imaging modules, the image acquisition time may range from about 0.001 sec to about 1 sec. In some instances, the image acquisition time may be at least 0.001 sec, at least 0.01 sec, at least 0.1 sec, or at least 1 sec. in some instances, the image acquisition time may be at most 1 sec, at most 0.1 sec, at most 0.01 sec, or at most 0.001 sec. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the image acquisition time may range from about 0.01 sec to about 0.1 sec. Those of skill in the art will recognize that the image acquisition time may have any value within this range, *e.g.*, about 0.250 seconds.

[0401] *Imaging time per FOV:* In some instances, the imaging times may range from about 0.5 seconds to about 3 seconds per field-of-view. In some instances, the imaging time within a sequencing cycle (i.e., imaging cycle time) may range from 0.1 seconds to 60 seconds per field-of-view. In some instances, the imaging time within a sequencing cycle (i.e., imaging cycle time) may range from 0.5 seconds to 30 seconds per field-of-view. In some instances, the imaging time within a sequencing cycle (i.e., imaging cycle time) may range from 0.5 seconds to 20 seconds per field-of-view. In some instances, the imaging time within a sequencing cycle (i.e., imaging cycle time) may range from 0.5 seconds to 10 seconds per field-of-view. In some instances, the imaging time may be at least 0.5 seconds, at least 1 second, at least 1.5 seconds, at

least 2 seconds, at least 2.5 seconds, or at least 3 seconds per FOV. In some instances, the imaging time may be at most 3 seconds, at most 2.5 seconds, at most 2 seconds, at most 1.5 seconds, at most 1 second, or at most 0.5 seconds per FOV. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the imaging time may range from about 1 second to about 2.5 seconds. Those of skill in the art will recognize that the imaging time may have any value within this range, *e.g.*, about 1.85 seconds.

[0402] *Flatness of field:* In some instances, images across 80%, 90%, 95%, 98%, 99%, or 100% percent of the field-of-view are acquired within ± 200 nm, ± 175 nm, ± 150 nm, ± 125 nm, ± 100 nm, ± 75 nm, or ± 50 nm relative to the best focal plane for each fluorescence (or other imaging mode) detection channel.

[0403] *Systems and system components for genomics and other applications:* As noted above, in some implementations, the disclosed optical imaging modules may function as modules, components, sub-assemblies, or sub-systems of larger systems configured for performing, *e.g.*, genomics applications (*e.g.*, genetic testing and/or nucleic acid sequencing applications) or other chemical analysis, biochemical analysis, nucleic acid analysis, cell analysis or tissue analysis applications. FIG. 39 provides a non-limiting example of a block diagram for, *e.g.*, a sequencing system as disclosed herein. In addition to one, two, three, four, or more than four imaging modules as disclosed herein (each of which may comprise one or more illumination optical paths and/or one or more detection optical paths (*e.g.*, one or more detection channels configured for imaging fluorescence emission within a specified wavelength range onto an image sensor)), such systems may comprise one or more X-Y translation stages, one or more X-Y-Z translation stages, flow cells or cartridges, fluidics systems and fluid flow control modules, reagent cartridges, temperature control modules, fluid dispensing robotics, cartridge- and/or microplate-handling (pick-and-place) robotics, light-tight housings and/or environmental control chambers, one or more processors or computers, data storage modules, data communication modules (*e.g.*, Bluetooth, WiFi, intranet, or internet communication hardware and associated software), display modules, one or more local and/or cloud-based software packages (*e.g.*, instrument / system control software packages, image processing software packages, data analysis software packages), *etc.*, or any combination thereof.

Translation stages

[0404] In some implementations of the imaging and analysis systems (*e.g.*, nucleic acid sequencing systems) disclosed herein, the system may comprise one or more (*e.g.*, one, two, three, four, or more than four) high precision X-Y (or in some cases, X-Y-Z) translation stage(s)

for re-positioning one or more sample support structure(s) (*e.g.*, flow cell(s)) in relation to the one or more imaging modules, for example, in order to tile one or more images, each corresponding to a field-of-view of the imaging module, to reconstruct composite image(s) of an entire flow cell surface. In some implementations of the imaging systems and genomics analysis systems (*e.g.*, nucleic acid sequencing systems) disclosed herein, the system may comprise one or more (*e.g.*, one, two, three, four, or more than four) high precision X-Y (or in some cases, X-Y-Z) translation stage(s) for re-positioning the one or more imaging modules in relation to one or more sample support structure(s) (*e.g.*, flow cell(s)), for example, in order to tile one or more images, each corresponding to a field-of-view of the imaging module, to reconstruct composite image(s) of an entire flow cell surface.

[0405] Suitable translation stages are commercially available from a variety of vendors, for example, Parker Hannifin. Precision translation stage systems typically comprise a combination of several components including, but not limited to, linear actuators, optical encoders, servo and/or stepper motors, and motor controllers or drive units. High precision and repeatability of stage movement is required for the systems and methods disclosed herein in order to ensure accurate and reproducible positioning and imaging of, *e.g.*, fluorescence signals when interspersing repeated steps of reagent delivery and optical detection.

[0406] Consequently, the systems disclosed herein may comprise specifying the precision with which the translation stage is configured to position a sample support structure in relation to the illumination and/or imaging optics (or *vice versa*). In one aspect of the present disclosure, the precision of the one or more translation stages is between about 0.1 μm and about 10 μm. In other aspects, the precision of the translation stage is about 10 μm or less, about 9 μm or less, about 8 μm or less, about 7 μm or less, about 6 μm or less, about 5 μm or less, about 4 μm or less, about 3 μm or less, about 2 μm or less, about 1 μm or less, about 0.9 μm or less, about 0.8 μm or less, about 0.7 μm or less, about 0.6 μm or less, about 0.5 μm or less, about 0.4 μm or less, about 0.3 μm or less, about 0.2 μm or less, or about 0.1 μm or less. Those of skill in the art will appreciate that, in some instances, the positioning precision of the translation stage may fall within any range bounded by any of two of these values (*e.g.*, from about 0.5 μm to about 1.5 μm). In some instances, the positioning precision of the translation stage may have any value within the range of values included in this paragraph, *e.g.*, about 0.12 μm.

Flow cells, microfluidic devices, and cartridges

[0407] *Flow cell devices:* Disclosed herein, in some embodiments, are flow cell devices that can be employed for performing or facilitating DNA sequencing analysis. Flow cell devices herein can be used to immobilize template nucleic acid molecules derived from biological samples and then introduce a repetitive flow of sequencing reagents (e.g., sequencing-by-binding,

sequencing-by-synthesis, and/or sequencing-by-avidite) to attach labeled nucleotides to specific positions in the template sequences. A series of label signals are detected and decoded to reveal the nucleotide sequences of the template molecules, e.g., immobilized and/or amplified nucleic acid template molecules attached to a surface of the flow cell.

[0408] In some embodiments, a flow cell device disclosed herein can comprise a support having one or more substrates, a number of channels, an inlet, and outlet. FIGS. 41, 45, 47B, 53A, and 64A-64F show exemplary embodiments of the flow cell devices. FIGS. 45, 47B, and 53A shows exemplary embodiments of flow cell devices with one or two surfaces that can hold samples to be images. FIGS. 64A-64Fshow exemplary embodiments of the flow cell devices with one or more surfaces, in particular, four surfaces for holding imaging samples. FIG. 64C shows a cross section view at line AA' in FIG. 64A.

[0409] In some embodiments, the flow cell device 6400 can include a support 6410. The support 6410 can be solid. At least part of the support 6410 can be transparent so that light transmitting from a light source of the imaging system 4100 can travel through the transparent portion of the support and reach the samples located on the flow cell device 6400.

[0410] The support 6410 can comprise one or more substrates 6411, 6412, 6413. As shown in FIG. 64B, the one or more substrates can include a top substrate 6411 and a bottom substrate 6412. When the flow cell device is positioned with respect to the optical system for imaging, the top substrate 6411 can be closer to objective lens 4185, along the z direction, than the bottom substrate 6412. The bottom substrate 6412 can be closer to a translation stage of the imaging system for holding and supporting the flow cell during sequencing than the top substrate.

[0411] In some embodiments, the flow cell device 6400 can further include an interposer substrate, 6413 in between the top and bottom substrate 6411, 6412.

[0412] In some embodiments, the flow cell device 6400 can further include one or more middle substrates 6414, 6415 in between the top and bottom substrate 6411, 6412. In particular, a middle substrate 6414, 6415 can be in between the interposer substrate 6413 and one of the top or bottom substrates as shown in FIG. 64B.

[0413] Each substrate can have a predetermined thickness, and different substrate can have different thickness. In some embodiments, each substrate can have a uniform thickness along the z direction. In some embodiments, each substrate can have a uniform thickness along the z direction in at least a portion of the substrate. For example, the portion with uniform thickness can encompass the channel(s) or the imaging areas of the flow cell device.

[0414] In some embodiments, the top or bottom substrate can have a thickness of about 0.3 mm to about 3 mm. In some embodiments, the top or bottom substrate can have a thickness of about 0.6 mm to about 2 mm. In some embodiments, the top or bottom substrate can have a thickness

of about 0.8 mm to about 2 mm. In some embodiments, the top or bottom substrate can have a thickness of about 0.8 mm to about 1.5 mm. In some embodiments, the top or bottom substrate can have a thickness of about 0.85, 0.9, 0.93, 0.95, 0.98 mm.

[0415] In some embodiments, the interposer substrate can have a thickness of about 40 um to 250 um. In some embodiments, the interposer substrate can have a thickness of about 50 um to 160 um. In some embodiments, the interposer substrate can have a thickness of about 50 um to 70 um. In some embodiments, the interposer substrate can have a thickness of about 90 um to 160 um. In some embodiments, the interposer substrate can have a thickness of about 95, 100, 110, 120, 130, 140, 145, 155, or 160 um.

[0416] In some embodiments, the middle substrate can have a thickness of about 30 um to 250 um. In some embodiments, the interposer substrate can have a thickness of about 40 um to 150 um. In some embodiments, the interposer substrate can have a thickness of about 40 um to 70 um. In some embodiments, the interposer substrate can have a thickness of about 90 um to 120 um. In some embodiments, the interposer substrate can have a thickness of about 60, 70, 80, 90, 100, or 110 um.

[0417] In some embodiments, the flow cell device has a total thickness of about 220 um to about 360 um. The thickness can be orthogonal to the multiple surfaces when the multiple surfaces are planar and parallel to each other. The thickness can be along an optical path from the objective lens to the target FOV when the surfaces are non-planar, e.g., a curved surface.

[0418] In some embodiments, the flow cell device has a total thickness of about 0.33, 0.335, 0.34, 0.345, 0.35, 0.355, or 0.36 mm along a direction orthogonal to the x-y plane. In some embodiments, the flow cell has a total thickness of about 0.22, 0.225, 0.23, 0.235, 0.24, 0.245, 0.25, or 0.255 mm along a direction orthogonal to the x-y plane.

[0419] In some embodiments, for optical systems with a larger NA, e.g., NA of 0.5, the NA of the optical system can remain unaltered and the total thickness of the flow cell device can be selected to be smaller than traditional one or dual surface flow cells to achieve the desired image quality of the flow cell with three or more axially-displaced surfaces. For example, for a quad surface flow cell, the total thickness of the flow cell device can be 0.235 mm, 0.24 mm, or 0.245 mm. In some embodiments, when the total thickness of the multiple- surface flow cell devices herein remain comparable to that of the traditional flow cells, e.g., about 350 um, the NA of the optical system can be reduced, e.g., from 0.5 to 0.4 to achieve the desired image quality of the quad surface flow cell. The surface coating and chemistry disclosed herein can facilitate maintaining image quality of each surface of the multiple surface flow cells.

[0420] In some embodiments, the substrate(s) can have an elongate shape extending along the y axis. In some embodiments, the substrate(s) can have various shapes such as rectangular, square, etc., within the x-y plane.

[0421] In some embodiments, the one or more substrates can be planar. In some embodiments, the one or more substrates contains no curvature perceivable to naked eyes, e.g., as shown in FIGS. 64A-64F, so that the one or more substrates can have planar surfaces. However, the substrates do not have to be planar in certain embodiments. Alternatively, a part or the entirety of one or more substrates can be curved.

[0422] In some embodiments, the support or the one or more substrates can comprise glass or plastic. In some embodiments, the support or one or more substrates are all-glass or all-plastic. In some embodiments, the support or the one or more substrates can comprise a tape such as a pressure sensitive adhesive (PSA) tape. For example, the interposer substrate, or other substrate in between the top and bottom substrate can be made from PSA tape and can conveniently tap the top and bottom substrates to it fixedly.

[0423] The substrate(s) can define one or more channels 6416 of the flow cell devices. The channels can allow fluid, e.g., liquid or gas, to flow therethrough. The flow cell device can have one or more channels distributed in the x-y plane, as shown in FIG. 64B. In some embodiments, the flow cell device can have more than one channels 6416, 6417 distributed along the z direction as shown in Fig. 64C. The two adjacent channels can be separated by an interposer substrate 6413. FIG. 64C shows 2 channels along z direction, but 3, 4, or even more channels can be included in a flow cell device in certain embodiments.

[0424] The gas herein can comprise one type of gas or a combination of different type of gases. In some embodiments, the gas comprises air. The gas can comprise dry air. In some embodiments, the gas comprises one or more inert gases. In some embodiments, the gas comprises one or more active gases.

[0425] The reagents herein can comprise liquid. In some embodiments, the reagents are deprived of air bubbles that are greater than a predetermined size. In some embodiments, the first reagent is configured to wet the first coating of the surface of the one or more channels. In some embodiments, the second reagent is configured to rewet the surface of the one or more channels after at least partly drying the surface by the gas gap.

[0426] In some embodiments, each channel(s) 6416, 6417 can be defined by a top surface 6418, 6420 and a bottom surface 6419, 6421 of the substrates. The channels 6416, 6417 can each include a lumen. The lumen can be defined by the top and bottom surface of the substrates surrounding the lumen, and a grove in one or more of the substrates. In some embodiments, the top surface and the bottom surface are interior surfaces facing the corresponding lumen.

[0427] In some embodiments, the middle substrate 6414, 6415 can include a void, e.g., an elongated void, extending along a longitudinal axis, or y axis, of the middle substrate. The void's width can define the width of the channel 6416, 6417 along the x axis, and the void's length, along the y direction, can define the length of the channel.

[0428] In some embodiments, the channels are microfluidic channels. In some embodiments, a gap or height between the top interior surface and the bottom interior surface of the substrates that defines the channels, along the z direction, is about 150 um, 130um, 120 um, 110 um, 100 um, 90 um, 80 um, 70 um, 60 um, 50 um, or 40 um. In some embodiments, the gap or height of the channel is no more than about 100 um. In some embodiments, the gap or height of the channel is no more than about 70, 60 um, 50um, or 40 um.

[0429] In some embodiments, a length of the channel, along the y direction, is about 120 mm, 100 mm, 90 mm, 80 mm, 70 mm, 60 mm, 50 mm, 40 mm, or 30 mm. In some embodiments, the length of the channel is no more than about 100 um. In some embodiments, the length of the channel is no more than about 80 mm, 75 mm, 70 mm, 65 mm, 60 mm, 55 mm, or 50 mm.

[0430] In some embodiments, a width of the channel, along the x direction, is about 50 mm, 40 mm, 30 mm, 25 mm, 20 mm, 10 mm, 15 mm, 8 mm, or 5 mm. In some embodiments, the length of the channel is no more than about 10 mm or about 7 mm. In some embodiments, the width of the channel is no more than about 40 mm, 35 mm, 30 mm, 25 mm, 20 mm, or 15 mm.

[0431] In some embodiments, the distance between two adjacent channels or the distance from an edge of the channel to the edge of the flow cell device, along the x axis, is about 0.5 mm to about 15 mm. In some embodiments, the distance between two adjacent channels or the distance from an edge of the channel to the edge of the flow cell device, along the x axis, is about 1 mm to about 5 mm.

[0432] In some embodiments, the flow cell devices can have more than one channels, and all the channels can have a unform size and shape. FIG. 64B show exemplary embodiments of flow cell devices with two channels of the identical size and shape. In some embodiments, the flow cell devices can have channels of different sizes and/or shapes, for example, with similar channel length but different channel widths.

[0433] In some embodiments, the flow cell devices can include three or more axially-displaced surfaces, and each surface has an equal size of imaging area, e.g., the active area or ROI, on which sample(s) are disposed, and images are acquired. The equal size of imaging area or ROI can be identical to that of the traditional one or dual surface flow cells, so that a quad surface flow cell can have doubled surface area of that in a traditional dual surface flow cell. The doubled or otherwise increase surface area can allow more samples (e.g., increased sample

volume and/or sample varieties) to be disposed on a single flow cell device herein than a traditional one or dual surface flow cell.

[0434] In some embodiments, the flow cell device can include two or more surfaces in the x-y plane orthogonal to the axial direction. As shown in FIG. 64B, the flow cell device 6400 has 2 surfaces in the x-y plane, and for each surface in the x-y plane, there are 4 axially-displaced surfaces. The total surfaces of the flow cell device is 8.

[0435] The flow cell device 6400 can include one or more inlet 6422 and one or more outlets 6423. A channel 6416,6417 can run from its corresponding inlet 6422 to its corresponding outlet 6423 thereby allowing fluidic communication from the inlet to the outlet. Sequencing reagents can be introduced to the flow cell device via the inlet 6423, flow through the channels 6416, 6417 and interact with samples located therein, and then exit from the outlet 6423. In some embodiments, the channels along the z direction, 6416, 6417 can share the same inlet and/or outlet. FIGS. 64C and 64D show exemplary embodiments with shared inlet and outlet for two channels displaced from each other in the z direction. The shared inlet and outlet can be through the top or bottom substrate. In some embodiments, the channels along the z direction, 6416, 6417 can each have a different inlet and outlet as shown in FIG.64E. In some embodiments, the inlet and outlet can be a side port in the middle substrate(s) instead of a port in the top or bottom substrate.

[0436] The size and shape of the inlet and outlet can be customized to suite various sequencing applications. For example, the size and shape can be determined based on the specific sequencing application(s), such as, a minimal flush volume, a contamination threshold, the parameters of the flow cell, e.g., the size of the flow cell channels, or the parameters of the dispenser, e.g., the size of the dispensing tip. As a nonlimiting example, the inlet can be cylindrical with walls extending along the z direction and orthogonal to the substrates.

[0437] The diameter of the inlet, e.g., the widest dimension in the x-y plane, can be in the range of about 30 um to about 3 mm. The diameter of the inlet, e.g., the widest dimension in the x-y plane, can be in the range of about 50 um to about 1 mm. The height of the inlet, along the z direction, can be the total height of the top substrate and the middle substrate, and it can be in the range of 1 mm to 5 mm. As yet another example, the diameter of the outlet, in the x-y plane, can be in the range of about 30 um mm to about 3 mm. In some embodiments, the diameter of the outlet can be in the range of about 50 um to about 1 mm, and the outlet can be a cylindrical shape.

[0438] The size and shape of the inlet and outlet can be customized to suite various sequencing applications. For example, the size and shape can be determined based on the specific sequencing application(s), such as, a minimal flush volume, a contamination threshold, the

parameters of the flow cell, e.g., the size of the flow cell channels, or the parameters of the dispenser, e.g., the size of the dispensing tip. As a nonlimiting example, the inlet can be cylindrical as shown in FIG. 64C with walls extending along the z direction and orthogonal to the substrates. At the bottom of the cylindrical void/ hole, the inlet 6422 can be connected to a cleaning outlet 6424 in FIG. 64F. The inlet can be shaped differently. For example, the inlet can have an inverted cone shape with wider openings at the top and narrows down toward the channel to reduce the residuals of reagents that can remain in the inlet.

[0439] FIGS. 64A – 64B show exemplary flow cell devices with 5 substrates forming 4 channels, and each channel having a corresponding inlet and outlet. However, the number of substrates, channels, inlets, and outlets can vary in different embodiments. In some embodiments, the number of substrates, channels, inlets and outlets can be any integer number that is greater than 0. In some embodiments, the flow cell devices herein have 2, 4, 6, 8, 10, or even more channels in the same x-y plane. In some embodiments, the flow cell devices herein have 2, 3, 4, or even more channels in the z direction.

[0440] The flow cell devices and flow cell cartridges disclosed herein may be used as components of systems designed for a variety of chemical analysis, biochemical analysis, nucleic acid analysis, cell analysis, or tissue analysis application. In general, such systems may comprise one or more one or more of the disclosed single capillary flow cell devices, multiple capillary flow cell devices, capillary flow cell cartridges, and/or microfluidic devices and cartridges described herein. Additional description of the disclosed flow cell devices and cartridges may be found in PCT Patent Application Publication WO 2020/118255, which is incorporated herein by reference in its entirety.

[0441] In some instances, the systems disclosed herein may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 single capillary flow cell devices, multiple capillary flow cell devices, capillary flow cell cartridges, and/or microfluidic devices and cartridges. In some instances, the single capillary flow cell devices, multiple capillary flow cell devices, and/or microfluidic devices and cartridges may be fixed components of the disclosed systems. In some instances, the single capillary flow cell devices, multiple capillary flow cell devices, and/or microfluidic devices and cartridges may be removable, exchangeable components of the disclosed systems. In some instances, the single capillary flow cell devices, multiple capillary flow cell devices, and/or microfluidic devices and cartridges may be disposable or consumable components of the disclosed systems.

[0442] In some implementations, the disclosed single capillary, i.e., channel, flow cell devices (or single capillary flow cell cartridges) comprise a single capillary/channel, *e.g.*, a glass or

fused-silica capillary, the lumen of which forms a fluid flow path through which reagents or solutions may flow, and the interior surface of which may form a sample support structure to which samples of interest are bound or tethered. In some implementations, the multi-capillary capillary flow cell devices (or multi-capillary flow cell cartridges) disclosed herein may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more than 20 capillaries configured for performing an analysis technique that further comprises imaging as a detection method.

[0443] In some instances, one or more capillaries may be packaged within a chassis to form a cartridge that facilitates ease-of-handling, incorporates adapters or connectors for making external fluid connections, and may optionally include additional integrated functionality such as reagent reservoirs, waste reservoirs, valves (*e.g.*, microvalves), pumps (*e.g.*, micropumps), *etc.*, or any combination thereof.

[0444] FIG. 29 illustrates one non-limiting example of a single glass capillary flow cell device that comprises two fluidic adaptors – one affixed to each end of the piece of glass capillary – that are designed to mate with standard OD fluidic tubing to provide for convenient, interchangeable fluid connections with an external fluid flow control system. The fluidic adaptors can be attached to the capillary using any of a variety of techniques known to those of skill in the art including, but not limited to, press fit, adhesive bonding, solvent bonding, laser welding, *etc.*, or any combination thereof.

[0445] In general, the capillaries used in the disclosed capillary flow cell devices and capillary flow cell cartridges will have at least one internal, axially-aligned fluid flow channel (or "lumen") that runs the full length of the capillary. In some instances, the capillary may have two, three, four, five, or more than five internal, axially-aligned fluid flow channels (or "lumen").

[0446] A number specified cross-sectional geometries for suitable capillaries (or the lumen thereof) are consistent with the disclosure herein including, but not limited to, circular, elliptical, square, rectangular, triangular, rounded square, rounded rectangular, or rounded triangular cross-sectional geometries. In some instances, the capillary (or lumen thereof) may have any specified cross-sectional dimension or set of dimensions. For example, in some instances the largest cross-sectional dimension of the capillary lumen (*e.g.,* the diameter if the lumen is circular in shape, or the diagonal if the lumen is square or rectangular in shape) may range from about 10 μm to about 10 mm. In some aspects, the largest cross-sectional dimension of the capillary lumen may be at least 10 μm, at least 25 μm, at least 50 μm, at least 75 μm, at least 100 μm, at least 200 μm, at least 300 μm, at least 400 μm, at least 500 μm, at least 600 μm, at least 700 μm, at least 800 μm, at least 900 μm, at least 1 mm, at least 2 mm, at least 3 mm, at least 4 mm, at

least 5 mm, at least 6 mm, at least 7 mm, at least 8 mm, at least 9 mm, or at least 10 mm. In some aspects, the largest cross-sectional dimension of the capillary lumen may be at most 10 mm, at most 9 mm, at most 8 mm, at most 7 mm, at most 6 mm, at most 5 mm, at most 4 mm, at most 3 mm, at most 2 mm, at most 1 mm, at most 900 μm, at most 800 μm, at most 700 μm, at most 600 μm, at most 500 μm, at most 400 μm, at most 300 μm, at most 200 μm, at most 100 μm, at most 75 μm, at most 50 μm, at most 25 μm, or at most 10 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the largest cross-sectional dimension of the capillary lumen may range from about 100 μm to about 500 μm. Those of skill in the art will recognize that the largest cross-sectional dimension of the capillary lumen may have any value within this range, *e.g.*, about 124 μm.

[0447] In some instances, *e.g.*, wherein the lumen of the one or more capillaries in a flow cell device or cartridge has a square or rectangular cross-section, the distance between a first interior surface (*e.g.*, a top or upper surface) and a second interior surface (*e.g.*, a bottom or lower surface) that defines the gap height or thickness of a fluid flow channel may range from about 10 μm to about 500 μm. In some instances, the gap height may be at least 10 μm, at least 20 μm, at least 30 μm, at least 40 μm, at least 50 μm, at least 60 μm, at least 70 μm, at least 80 μm, at least 90 μm, at least 100 μm, at least 125 μm, at least 150 μm, at least 175 μm, at least 200 μm, at least 225 μm, at least 250 μm, at least 275 μm, at least 300 μm, at least 325 μm, at least 350 μm, at least 375 μm, at least 400 μm, at least 425 μm, at least 450 μm, at least 475 μm, or at least 500 μm. In some instances, the gap height may be at most 500 μm, at most 475 μm, at most 450 μm, at most 425 μm, at most 400 μm, at most 375 μm, at most 350 μm, at most 325 μm, at most 300 μm, at most 275 μm, at most 250 μm, at most 225 μm, at most 200 μm, at most 175 μm, at most 150 μm, at most 125 μm, at most 100 μm, at most 90 μm, at most 80 μm, at most 70 μm, at most 60 μm, at most 50 μm, at most 40 μm, at most 30 μm, at most 20 μm, or most 10 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the gap height may range from about 40 μm to about 125 μm. Those of skill in the art will recognize that the gap height may have any value within the range of values in this paragraph, *e.g.*, about 122 μm.

[0448] In some instances, the length of the one or more capillaries used to fabricate the disclosed capillary flow cell devices or flow cell cartridges may range from about 5 mm to about 5 cm or greater. In some instances, the length of the one or more capillaries may be less than 5 mm, at least 5 mm, at least 1 cm, at least 1.5 cm, at least 2 cm, at least 2.5 cm, at least 3 cm, at least 3.5 cm, at least 4 cm, at least 4.5 cm, or at least 5 cm. In some instances, the length of the one or more capillaries may be at most 5 cm, at most 4.5 cm, at most 4 cm, at most 3.5 cm, at

most 3 cm, at most 2.5 cm, at most 2 cm, at most 1.5 cm, at most 1 cm, or at most 5 mm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the length of the one or more capillaries may range from about 1.5 cm to about 2.5 cm. Those of skill in the art will recognize that the length of the one or more capillaries may have any value within this range, *e.g.*, about 1.85 cm. In some instances, devices or cartridges may comprise a plurality of two or more capillaries that are the same length. In some instances, devices or cartridges may comprise a plurality of two or more capillaries that are of different lengths.

[0449] The capillaries used for constructing the disclosed capillary flow cell devices or capillary flow cell cartridges may be fabricated from any of a variety of materials known to those of skill in the art including, but not limited to, glass (*e.g.*, borosilicate glass, soda lime glass, *etc.*), fused silica (quartz), polymer (*e.g.*, polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), polydimethylsiloxane (PDMS), *etc.*), polyetherimide (PEI) and perfluoroelastomer (FFKM) as more chemically inert alternatives, or any combination thereof. PEI is somewhere between polycarbonate and PEEK in terms of both cost and chemical compatibility. FFKM is also known as Kalrez.

[0450] The one or more materials used to fabricate the capillaries are often optically transparent to facilitate use with spectroscopic or imaging-based detection techniques. In some instances, the entire capillary will be optically transparent. Alternatively, in some instances, only a portion of the capillary (*e.g.*, an optically transparent "window") will be optically transparent.

[0451] The capillaries used for constructing the disclosed capillary flow cell devices and capillary flow cell cartridges may be fabricated using any of a variety of techniques known to those of skill in the art, where the choice of fabrication technique is often dependent on the choice of material used, and *vice versa*. Examples of suitable capillary fabrication techniques include, but are not limited to, extrusion, drawing, precision computer numerical control (CNC) machining and boring, laser photoablation, and the like.

[0452] In some implementations, the capillaries used in the disclosed capillary flow cell devices and cartridges may be off-the-shelf commercial products. Examples of commercial vendors that provide precision capillary tubing include Accu-Glass (St. Louis, MO; precision glass capillary tubing), Polymicro Technologies (Phoenix, AZ; precision glass and fused-silica capillary tubing), Friedrich & Dimmock, Inc. (Millville, NJ; custom precision glass capillary tubing), and Drummond Scientific (Broomall, PA; OEM glass and plastic capillary tubing).

[0453] The fluidic adapters that are attached to the capillaries of the capillary flow cell devices and cartridges disclosed herein, and other components of the capillary flow cell devices or cartridges, may be fabricated using any of a variety of suitable techniques (*e.g.*, extrusion molding, injection molding, compression molding, precision CNC machining, *etc.*) and materials (*e.g.*, glass, fused-silica, ceramic, metal, polydimethylsiloxane, polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), *etc.*), where again the choice of fabrication technique is often dependent on the choice of material used, and *vice versa*.

[0454] FIG. 30 provides a non-limiting example of capillary flow cell cartridge that comprises two glass capillaries, fluidic adaptors (two per capillary in this example), and a cartridge chassis that mates with the capillaries and/or fluidic adapters such that the capillaries are held in a fixed orientation relative to the cartridge. In some instances, the fluidic adaptors may be integrated with the cartridge chassis. In some instances, the cartridge may comprise additional adapters that mate with the capillaries and/or capillary fluidic adapters. As noted elsewhere herein, in some instances, the cartridge may comprise additional functional components. In some instances, the capillaries are permanently mounted in the cartridge. In some instances, the cartridge chassis is designed to allow one or more capillaries of the flow cell cartridge to be interchangeably removed and replaced. For example, in some instances, the cartridge chassis may comprise a hinged "clamshell" configuration which allows it to be opened so that one or more capillaries may be removed and replaced. In some instances, the cartridge chassis is configured to mount on, for example, the stage of a fluorescence microscope or within a cartridge holder of a fluorescence imaging module or instrument system of the present disclosure.

[0455] In some instances, the disclosed flow cell devices may comprise microfluidic devices (or "microfluidic chips") and cartridges, where the microfluidic devices are fabricated by forming fluid channels in one or more layers of a suitable material and comprise one or more fluid channels (*e.g.*, "analysis" channels) configured for performing an analysis technique that further comprises imaging as a detection method. In some implementations, the microfluidic devices or cartridges disclosed herein may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more than 20 fluid channels (*e.g.*, "analysis" fluid channels) configured for performing an analysis technique that further comprises imaging as a detection method. In some instances, the disclosed microfluidic devices may further comprise additional fluid channels (*e.g.*, for dilution or mixing of reagents), reagent reservoirs, waste reservoirs, adapters for

making external fluid connections, and the like, to provide integrated "lab-on-a-chip" functionality within the device.

[0456] A non-limiting example of microfluidic flow cell cartridge comprises a chip having two or more parallel glass channels formed on the chip, fluidic adaptors coupled to the chip, and a cartridge chassis that mates with the chip and/or fluidic adapters such that the chip is posited in a fixed orientation relative to the cartridge. In some instances, the fluidic adaptors may be integrated with the cartridge chassis. In some instances, the cartridge may comprise additional adapters that mate with the chip and/or fluidic adapters. In some instances, the chip is permanently mounted in the cartridge. In some instances, the cartridge chassis is designed to allow one or more chips of the flow cell cartridge to be interchangeably removed and replaced. For example, in some instances, the cartridge chassis may comprise a hinged "clamshell" configuration which allows it to be opened so that one or more chips may be removed and replaced. In some instances, the cartridge chassis is configured to mount on, for example, the stage of a microscope system or within a cartridge holder of an imaging system. Even though only one chip is described in the non-limiting example, it is understood that more than one chip can be used in the microfluidic flow cell cartridge. The flow cell cartridges of the present disclosure may comprise a single microfluidic chip or a plurality of microfluidic chips. In some instances, the flow cell cartridges of the present disclosure may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more than 20 microfluidic chips. The packaging of one or more microfluidic devices within a cartridge may facilitate ease-of-handling and correct positioning of the device within the optical imaging system.

[0457] The fluid channels within the disclosed microfluidic devices and cartridges may have an of a variety of cross-sectional geometries including, but not limited to, circular, elliptical, square, rectangular, triangular, rounded square, rounded rectangular, or rounded triangular cross-sectional geometries. In some instances, the fluid channels may have any specified cross-sectional dimension or set of dimensions. For example, in some instances, the height (*e.g.*, gap height), width, or largest cross-sectional dimension of the fluid channels (*e.g.*, the diagonal if the fluid channel has a square, rounded square, rectangular, or rounded rectangular cross-section) may range from about 10 μm to about 10 mm. In some aspects, the height (*e.g.*, gap height), width, or largest cross-sectional dimension of the fluid channels may be at least 10 μm, at least 25 μm, at least 50 μm, at least 75 μm, at least 100 μm, at least 200 μm, at least 300 μm, at least 400 μm, at least 500 μm, at least 600 μm, at least 700 μm, at least 800 μm, at least 900 μm, at least 1 mm, at least 2 mm, at least 3 mm, at least 4 mm, at least 5 mm, at least 6 mm, at least 7 mm, at least 8 mm, at least 9 mm, or at least 10 mm. In some aspects, the height (*e.g.*, gap height), width, or largest cross-sectional dimension of the fluid channels may be at most 10 mm,

at most 9 mm, at most 8 mm, at most 7 mm, at most 6 mm, at most 5 mm, at most 4 mm, at most 3 mm, at most 2 mm, at most 1 mm, at most 900 μm, at most 800 μm, at most 700 μm, at most 600 μm, at most 500 μm, at most 400 μm, at most 300 μm, at most 200 μm, at most 100 μm, at most 75 μm, at most 50 μm, at most 25 μm, or at most 10 μm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the height (*e.g.*, gap height), width, or largest cross-sectional dimension of the fluid channels may range from about 20 μm to about 200 μm. Those of skill in the art will recognize that the height (*e.g.*, gap height), width, or largest cross-sectional dimension of the fluid channels may have any value within this range, *e.g.*, about 122 μm.

[0458] In some instances, the length of the fluid channels in the disclosed microfluidic devices and cartridges may range from about 5 mm to about 10 cm or greater. In some instances, the length of the fluid channels may be less than 5 mm, at least 5 mm, at least 1 cm, at least 1.5 cm, at least 2 cm, at least 2.5 cm, at least 3 cm, at least 3.5 cm, at least 4 cm, at least 4.5 cm, at least 5 cm, at least 6 cm, at least 7 cm, at least 8 cm, at least 9 cm, or at least 10 cm. In some instances, the length of the fluid channels may be at most 10 cm, at most 9 cm, at most 8 cm, at most 7 cm, at most 6 cm, at most 5 cm, at most 4.5 cm, at most 4 cm, at most 3.5 cm, at most 3 cm, at most 2.5 cm, at most 2 cm, at most 1.5 cm, at most 1 cm, or at most 5 mm. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the length of the fluid channels may range from about 1.5 cm to about 2.5 cm. Those of skill in the art will recognize that the length of the fluid channels may have any value within this range, *e.g.*, about 1.35 cm. In some instances, the microfluidic devices or cartridges may comprise a plurality of fluid channels that are the same length. In some instances, the microfluidic devices or cartridges may comprise a plurality of fluid channels that are of different lengths.

[0459] The disclosed microfluidic devices can comprise at least one layer of material having one or more fluid channels formed therein. In some instances, the microfluidic chip may include two layers bonded together to form one or more fluid channels. In some instances, the microfluidic chip may include three or more layers bonded together to form one or more fluid channels. In some instances, the microfluidic fluid channels may have an open top. In some instances, the microfluidic fluid channels may be fabricated within one layer, *e.g.*, the top surface of a bottom layer, and sealed by bonding the top surface of the bottom layer to the bottom surface of a top layer of material. In some instances, the microfluidic channels may be fabricated within one layer, *e.g.*, as patterned channels the depth of which extends through the full thickness of the layer, which is then sandwiched between and bonded to two non-patterned layers to seal the fluid channels. In some instances, the microfluidic channels are fabricated by the removal of a

sacrificial layer on the surface of a substrate. This method does not require the bulk substrate (*e.g.*, a glass or silicon wafer) to be etched away. Instead, the fluid channels are located on the surface of the substrate. In some instances, the microfluidic channels may be fabricated in or on the surface of a substrate and then sealed by deposition of a conformal film or layer on the surface of the substrate to create sub-surface or buried fluid channels in the chip.

[0460] The microfluidic chips can be manufactured using a combination of microfabrication processes. Because the devices are microfabricated, substrate materials will typically be selected based upon their compatibility with known microfabrication techniques, *e.g.*, photolithography, wet chemical etching, laser ablation, laser irradiation, air abrasion techniques, injection molding, embossing, and other techniques. The substrate materials are also generally selected for their compatibility with the full range of conditions to which the microfluidic devices may be exposed, including extremes of pH, temperature, salt concentration, and application of electromagnetic (*e.g.*, light) or electric fields.

[0461] The disclosed microfluidic chips may be fabricated from any of a variety of materials known to those of skill in the art including, but not limited to, glass (*e.g.*, borosilicate glass, soda lime glass, *etc.*), fused-silica (quartz), silicon, a polymer (*e.g.*, polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), polydimethylsiloxane (PDMS), *etc.*), polyetherimide (PEI) and perfluoroelastomer (FFKM) (as more chemically inert alternatives), or any combination thereof. In some preferred instances, the substrate material(s) may include silica-based substrates, such as borosilicate glass, and quartz, as well as other substrate materials.

[0462] The disclosed microfluidic devices may be fabricated using any of a variety of techniques known to those of skill in the art, where the choice of fabrication technique is often dependent on the choice of material used, and *vice versa*. The microfluidic channels on the chip can be constructed using techniques suitable for forming micro-structures or micro-patterns on the surface of a substrate. In some instances, the fluid channels are formed by laser irradiation. In some instances, the microfluidic channels are formed by focused femtosecond laser radiation. In some instances, the microfluidic channels are formed by photolithography and etching including, but not limited to, chemical etching, plasma etching, or deep reactive ion etching. In some instances, the microfluidic channels are formed using laser etching. In some instances, the microfluidic channels are formed using a direct-write lithography technique. Examples of direct-write lithography include electron beam direct-write and focused ion beam milling.

[0463] In additional preferred instances, the substrate material(s) may comprise polymeric materials, *e.g.*, plastics, such as polymethylmethacrylate (PMMA), polycarbonate, polytetrafluoroethylene (TEFLON™), polyvinylchloride (PVC), polydimethylsiloxane (PDMS), polysulfone, and the like. Such polymeric substrates may be readily patterned or micromachined using available microfabrication techniques, such as those described above. In some instances, microfluidic chips may be fabricated from polymeric materials, *e.g.*, from microfabricated masters, using well known molding techniques, such as injection molding, embossing, stamping, or by polymerizing the polymeric precursor material within a mold (see, *e.g.*, U.S. Pat. No. 5,512,131). In some instances, such polymeric substrate materials are preferred for their ease of manufacture, low cost, and disposability, as well as their general inertness to most extreme reaction conditions. As with flow cell devices fabricated from other materials, *e.g.*, glass, flow cell devices fabricated from these polymeric materials may include treated surfaces, *e.g.*, derivatized or coated surfaces, to enhance their utility in the microfluidic system, as will be discussed in more detail below.

[0464] The fluid channels and/or fluid chambers of the microfluidic devices can be fabricated into the upper surface of a first substrate as microscale channels (*e.g.*, grooves, indentations, *etc.*) using the above described microfabrication techniques. The first substrate comprises a top side having a first planar surface and a bottom side. In the microfluidic devices prepared in accordance with the methods described herein, the plurality of fluid channels (*e.g.*, grooves and/or indentations) are formed on the first planar surface. In some instances, the fluid channels (*e.g.*, grooves and/or indentations) formed in the first planar surface (prior to bonding to a second substrate) have a bottom and side walls, with the top remaining open. In some instances, the fluid channels (*e.g.*, grooves and/or indentations) formed in the first planar surface (prior to bonding to a second substrate) have a bottom and side walls and the top remaining closed. In some instances, the fluid channels (*e.g.*, grooves and/or indentations) formed in the first planar surfaces (prior to bonding to a second substrate) have only side walls and no top or bottom surface (*e.g.*, the fluid channels span the full thickness of the first substrate.

[0465] Fluid channels and chambers may be sealed by placing the first planar surface of the first substrate in contact with, and bonding to, the planar surface of a second substrate to form the channels and/or chambers (*e.g.*, the interior portion) of the device at the interface of these two components. In some instances, after the first substrate is bonded to a second substrate, the structure may further be placed in contact with and bonded to a third substrate. In some instances, the third substrate may be placed in contact with the side of the first substrate that is not in contact with the second substrate. In some instances, the first substrate is placed between the second substrate and the third substrate. In some instances, the second substrate and the third

substrate can cover and/or seal the grooves, indentations, or apertures formed on the first substrate to form the channels and/or chambers (*e.g.*, the interior portion) of the device at the interface of these components.

[0466] The device can have openings that are oriented such that they are in fluid communication with at least one of the fluid channels and/or fluid chambers formed in the interior portion of the device, thereby forming fluid inlets and/or fluid outlets. In some instances, the openings are formed on the first substrate. In some instances, the openings are formed on the first and the second substrate. In some instances, the openings are formed on the first, the second, and the third substrate. In some instances, the openings are positioned at the top side of the device. In some instances, the openings are positioned at the bottom side of the device. In some instances, the openings are positioned at the first and/or the second ends of the device, and the channels run along the direction from the first end to the second end.

[0467] Conditions under which substrates may be bonded together are generally widely understood by those of skill in the art, and such bonding of substrates is generally carried out by any of a variety of methods, the choice of which may vary depending upon the nature of the substrate materials used. For example, thermal bonding of substrates may be applied to a number of substrate materials including, *e.g.*, glass or silica-based substrates, as well as some polymer based-substrates. Such thermal bonding techniques typically comprise mating the substrate surfaces that are to be bonded under conditions of elevated temperature and, in some cases, application of external pressure. The precise temperatures and pressures utilized will generally vary depending upon the nature of the substrate materials used.

[0468] For example, for silica-based substrate materials, *e.g.*, glass (borosilicate glass, Pyrex™, soda lime glass, *etc.*), fused-silica (quartz), and the like, thermal bonding of substrates is typically carried out at temperatures ranging from about 500 °C to about 1400 °C, and preferably, from about 500 °C to about 1200 °C. For example, soda lime glass is typically bonded at temperatures of around 550 °C, whereas borosilicate glass is typically thermally bonded at or near 800 °C. Quartz substrates, on the other hand, are typically thermally bonded at temperatures at or near 1200 °C. These bonding temperatures are typically achieved by placing the substrates to be bonded into high temperature annealing ovens.

[0469] Polymeric substrates that are thermally bonded, on the other hand, will typically utilize lower temperatures and/or pressures than silica-based substrates, in order to prevent excessive melting of the substrates and/or distortion, *e.g.*, flattening of the interior portion of the device (*e.g.*, the fluid channels or chambers). Generally, such elevated temperatures for bonding polymeric substrates will vary from about 80 °C to about 200 °C, depending upon the polymeric material used, and will preferably be between about 90 °C and about 150 °C. Because of the

significantly reduced temperatures required for bonding polymeric substrates, such bonding may typically be carried out without the need for the high temperature ovens used in the bonding of silica-based substrates. This allows incorporation of a heat source within a single integrated bonding system, as described in greater detail below.

[0470] Adhesives may also be used to bond substrates together according to well-known methods, which typically comprise applying a layer of adhesive between the substrates that are to be bonded and pressing them together until the adhesive sets. A variety of adhesives may be used in accordance with these methods, including, *e.g.*, UV curable adhesives, which are commercially available. Alternative methods may also be used to bond substrates together in accordance with the present disclosure, including *e.g.*, acoustic or ultrasonic welding and/or solvent welding of polymeric parts.

[0471] Typically, a number of the described microfluidic chips or devices will be manufactured at the same time, *e.g.*, using "wafer-scale" fabrication. For example, polymeric substrates may be stamped or molded in large separable sheets which can then be mated and bonded together. Individual devices or bonded substrates may then be separated from the larger sheet by cutting or dicing. Similarly, for silica-based substrates, individual devices can be fabricated from larger substrate wafers or plates, allowing higher throughput of the manufacturing process. Specifically, a plurality of fluid channel structures can be fabricated on a first substrate wafer or plate, which is then overlaid with and bonded to a second substrate wafer or plate, and optionally further overlaid with and bonded to a third substrate wafer or plate. The individual devices are then segmented from the larger substrates using known methods, such as sawing, scribing and breaking, and the like.

[0472] As noted above, the top or second substrate is overlaid upon the bottom or first substrate to seal the various channels and chambers. In carrying out the bonding process according to the methods of the present disclosure, the bonding of the first and second substrates may be carried out using vacuum and/or pressure to maintain the two substrate surfaces in optimal contact. In particular, the bottom substrate may be maintained in optimal contact with the top substrate by, *e.g.*, mating the planar surface of the bottom substrate with the planar surface of the top substrate and applying a vacuum through holes that are disposed through the top substrate. Typically, application of a vacuum to holes in the top substrate is carried out by placing the top substrate on a vacuum chuck, which typically comprises a mounting table or surface, having an integrated vacuum source. In the case of silica-based substrates, the bonded substrates are subjected to elevated temperatures in order to create an initial bond, so that the bonded substrates may then be transferred to the annealing oven, without any shifting relative to each other.

[0473] Alternate bonding systems for incorporation with the apparatus described herein include, *e.g.*, adhesive dispensing systems, for applying adhesive layers between the two planar surfaces of the substrates. This may be done by applying the adhesive layer prior to mating the substrates, or by placing an amount of the adhesive at one edge of the adjoining substrates and allowing the wicking action of the two mated substrates to draw the adhesive across the space between the two substrates.

[0474] In certain instances, the overall bonding system can include automatable systems for placing the top and bottom substrates on the mounting surface and aligning them for subsequent bonding. Typically, such systems include translation systems for moving either the mounting surface or one or more of the top and bottom substrates relative to each other. For example, robotic systems may be used to lift, translate and place each of the top and bottom substrates upon the mounting table, and within the alignment structures, in turn. Following the bonding process, such systems also can remove the finished product from the mounting surface and transfer these mated substrates to a subsequent operation, *e.g.*, a separation or dicing operation, an annealing oven for silica-based substrates, *etc.*, prior to placing additional substrates thereon for bonding.

[0475] In some instances, the manufacturing of the microfluidic chip includes the layering or laminating of two or more layers of substrate, *e.g.*, patterned and non-patterned polymeric sheets, in order to produce the chip. For example, in microfluidic devices, the microfluidic features of the device are typically produced by laser irradiation, etching, or otherwise fabricating features into the surface of a first layer. A second layer is then laminated or bonded to the surface of the first to seal these features and provide the fluidic elements of the device, *e.g.*, the fluid channels.

[0476] As noted above, in some instances one or more capillary flow cell devices or microfluidic chips may be mounted in a cartridge chassis to form a capillary flow cell cartridge or microfluidic cartridge. In some instances, the capillary flow cell cartridge or microfluidic cartridge may further comprise additional components that are integrated with the cartridge to provide enhanced performance for specific applications. Examples of additional components that may be integrated into the cartridge include, but are not limited to, adapters or connectors for making fluidic connections to other components of the system, fluid flow control components (*e.g.*, miniature valves, miniature pumps, mixing manifolds, *etc.*), temperature control components (*e.g.*, resistive heating elements, metal plates that serve as heat sources or sinks, piezoelectric (Peltier) devices for heating or cooling, temperature sensors), or optical components (*e.g.*, optical lenses, windows, filters, mirrors, prisms, fiber optics, and/or light-emitting diodes (LEDs) or other miniature light sources that may collectively be used to

facilitate spectroscopic measurements and/or imaging of one or more capillary or fluid flow channels.

[0477] The fluidic adaptors, cartridge chassis, and other cartridge components may be attached to the capillaries, capillary flow cell device(s), microfluidic chip(s) (or fluid channels within the chip) using any of a variety of techniques known to those of skill in the art including, but not limited to, press fit, adhesive bonding, solvent bonding, laser welding, *etc.*, or any combination thereof. In some instances, the inlet(s) and/or outlet(s) of the microfluidic channels in the microfluidic chip are apertures on the top surface of the chip, and the fluidic adaptors can be attached or coupled to the inlet(s) and/or outlet(s) of the microfluidic channels within the chip. In some instances, the cartridge may comprise additional adapters (*e.g.*, in addition to the fluidic adapters) that mate with the chip and/or fluidic adapters and help to position the chip within the cartridge. These adapters may be constructed using the same fabrication techniques and materials as those outlined above for the fluidic adapters.

[0478] The cartridge chassis (or "housing") may be fabricated from metal and/or polymer materials such as aluminum, anodized aluminum, polycarbonate (PC), acrylic (PMMA), or Ultem (PEI), while other materials are also consistent with the present disclosure. A housing may be fabricated using CNC machining and/or molding techniques, and designed so that one, two, or more than two capillaries or microfluidic chips are constrained by the chassis in a fixed orientation to create one or more independent flow channels. The capillaries or chips may be mounted in the chassis using, *e.g.*, a compression fit design, or by mating with compressible adapters made of silicone or a fluoroelastomer. In some instances, two or more components of the cartridge chassis (*e.g.*, an upper half and a lower half) are assembled using, *e.g.*, screws, clips, clamps, or other fasteners so that the two halves are separable. In some instances, two or more components of the cartridge chassis are assembled using, *e.g.*, adhesives, solvent bonding, or laser welding so that the two or more components are permanently attached.

Quality of the optical systems

[0479] In some embodiment, the root-mean-square (RMS) wavefront error of the optical systems herein is measured with a flow cell device, e.g., a flow cell device with two or more axially displaced surfaces and one or more axially displaced fluidic channels. In some embodiments, the RMS wavefront error of the optical systems herein is less than the diffraction limit of the optical system. The RMS wavefront error of one or more surfaces of the flow cell device is less than 0.09λ, 0.08λ, 0.07λ, 0.06λ, 0.05λ, 0.03λ, or $0.02\ \lambda$, wherein λ is the central wavelength of a light source. The light source can be predetermined and used to measure the RMS of the optical systems. The RMS wavefront error can be determined for one or more surfaces of the flow cell. The RMS can be determined for each surface in four different color

channels, wherein λ is the center wavelength of the light source. The RMS wavefront error is for a target FOV of about 1.5, 2, 2.5, 3, 3.5, or 4 mm in x or y direction. The RMS wavefront error is for a target FOV of greater than 1 mm^2, 5 mm^2, 10 mm^2, 20 mm^2, 30 mm^2, 40 mm^2, 50 mm^2, or more . In some embodiments, the RMS wavefront error is greater for the fourth surface than the first, second, or third surfaces. In some embodiments, the root-mean-square (RMS) wavefront error of one or more surfaces of the flow cell device is less than 20,30,40,50,60,70,80,90, or 100 milli-wavelengths across the FOV. In some embodiments, the RMS wavefront error is diffraction limited and varies by less than 20,30,40,50,60,70,80,90, or 100 milli-wavelengths across the FOV, wherein the wavelength is the central wavelength of a light source.

[0480] In an exemplary embodiment, the root-mean-square (RMS) wavefront error of the optical system herein with the NA of greater than 0.25 (e.g., NA of 0.5) is measured with a flow cell device with four axially displaced surfaces and two axially displaced fluidic channels and a total thickness of about 240um. In this particular embodiment, the RMS wavefront error of the optical system is less than the diffraction limit of the optical system. The root-mean-square (RMS) wavefront error of is less than 0.09λ or 0.07λ, wherein λ is the center wavelength of an light source. The RMS wavefront error is for the four surfaces of the flow cell and each surface in four different color channels. The RMS wavefront error is for a target FOV of about 1.5 mm in x or y direction.

[0481] In an exemplary embodiment, the root-mean-square (RMS) wavefront error of the optical system herein with the NA of 0.4, or any other NA that is greater than 0.25, is measured with a flow cell device with four axially displaced surfaces and two axially displaced fluidic channels and a total thickness of about 240 or about 350 um. In this particular embodiment, the RMS wavefront error of the optical system is less than the diffraction limit of the optical system. The root-mean-square (RMS) wavefront error of is less than 0.09λ or 0.07λ, wherein λ is the center wavelength of an light source. The RMS wavefront error is for the four surfaces of the flow cell and each surface in four different color channels. The RMS wavefront error is for a target FOV of about 1.5 mm in x or y direction.

[0482] *Flow cell shape*: Typically, flow cell shape is limited by standard microscopy systems that require flat surfaces that can reside within the focal depth of the FOV of the microscope imaging system. Such limitations limit flow cell design at its interfaces, create gradients of pressure, temperature, viscosity, or a combination thereof. Such gradients may cause a propensity to form bubbles, differential reaction kinetics across the cell, or a combination thereof. Additionally, typical solutions to such problems require flow cell designs that may not be effectively imaged by standard microscopy systems. For optimal imaging performance of

non-flat flow cell shapes infrared (IR) heating, conformable and transparent heaters, or a combination thereof, may be utilized to reduce gradients in binding, reaction kinetics or other assays factors. In some embodiments, the surface 5101 may be non-flat, or curved as illustrated in FIG. 52. The surface 5101 can include a concave (curving away from the optical system) or a convex curve (e.g., curving toward the optical system). In some cases, a non-flat surface can be a curved surface. For example, a curved surface described elsewhere herein may be a non-flat surface. A non-flat substrate can comprise features that deviate from flatness on a length scale comparable to the surface. For example, a non-flat surface can comprise one or more features that are at least about 1, 5, 10, 15, 20, 25, 30, 25, 40, 45, 50, 55, 60, or more percent of a dimension (e.g., length, width, thickness, etc.) of the non-flat surface. In some cases, a non-flat surface can comprise one or more features that are at most about 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 1, or less percent of a dimension of the non-flat surface. Examples of feature include, but are not limited to, curves (e.g., single curves, waveforms, etc.), triangular features, square features, other geometric features, or the like, or any combination thereof.

[0483] In some embodiments, the flow cell may comprise a capillary flow cell 5201 as illustrated in FIGS. 53A-53B and FIGS. 54A-54B. In FIGS. 53A-53B, the sample is flown through the capillary flow cell 5201, wherein the flow direction is along the x-axis. FIG. 53A illustrates a non-limiting example of a cross section of a capillary flow cell 5201, wherein sample sites 4902 are disposed on the interior surface(s) of the capillary flow cell 5201. The capillary flow cell 5201 can have one, two, three, or even more interior surfaces, for example, in certain embodiments where the capillary flow cell have concentric capillary channels, with the inner most capillary channel having a circular cross section in the yz plane, and outer capillary channel(s) having a cross section of a ring in the yz plane. In some embodiments, a light source 4901 may be directed toward the capillary flow cell, wherein the light is focused, creating an optimized imaging volume 4915 containing sample sites 4902 disposed on the far side of the interior surface of the capillary flow cell. In some embodiments, a plurality of optical subsystems 5001, each comprising a light source 4901 are distributed around the capillary flow cell, such that the optimal imaging volumes 4915 overlap, enabling optimized imaging of sample sites disposed on an area larger than an area corresponding to one optimal imaging volume 4915. In some embodiments, the optical subsystems may be rotated about the x-axis of the capillary flow cell 5201 as illustrated in FIGS. 53A-53B, thus enabling the overlapping optimized imaging volumes 4915 to be scanned across the entire inside surface of the capillary flow cell 5201. Alternatively, the capillary flow cell 5201 may be rotated about the x-axis and the optical subsystems 4914 may be held in constant position while imaging.

[0484] Another way to acquire images of sample sites 4902 disposed across the entire interior surface of the capillary flow cell 5201, with uniform image quality, is by incorporating a wedge block 4916 into each of the optical subsystems 4914, as illustrated in FIGS 53A-53B. In certain aspects, multiple optical subsystems 4914 are disposed about the x-axis of the capillary flow cell. In such cases, the multiple optical subsystems 4914 may image a portion of interior surface of the capillary flow cell, larger than one optimal imaging volume 4915 of one optical subsystem 4914 via overlapping optimal imaging volumes 4915 as described herein. In certain aspects, as described herein, curved surfaces can also be properly imaged by placing the optical subsystems 4914 such that their corresponding optical axes 4913 are at least approximately orthogonal to the region of the surface to be imaged. In such cases, the plurality of optical subsystems 5001 can provide optimized images of curved, large area surfaces. In some embodiments, the wedge block assembly 4916 of each optical subsystem 4914 is adjusted to provide focus on half of the interior surface, closest to the light sources as illustrated in FIG 54A. Alternatively, the wedge block assembly 4916 may be adjusted to focus on sample sites 4902 disposed on the opposite side of the interior surface of the capillary flow cell 5201 as illustrated in FIG. 54B. In such cases, there is no need to rotate the capillary flow cell 5201 or plurality of optical subsystems 5001 since refocusing and acquiring images, using the multiple optimal imaging volumes 4914 provides imaging coverage of the entire interior surface of the capillary flow cell. In some embodiments, the capillary flow cell is translated along the x-axis in order to provide images along the entire length of the capillary flow cell 5201. In some embodiments, the large area surface may comprise an area of at least about 5 square millimeters.

Flow cell surface coatings

[0485] In some instances, one or more interior surfaces of the capillary lumens or microfluidic channels in the disclosed flow cell devices may be coated using any of a variety of surface modification techniques or polymer coatings known to those of skill in the art. In some instances, the coatings may be formulated to increase or maximize the number of available binding sites (*e.g.*, tethered oligonucleotide adapter/ primer sequences) on the one or more interior surfaces to increase or maximize a foreground signal, *e.g.*, a fluorescence signal arising from labeled nucleic acid molecules hybridized to tethered oligonucleotide adapter/ primer sequences. In some instances, the coatings may be formulated to decrease or minimize nonspecific binding of fluorophores and other small molecules, or labeled or unlabeled nucleotides, proteins, enzymes, antibodies, oligonucleotides, or nucleic acid molecules (*e.g.*, DNA, RNA, *etc.*), in order to decrease or minimize a background signal, *e.g.*, background fluorescence arising from the nonspecific binding of labeled biomolecules or from

autofluorescence of a sample support structure. The combination of increased foreground signal and reduced background signal that may be achieved in some instances through the use of the disclosed coatings may thus provide improved signal-to-noise ratio (SNR) in spectroscopic measurements or improved contrast-to-noise ratio (CNR) in imaging methods.

[0486] As will be discussed in more detail below, the disclosed hydrophilic, polymer-coated flow cell devices, optionally used in combination with the improved hybridization and/or amplification protocols, yield solid-phase bioassay reactions that exhibit: (i) negligible non-specific binding of protein and other reaction components (thus reducing or minimizing substrate background), (ii) negligible non-specific nucleic acid amplification product, and (iii) provide tunable nucleic acid amplification reactions. Although described herein primarily in the context of nucleic acid hybridization, amplification, and sequencing assays, it will be understood by those of skill in the art that the disclosed low-binding supports may be used in any of a variety of other bioassay formats including, but not limited to, sandwich immunoassays, enzyme-linked immunosorbent assays (ELISAs), *etc.*

[0487] In a preferred aspect, one or more layers of a coating material may be applied to the interior flow cell device surfaces, where the number of layers and/or the material composition of each layer is chosen to adjust one or more surface properties of the interior flow cell device surfaces, as noted in U.S. Patent Application No. 16/363,842, the disclosure of which is incorporated by reference in its entirety. Examples of surface properties that may be adjusted include, but are not limited to, surface hydrophilicity / hydrophobicity, overall coating thickness, the surface density of chemically-reactive functional groups, the surface density of grafted linker molecules or oligonucleotide adapters/primers, *etc.* In some preferred applications, one or more surface properties of the capillary or channel lumen are adjusted to, for example, (i) provide for very low non-specific binding of proteins, oligonucleotides, fluorophores, and other molecular components of chemical or biological analysis applications, including solid-phase nucleic acid amplification and/or sequencing applications, (ii) provide for improved solid-phase nucleic acid hybridization specificity and efficiency, and (iii) provide for improved solid-phase nucleic acid amplification rate, specificity, and efficiency.

[0488] Any of a variety of molecules known to those of skill in the art including, but not limited to, silanes, amino acids, peptides, nucleotides, oligonucleotides, other monomers or polymers, or combinations thereof may be used in creating the one or more chemically-modified layers on the interior flow cell device surfaces, where the choice of components used may be varied to alter one or more properties of the support surface, *e.g.*, the surface density of functional groups and/or tethered oligonucleotide primers, the hydrophilicity/hydrophobicity of the support surface, or the three three-dimensional nature (*e.g.*, "thickness") of the support surface.

[0489] The attachment chemistry used to graft a first chemically-modified layer to an interior surface of the flow cell (capillary or channel) will generally be dependent on both the material from which the flow cell device is fabricated and the chemical nature of the layer. In some instances, the first layer may be covalently attached to the interior flow cell device surfaces. In some instances, the first layer may be non-covalently attached, *e.g.*, adsorbed to the surface through non-covalent interactions such as electrostatic interactions, hydrogen bonding, or van der Waals interactions between the surface and the molecular components of the first layer. In either case, the substrate surface may be treated prior to attachment or deposition of the first layer. Any of a variety of surface preparation techniques known to those of skill in the art may be used to clean or treat the support surface. For example, glass or silicon surfaces may be acid-washed using a Piranha solution (a mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2)) and/or cleaned using an oxygen plasma treatment method.

[0490] Silane chemistries constitute one non-limiting approach for covalently modifying the silanol groups on glass or silicon surfaces to attach more reactive functional groups (*e.g.*, amines or carboxyl groups), which may then be used in coupling linker molecules (*e.g.*, linear hydrocarbon molecules of various lengths, such as C6, C12, C18 hydrocarbons, or linear polyethylene glycol (PEG) molecules) or layer molecules (*e.g.*, branched PEG molecules or other polymers) to the surface. Examples of suitable silanes that may be used in creating any of the disclosed low binding support surfaces include, but are not limited to, (3-Aminopropyl) trimethoxysilane (APTMS), (3-Aminopropyl) triethoxysilane (APTES), any of a variety of PEG-silanes (*e.g.*, comprising molecular weights of 1K, 2K, 5K, 10K, 20K, *etc.*), amino-PEG silane (*e.g.*, comprising a free amino functional group), maleimide-PEG silane, biotin-PEG silane, and the like.

[0491] Examples of preferred polymers that may be used to create one or more layers of low non-specific binding material in any of the disclosed support surfaces include, but are not limited to, polyethylene glycol (PEG) of various molecular weights and branching structures, streptavidin, polyacrylamide, polyester, dextran, poly-lysine, and poly-lysine copolymers, or any combination thereof. Examples of conjugation chemistries that may be used to graft one or more layers of material (*e.g.* polymer layers) to the support surface and/or to cross-link the layers to each other include, but are not limited to, biotin-streptavidin interactions (or variations thereof), His tag – Ni/NTA conjugation chemistries, methoxy ether conjugation chemistries, carboxylate conjugation chemistries, amine conjugation chemistries, NHS esters, maleimides, thiol, epoxy, azide, hydrazide, alkyne, isocyanate, and silane.

[0492] In some instances, the number of layers of polymer or other chemical layers on the interior flow cell device surfaces may range from 1 to about 10, or greater than 10. In some

instances, the number of layers is at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10. In some instances, the number of layers may be at most 10, at most 9, at most 8, at most 7, at most 6, at most 5, at most 4, at most 3, at most 2, or at most 1. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the number of layers may range from about 2 to about 4. In some instances, the one or more layers may all comprise the same material. In some instances, each layer may comprise a different material. In some instances, a plurality of layers may comprise a plurality of materials.

[0493] One or more layers of a multi-layered surface may comprise a branched polymer or may be linear. Examples of suitable branched polymers include, but are not limited to, branched PEG, branched poly(vinyl alcohol) (branched PVA), branched poly(vinyl pyridine), branched poly(vinyl pyrrolidone) (branched PVP), branched), poly(acrylic acid) (branched PAA), branched polyacrylamide, branched poly(N-isopropylacrylamide) (branched PNIPAM), branched poly(methyl methacrylate) (branched PMA), branched poly(2-hydroxylethyl methacrylate) (branched PHEMA), branched poly(oligo(ethylene glycol) methyl ether methacrylate) (branched POEGMA), branched polyglutamic acid (branched PGA), branched poly-lysine, branched poly-glucoside, and dextran.

[0494] In some instances, the branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may comprise at least 4 branches, at least 5 branches, at least 6 branches, at least 7 branches, at least 8 branches, at least 9 branches, at least 10 branches, at least 12 branches, at least 14 branches, at least 16 branches, at least 18 branches, at least 20 branches, at least 22 branches, at least 24 branches, at least 26 branches, at least 28 branches, at least 30 branches, at least 32 branches, at least 34 branches, at least 36 branches, at least 38 branches, or at least 40 branches. Molecules often exhibit a 'power of 2' number of branches, such as 2, 4, 8, 16, 32, 64, or 128 branches.

[0495] In some instances, the resulting functional end groups distal from the surface following the deposition of one or more layers, *e.g.*, polymer layers can include, but are not limited to, biotin, methoxy ether, carboxylate, amine, NHS ester, maleimide, and bis-silane.

[0496] Linear, branched, or multi-branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may have a molecular weight of at least 500 Daltons, at least 1,000 Daltons, at least 1,500 Daltons, at least 2,000 Daltons, at least 2,500 Daltons, at least 3,000 Daltons, at least 3,500 Daltons, at least 4,000 Daltons, at least 4,500 Daltons, at least 5,000 Daltons, at least 7,500 Daltons, at least 10,000 Daltons, at least 12,500 Daltons, at least 15,000 Daltons, at least 17,500 Daltons, at least 20,000 Daltons, at least 25,000 Daltons, at least 30,000 Daltons, at least 35,000 Daltons, at least 40,000 Daltons, at least 45,000 Daltons, or at

least 50,000 Daltons. In some instances, the linear, branched, or multi-branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may have a molecular weight of at most 50,000 Daltons, at most 45,000 Daltons, at most 40,000 Daltons, at most 35,000 Daltons, at most 30,000 Daltons, at most 25,000 Daltons, at most 20,000 Daltons, at most 17,500 Daltons, at most 15,000 Daltons, at most 12,500 Daltons, at most 10,000 Daltons, at most 7,500 Daltons, at most 5,000 Daltons, at most 4,500 Daltons, at most 4,000 Daltons, at most 3,500 Daltons, at most 3,000 Daltons, at most 2,500 Daltons, at most 2,000 Daltons, at most 1,500 Daltons, at most 1,000 Daltons, or at most 500 Daltons. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the molecular weight of linear, branched, or multi-branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may range from about 1,500 Daltons to about 20,000 Daltons. Those of skill in the art will recognize that the molecular weight of linear, branched, or multi-branched polymers used to create one or more layers of any of the multi-layered surfaces disclosed herein may have any value within this range, *e.g.*, about 1,260 Daltons.

[0497] In some instances, two or more layers may be covalently coupled to each other or internally cross-linked to improve the stability of the resulting surface. In some instances, *e.g.*, wherein at least one layer of a multi-layered surface comprises a branched polymer, the number of covalent bonds between a branched polymer molecule of the layer being deposited and molecules of the previous layer may range from about one covalent linkage per molecule and about 32 covalent linkages per molecule. In some instances, the number of covalent bonds between a branched polymer molecule of the new layer and molecules of the previous layer may be at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 14, at least 16, at least 18, at least 20, at least 22, at least 24, at least 26, at least 28, at least 30, or at least 32, or more than 32 covalent linkages per molecule. In some instances, the number of covalent bonds between a branched polymer molecule of the new layer and molecules of the previous layer may be at most 32, at most 30, at most 28, at most 26, at most 24, at most 22, at most 20, at most 18, at most 16, at most 14, at most 12, at most 10, at most 9, at most 8, at most 7, at most 6, at most 5, at most 4, at most 3, at most 2, or at most 1. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the number of covalent bonds between a branched polymer molecule of the new layer and molecules of the previous layer may range from about 4 to about 16. Those of skill in the art will recognize that the number of covalent bonds between a branched polymer molecule of the new layer and

molecules of the previous layer may have any value within this range, *e.g.*, about 11 in some instances, or an average number of about 4.6 in other instances.

[0498] Any reactive functional groups that remain following the coupling of a material layer to the interior flow cell device surfaces may optionally be blocked by coupling a small, inert molecule using a high yield coupling chemistry. For example, in the case that amine coupling chemistry is used to attach a new material layer to the previous one, any residual amine groups may subsequently be acetylated or deactivated by coupling with a small amino acid such as glycine.

[0499] In order to scale binding site surface density, *e.g.*, oligonucleotide adapter/primer surface density, and add additional dimensionality to hydrophilic or amphoteric surfaces, substrates comprising multi-layer coatings of PEG and other hydrophilic polymers have been developed. By using hydrophilic and amphoteric surface layering approaches that include, but are not limited to, the polymer/co-polymer materials described below, it is possible to increase adapter/primer loading density on the surface significantly. Traditional PEG coating approaches use monolayer primer deposition, which has been tested and reported for single molecule sequencing applications but do not yield high copy numbers for nucleic acid amplification applications. As described herein, "layering" can be accomplished using traditional crosslinking approaches with any compatible polymer or monomer subunits such that a surface comprising two or more highly crosslinked layers can be built sequentially. Examples of suitable polymers include, but are not limited to, streptavidin, polyacrylamide, polyester, dextran, poly-lysine, and copolymers of poly-lysine and PEG. In some instances, the different layers may be cross-linked to each other through any of a variety of conjugation reactions including, but not limited to, biotin-streptavidin binding, azide-alkyne click reaction, amine-NHS ester reaction, thiol-maleimide reaction, and ionic interactions between positively charged polymer and negatively charged polymer. In some instances, high adapter/primer density materials may be constructed in solution and subsequently layered onto the surface in multiple steps.

[0500] In some cases, PEG multilayers include PEG (8 arm, 16 arm, 8 arm) on PEG-amine-APTES. Similar concentrations were observed for 3-layer multi-arm PEG (8 arm,16 arm, 8arm) and (8arm, 64arm, 8arm) on PEG-amine-APTES exposed to 8uM primer, and 3-layer multi-arm PEG (8arm, 8arm, 8arm) using star-shape PEG-amine to replace 16 arm and 64 arm. PEG multilayers having comparable first, second and third PEG layers are also contemplated.

[0501] In some instances, the resultant surface density of binding sites on the interior flow cell device surfaces, *e.g.*, oligonucleotide adapter/primer surface densities, may range from about 100 primer molecules per μm^2 to about 1,000,000 primer molecules per μm^2. In some instances, the surface density of binding sites may be at least 100, at least 200, at least 300, at least 400, at

least 500, at least 600, at least 700, at least 800, at least 900, at least 1,000, at least 1,500, at least 2,000, at least 2,500, at least 3,000, at least 3,500, at least 4,000, at least 4,500, at least 5,000, at least 5,500, at least 6,000, at least 6,500, at least 7,000, at least 7,500, at least 8,000, at least 8,500, at least 9,000, at least 9,500, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, at least 60,000, at least 65,000, at least 70,000, at least 75,000, at least 80,000, at least 85,000, at least 90,000, at least 95,000, at least 100,000, at least 150,000, at least 200,000, at least 250,000, at least 300,000, at least 350,000, at least 400,000, at least 450,000, at least 500,000, at least 550,000, at least 600,000, at least 650,000, at least 700,000, at least 750,000, at least 800,000, at least 850,000, at least 900,000, at least 950,000, or at least 1,000,000 molecules per μm^2. In some instances, the surface density of binding sites may be at most 1,000,000, at most 950,000, at most 900,000, at most 850,000, at most 800,000, at most 750,000, at most 700,000, at most 650,000, at most 600,000, at most 550,000, at most 500,000, at most 450,000, at most 400,000, at most 350,000, at most 300,000, at most 250,000, at most 200,000, at most 150,000, at most 100,000, at most 95,000, at most 90,000, at most 85,000, at most 80,000, at most 75,000, at most 70,000, at most 65,000, at most 60,000, at most 55,000, at most 50,000, at most 45,000, at most 40,000, at most 35,000, at most 30,000, at most 25,000, at most 20,000, at most 15,000, at most 10,000, at most 9,500, at most 9,000, at most 8,500, at most 8,000, at most 7,500, at most 7,000, at most 6,500, at most 6,000, at most 5,500, at most 5,000, at most 4,500, at most 4,000, at most 3,500, at most 3,000, at most 2,500, at most 2,000, at most 1,500, at most 1,000, at most 900, at most 800, at most 700, at most 600, at most 500, at most 400, at most 300, at most 200, or at most 100 molecules per μm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of binding sites may range from about 10,000 molecules per μm^2 to about 100,000 molecules per μm^2. Those of skill in the art will recognize that the surface density of binding sites may have any value within this range, *e.g.*, about 3,800 molecules per μm^2 in some instances, or about 455,000 molecules per μm^2 in other instances. In some instances, as will be discussed further below for nucleic acid sequencing applications, the surface density of template library nucleic acid sequences (*e.g.*, sample DNA molecules) initially hybridized to adapter or primer sequences tethered to the interior flow cell device surfaces may be less than or equal to that indicated for the surface density of binding sites. In some instances, as will also be discussed further below, the surface density of clonally-amplified template library nucleic acid sequences hybridized to adapter or primer sequences on the interior flow cell device surfaces may span the same range or a different range as that indicated for the surface density of tethered oligonucleotide adapters or primers.

[0502] Local surface densities of binding sites on the interior flow cell device surfaces as listed above do not preclude variation in density across a surface, such that a surface may comprise a region having a binding site density of, for example, 500,000 / um^2, while also comprising at least a second region having a substantially different local surface density.

[0503] In some instances, capture probes, *e.g.*, oligonucleotide primers with different base sequences and base modifications (or other biomolecules, *e.g.*, enzymes or antibodies) may be tethered to one or more layers of the resulting surface at various surface densities. In some instances, for example, both surface functional group density and the capture probe concentration used for coupling may be varied to target a certain capture probe surface density range. Additionally, capture probe surface density may be controlled by diluting capture probes with other "inert" molecules that carry the same reactive functional group for coupling to the surface. For example, amine-labeled oligonucleotide probes can be diluted with amine-labeled polyethylene glycol in a reaction with an NHS-ester coated surface to reduce the final primer density. In the case of oligonucleotide adapters/primers, probe sequences with different lengths of linker between the hybridization region and the surface attachment functional group may also be applied to vary surface density. Example of suitable linkers include poly-T and poly-A strands at the 5' end of the primer (*e.g.*, 0 to 20 bases), PEG linkers (*e.g.*, 3 to 20 monomer units), and carbon-chain (*e.g.*, C6, C12, C18, *etc.*). To measure or estimate the capture probe surface density, fluorescently labeled capture probes may be tethered to the surface and a fluorescence reading then compared with that for a calibration solution comprising a known concentration of the fluorophore.

[0504] In some instances, the degree of hydrophilicity (or "wettability" with aqueous solutions) of the disclosed support surfaces, *e.g.*, interior flow cell device surfaces, may be assessed, for example, through the measurement of water contact angles in which a small droplet of water is placed on the surface and its angle of contact with the surface is measured using, *e.g.*, an optical tensiometer. In some instances, a static contact angle may be determined. In some instances, an advancing or receding contact angle may be determined. In some instances, the water contact angle for the hydrophilic, low-binding support surface(s)disclosed herein may range from about 0 degrees to about 50 degrees. In some instances, the water contact angle for the hydrophilic, low-binding support surface(s)disclosed herein may no more than 50 degrees, 45 degrees, 40 degrees, 35 degrees, 30 degrees, 25 degrees, 20 degrees, 18 degrees, 16 degrees, 14 degrees, 12 degrees, 10 degrees, 8 degrees, 6 degrees, 4 degrees, 2 degrees, or 1 degree. In many cases the contact angle is no more than any value within this range, *e.g.*, no more than 40 degrees. Those of skill in the art will realize that a given hydrophilic, low-binding support surface of the present disclosure may exhibit a water contact angle having a value of anywhere within this range, *e.g.*,

about 27 degrees. In some instances, the disclosed low nonspecific binding surfaces have a water contact angle of less than 45 degrees. In some instances, the disclosed low nonspecific binding surfaces have a water contact angle of less than 35 degrees.

[0505] As noted, the hydrophilic coated interior flow cell device surfaces of the present disclosure exhibit reduce non-specific binding of proteins, nucleic acids, fluorophores, and other components of biological and biochemical assay methods. The degree of non-specific binding exhibited by a given support surface, *e.g.*, an interior flow cell device surface, may be assessed either qualitatively or quantitatively. For example, in some instances, exposure of the surface to fluorescent dyes (*e.g.*, cyanine dye 3 (Cy3), cyanine dye 5 (Cy5), *etc.*), fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (*e.g.* polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging may be used as a qualitative tool for comparison of non-specific binding on supports comprising different surface formulations. In some instances, exposure of the surface to fluorescent dyes, fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (*e.g.* polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging may be used as a quantitative tool for comparison of non-specific binding on supports comprising different surface formulations – provided that care has been taken to ensure that the fluorescence imaging is performed under conditions where fluorescence signal is linearly related (or related in a predictable manner) to the number of fluorophores on the support surface (*e.g.*, under conditions where signal saturation and/or self-quenching of the fluorophore is not an issue) and suitable calibration standards are used. In some instances, other techniques known to those of skill in the art, for example, radioisotope labeling and counting methods may be used for quantitative assessment of the degree to which non-specific binding is exhibited by the different support surface formulations of the present disclosure.

[0506] In some instances, the degree of non-specific binding exhibited by the disclosed low nonspecific binding support surfaces may be assessed using a standardized protocol for contacting the surface with a labeled protein (*e.g.*, bovine serum albumin (BSA), streptavidin, a DNA polymerase, a reverse transcriptase, a helicase, a single-stranded binding protein (SSB), *etc.*, or any combination thereof), a labeled nucleotide, a labeled oligonucleotide, *etc.*, under a standardized set of incubation and rinse conditions, followed be detection of the amount of label remaining on the surface and comparison of the signal resulting therefrom to an appropriate calibration standard. In some instances, the label may comprise a fluorescent label. In some instances, the label may comprise a radioisotope. In some instances, the label may comprise any other detectable label known to one of skill in the art. In some instances, the degree of non-

specific binding exhibited by a given support surface formulation may thus be assessed in terms of the number of non-specifically bound protein molecules (or other molecules) per unit area. In some instances, the low nonspecific binding supports of the present disclosure may exhibit nonspecific protein binding (or nonspecific binding of other specified molecules, *e.g.*, cyanine dye 3 (Cy3) of less than 0.001 molecule per μm^2, less than 0.01 molecule per μm^2, less than 0.1 molecule per μm^2, less than 0.25 molecule per μm^2, less than 0.5 molecule per μm^2, less than 1 molecule per μm^2, less than 10 molecules per μm^2, less than 100 molecules per μm^2, or less than 1,000 molecules per μm^2. Those of skill in the art will realize that a given support surface of the present disclosure may exhibit nonspecific binding falling anywhere within this range, for example, of less than 86 molecules per μm^2. For example, some modified surfaces disclosed herein exhibit nonspecific protein binding of less than 0.5 molecule / μm^2 following contact with a 1 uM solution of Cy3 labeled streptavidin (GE Amersham) in phosphate buffered saline (PBS) buffer for 15 minutes, followed by 3 rinses with deionized water. Some modified surfaces disclosed herein exhibit nonspecific binding of Cy3 dye molecules of less than 0.25 molecules per μm2. In independent nonspecific binding assays, 1 μM labeled Cy3 SA (ThermoFisher), 1 uM Cy5 SA dye (ThermoFisher), 10 uM Aminoallyl-dUTP - ATTO-647N (Jena Biosciences), 10 uM Aminoallyl-dUTP - ATTO-Rho11 (Jena Biosciences), 10 uM Aminoallyl-dUTP - ATTO-Rho11 (Jena Biosciences), 10 uM 7-Propargylamino-7-deaza-dGTP - Cy5 (Jena Biosciences, and 10 uM 7-Propargylamino-7-deaza-dGTP – Cy3 (Jena Biosciences) were incubated on the low nonspecific binding substrates at 37°C for 15 minutes in a 384 well plate format. Each well was rinsed 2-3 x with 50 ul deionized RNase/DNase Free water and 2-3 x with 25 mM ACES buffer pH 7.4. The 384 well plates were imaged on a GE Typhoon(GE Healthcare Lifesciences, Pittsburgh, PA) instrument using the Cy3, AF555, or Cy5 filter sets (according to dye test performed) as specified by the manufacturer at a PMT gain setting of 800 and resolution of 50-100 μm. For higher resolution imaging, images were collected on an Olympus IX83 microscope (Olympus Corp., Center Valley, PA) with a total internal reflectance fluorescence (TIRF) objective (20x, 0.75 NA or 100X, 1.5 NA, Olympus), an sCMOS Andor camera (Zyla 4.2. Dichroic mirrors were purchased from Semrock (IDEX Health & Science, LLC, Rochester, New York), *e.g.*, 405, 488, 532, or 633 nm dichroic reflectors/beamsplitters, and band pass filters were chosen as 532 LP or 645 LP concordant with the appropriate excitation wavelength. Some modified surfaces disclosed herein exhibit nonspecific binding of dye molecules of less than 0.25 molecules per μm^2.

[0507] In some instances, the coated flow cell device surfaces disclosed herein may exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8,

9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

[0508] In some instances, one or more surface modification and/or polymer layers may be applied to the interior flow cell device surfaces using a technique such as chemical vapor deposition (CVD). In some instances, one or more surface modification and/or polymer layers may be applied to the interior flow cell device surfaces by flowing one or more appropriate chemical coupling or coating reagents through the capillaries or fluid channels prior to use for their intended application. In some instances, one or more coating reagents may be added to a buffer used, *e.g.*, a nucleic acid hybridization, amplification reaction, and/or sequencing reaction buffer to provide for dynamic coating of the interior flow cell device surfaces.

[0509] In some instances, the chemical modification layers may be applied uniformly across the surface of the substrate or support structure. Alternatively, the surface of the substrate or support structure may be non-uniformly distributed or patterned, such that the chemical modification layers are confined to one or more discrete regions of the substrate. For example, the substrate surface may be patterned using photolithographic techniques to create an ordered array or random pattern of chemically-modified regions on the surface. Alternatively or in combination, the substrate surface may be patterned using, *e.g.*, contact printing and/or ink-jet printing techniques. In some instances, an ordered array or random pattern of chemically-modified discrete regions may comprise at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10,000 or more discrete regions, or any intermediate number of discrete regions spanned by the range herein.

[0510] In some instances, fluorescence images of the disclosed low nonspecific binding surfaces when used, *e.g.*, in nucleic acid hybridization or amplification applications to create clusters of hybridized or clonally-amplified nucleic acid molecules (*e.g.*, "discrete regions" that have been directly or indirectly labeled with a fluorophore) exhibit contrast-to-noise ratios (CNRs) of at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 20, 210, 220, 230, 240, 250, or greater than 250 when the nucleic acid molecules are labeled with Cy3 and the images are acquired using an Olympus IX83 inverted fluorescence microscope equipped with a 20x, 0.75 NA objective, a 532 nm light source, a bandpass and dichroic mirror filter set adapted or optimized for 532 nm long-pass excitation and Cy3 fluorescence emission filter, a Semrock 532 nm dichroic reflector, and a camera (Andor sCMOS, Zyla 4.2) where the excitation light intensity is adjusted to avoid signal saturation, and the surface is immersed in a buffer (*e.g.*, 25 mM ACES, pH 7.4 buffer) while the image is acquired. As used herein, contrast-to-noise ratio (CNR) is calculated as:

$$CNR = (S - B) / Noise$$

[0511] where S = foreground signal (*e.g.*, the fluorescence signal as measured in the image that arises from a labeled nucleic acid colony or cluster on a sample support surface), B = background signal (where $B = B_{inter} + B_{intra}$), B_{inter} = background signal measured at a location on the sample support surface that is between labeled nucleic acid colonies or clusters, B_{intra} = background signal measured at the location of a nucleic acid colony or cluster (determined, *e.g.*, by contacting the sample support surface with a labeled, non-complementary oligonucleotide and measuring the resulting fluorescence), and Noise = the signal noise. The contrast-to-noise ratio (CNR) of images of sequencing surfaces, for example, provides a key metric in assessing nucleic acid amplification specificity and non-specific binding on the support. While signal-to-noise ratio (SNR) is often considered to be a benchmark of overall signal quality, it can be shown that improved CNR can provide a significant advantage over SNR as a benchmark for signal quality in imaging applications that require rapid image capture (*e.g.*, nucleic acid sequencing applications for which cycle times can be minimized). Further description of low nonspecific binding surfaces can be found in U.S. Patent Nos. 10,876,148, 10,704,094 and 10,982,280 which are incorporated herein by reference in their entirety.

[0512] In some instances, polymer-coated sample support structures, *e.g.*, interior flow cell device surfaces comprising the disclosed hydrophilic polymer coatings, may exhibit improved stability to repetitive exposure to solvents, changes in temperature, changes in pH, or long-term storage.

Fluidics systems and fluid flow control modules

[0513] In some implementations, the disclosed imaging and/or analysis systems may provide fluid flow control capability for delivering samples or reagents to the one or more flow cell devices or flow cell cartridges (*e.g.*, single capillary flow cell device or microfluidic channel flow cell device) connected to the system. Reagents and buffers may be stored in bottles, reagent and buffer cartridges, or other suitable containers that are connected to the flow cell inlets by tubing and valve manifolds. The disclosed systems may also include processed sample and waste reservoirs in the form of bottles, cartridges, or other suitable containers for collecting fluids downstream of the capillary flow cell devices or capillary flow cell cartridges. In some embodiments, the fluid flow (or "fluidics") control module may provide programmable switching of flow between different sources, *e.g.,* sample or reagent reservoirs or bottles located in the instrument, and the inlet(s) to a central region (*e.g.*, a capillary flow cell or microfluidic device, or a large fluid chamber such as a large fluid chamber within a microfluidic device). In some instances, the fluid flow control module may provide programmable switching of flow

between outlet(s) from the central region (*e.g.*, a capillary flow cell or microfluidic device) and different collection points, *e.g.*, processed sample reservoirs, waste reservoirs, *etc.*, connected to the system. In some instances, samples, reagents, and/or buffers may be stored within reservoirs that are integrated into the flow cell cartridge or microfluidic cartridge itself. In some instances, processed samples, spent reagents, and/or used buffers may be stored within reservoirs that are integrated into the flow cell cartridge or microfluidic device cartridge itself.

[0514] In some implementations, one or more fluid flow control modules may be configured to control the delivery of fluids to one or more capillary flow cells, capillary flow cell cartridges, microfluidic devices, microfluidic cartridges, or any combination thereof. In some instances, the one or more fluidics controllers may be configured to control volumetric flow rates for one or more fluids or reagents, linear flow velocities for one or more fluids or reagents, mixing ratios for one or more fluids or reagents, or any combination thereof. Control of fluid flow through the disclosed systems will typically be performed using pumps (or other fluid actuation mechanisms) and valves (*e.g.*, programmable pumps and valves). Examples of suitable pumps include, but are not limited to, syringe pumps, programmable syringe pumps, peristaltic pumps, diaphragm pumps, and the like. Examples of suitable valves include, but are not limited to, check valves, electromechanical two-way or three-way valves, pneumatic two-way and three-way valves, and the like. In some instances, fluid flow through the system may be controlled by applying positive pneumatic pressure to one or more inlets of the reagent and buffer containers, or to inlets incorporated into flow cell cartridge(s) (*e.g.*, capillary flow cell or microfluidic cartridges). In some embodiments, fluid flow through the system may be controlled by drawing a vacuum at one or more outlets of waste reservoir(s), or at one or more outlets incorporated into flow cell cartridge(s) (*e.g.*, capillary flow cell or microfluidic cartridges).

[0515] In some instances, different modes of fluid flow control are utilized at different points in an assay or analysis procedure, *e.g.*, forward flow (relative to the inlet and outlet for a given capillary flow cell device), reverse flow, oscillating or pulsatile flow, or combinations thereof. In some applications, oscillating or pulsatile flow may be applied, for example, during assay wash/rinse steps to facilitate complete and efficient exchange of fluids within the one or more flow cell devices or flow cell cartridges (*e.g.*, capillary flow cell devices or cartridges, and microfluidic devices or cartridges).

[0516] Similarly, in some cases different fluid flow rates may be utilized at different locations within a flow cell device or at different points in the assay or analysis process workflow, for example, in some instances, the volumetric flow rate may vary from -100 ml/sec to +100 ml/sec. In some embodiment, the absolute value of the volumetric flow rate may be at least 0.001 ml/sec, at least 0.01 ml/sec, at least 0.1 ml/sec, at least 1 ml/sec, at least 10 ml/sec, or at least

100 ml/sec. In some embodiments, the absolute value of the volumetric flow rate may be at most 100 ml/sec, at most 10 ml/sec, at most 1 ml/sec, at most 0.1 ml/sec, at most 0.01 ml/sec, or at most 0.001 ml/sec. The volumetric flow rate at a given location with the flow cell device or at a given point in time may have any value within this range, *e.g.*, a forward flow rate of 2.5 ml/sec, a reverse flow rate of -0.05 ml/sec, or a value of 0 ml/sec (*e.g.*, stopped flow).

[0517] In some implementations, the fluidics system may be designed to minimize the consumption of key reagents (*e.g.*, expensive reagents) required for performing, *e.g.*, genomic analysis applications. For example, in some implementations the disclosed fluidics systems may comprise a first reservoir housing a first reagent or solution, a second reservoir housing a second reagent or solution, and a central region, *e.g.*, a central capillary flow cell or microfluidic device, where an outlet from the first reservoir and an outlet from the second reservoir are fluidically coupled to an inlet of the central capillary flow cell or microfluidic device through at least one valve such that the volume of the first reagent or solution flowing per unit time from the outlet of the first reservoir to the inlet of the central capillary flow cell or microfluidic device is less than the volume of the second reagent or solution flowing per unit time from the outlet of the second reservoir to the inlet of the central region. In some implementations, the first reservoir and second reservoir may be integrated into a capillary flow cell cartridge or microfluidic cartridge. In some instances, the at least one valve may also be integrated into the capillary flow cell cartridge or microfluidic cartridge.

[0518] In some instances, the first reservoir is fluidically coupled to the central capillary flow cell or microfluidic device through a first valve, and the second reservoir is fluidically coupled to the central capillary flow cell or microfluidic device through a second valve. In some instances, the first and/or second valves may be, *e.g.*, a diaphragm valve, pinch valve, gate valve, or other suitable valve. In some instances, the first reservoir is positioned in close proximity to the inlet of the central capillary flow cell or microfluidic device to reduce dead volume for delivery of the first reagent solution. In some instances, the first reservoir is placed in closer proximity to the inlet of the central capillary flow cell or microfluidic device than is the second reservoir. In some instances, the first reservoir is positioned in close proximity to the second valve so as to reduce the dead volume for delivery of the first reagent relative to that for delivery of a plurality of "second" reagents (*e.g.*, two, three, four, five, or six or more "second" reagents) from a plurality of "second" reservoirs (*e.g.*, two, three, four, five, or six or more "second" reservoirs).

[0519] The first and second reservoirs described above may be used to house the same or different reagents or solutions. In some instances, the first reagent that is housed in the first reservoir is different from the second reagent that is housed in the second reservoir, and the

second reagent comprises at least one reagent that is used in common by a plurality of reactions occurring in the central a central capillary flow cell or microfluidic device. In some instances, *e.g.*, in fluidics systems configured for performing nucleic acid sequencing chemistry within the central capillary flow cell or microfluidic device, the first reagent comprises at least one reagent selected from the group consisting of a polymerase, nucleotide, and a nucleotide analog. In some instances, the second reagent comprises a low-cost reagent, *e.g.*, a solvent.

[0520] In some instances, the interior volume of the central region, *e.g.*, a central capillary flow cell cartridge, or microfluidic device comprising one or more fluid channels or fluid chambers, can be adjusted based on the specific application to be performed, *e.g.*, nucleic acid sequencing. In some embodiments, the central region comprises an interior volume suitable for sequencing a eukaryotic genome. In some embodiments, the central region comprises an interior volume suitable for sequencing a prokaryotic genome. In some embodiments, the central region comprises an interior volume suitable for sequencing a viral genome. In some embodiments, the central region comprises an interior volume suitable for sequencing a transcriptome. For example, in some embodiments, the interior volume of the central region may comprise a volume of less than 0.05 μl, between 0.05 μl and 0.1 μl, between 0.05 μl and 0.2 μl, between 0.05 μl and 0.5 μl, between 0.05 μl and 0.8 μl, between 0.05 μl and 1 μl, between 0.05 μl and 1.2 μl, between 0.05 μl and 1.5 μl, between 0.1 μl and 1.5 μl, between 0.2 μl and 1.5 μl, between 0.5 μl and 1.5 μl, between 0.8 μl and 1.5 μl, between 1 μl and 1.5 μl, between 1.2 μl and 1.5 μl, or greater than 1.5 μl, or a range defined by any two of the foregoing. In some embodiments, the interior volume of the central region may comprise a volume of less than 0.5 μl, between 0.5 μl and 1 μl, between 0.5 μl and 2 μl, between 0.5 μl and 5 μl, between 0.5 μl and 8 μl, between 0.5 μl and 10 μl, between 0.5 μl and 12 μl, between 0.5 μl and 15 μl, between 1 μl and 15 μl, between 2 μl and 15 μl, between 5 μl and 15 μl, between 8 μl and 15 μl, between 10 μl and 15 μl, between 12 μl and 15 μl, or greater than 15 μl, or a range defined by any two of the foregoing. In some embodiments, the interior volume of the central region may comprise a volume of less than 5μl, between 5 μl and 10 μl, between 5 μl and 20 μl, between 5 μl and 500 μl, between 5 μl and 80 μl, between 5 μl and 100 μl, between 5 μl and 120 μl, between 5 μl and 150 μl, between 10 μl and 150 μl, between 20 μl and 150 μl, between 50 μl and 150 μl, between 80 μl and 150 μl, between 100 μl and 150 μl, between 120 μl and 150 μl, or greater than 150 μl, or a range defined by any two of the foregoing. In some embodiments, the interior volume of the central region may comprise a volume of less than 50μl, between 50 μl and 100 μl, between 50 μl and 200 μl, between 50 μl and 500 μl, between 50 μl and 800 μl, between 50 μl and 1000 μl, between 50 μl and 1200 μl, between 50 μl and 1500 μl, between 100 μl and 1500 μl, between 200 μl and 1500 μl, between 500 μl and 1500 μl, between 800 μl and 1500 μl, between

1000 µl and 1500 µl, between 1200 µl and 1500 µl, or greater than 1500 µl, or a range defined by any two of the foregoing. In some embodiments, the interior volume of the central region may comprise a volume of less than 500µl, between 500 µl and 1000 µl, between 500 µl and 2000 µl, between 500 µl and 5 ml, between 500 µl and 8 ml, between 500 µl and 10 ml, between 500 µl and 12 ml, between 500 µl and 15 ml, between 1 ml and 15 ml, between 2 ml and 15 ml, between 5 ml and 15 ml, between 8 ml and 15 ml, between 10ml and 15 ml, between 12 ml and 15 ml, or greater than 15 ml, or a range defined by any two of the foregoing. In some embodiments, the interior volume of the central region may comprise a volume of less than 5 ml, between 5 ml and 10 ml, between 5 ml and 20 ml, between 5 ml and 50 ml, between 5 ml and 80 ml, between 5 ml and 100 ml, between 5 ml and 120 ml, between 5 ml and 150 ml, between 10 ml and 150 ml, between 20 ml and 150 ml, between 50 ml and 150 ml, between 80 ml and 150 ml, between 100ml and 150 ml, between 120 ml and 150 ml, or greater than 150 ml, or a range defined by any two of the foregoing. In some embodiments, the systems described herein comprise an array or collection of flow cell devices or systems comprising multiple discrete capillaries, microfluidic channels, fluidic channels, chambers, or luminal regions, wherein the combined interior volume is, comprises, or includes one or more of the values within a range disclosed herein.

[0521] In some instances, the ratio of volumetric flow rate for the delivery of the first reagent to the central capillary flow cell or microfluidic device to that for delivery of the second reagent to the central capillary flow cell or microfluidic device may be less than 1:20, less than 1:16, least than 1:12, less than 1:10, less than 1:8, less than 1:6, or less than 1:2. In some instances, the ratio of volumetric flow rate for the delivery of the first reagent to the central capillary flow cell or microfluidic device to that for delivery of the second reagent to the central capillary flow cell or microfluidic device may have any value with the range spanned by these values, *e.g.*, less than 1:15.

[0522] As noted, the flow cell devices and/or fluidics systems disclosed herein may be configured to achieve a more efficient use of the reagents than that achieved by, *e.g.*, other sequencing devices and systems, particularly for the costly reagents used in a variety of sequencing chemistry steps. In some instances, the first reagent comprises a reagent that is more expensive than the second reagent. In some instances, the first reagent comprises a reaction-specific reagent and the second reagent comprises a nonspecific reagent common to all reactions performed in the central capillary flow cell or microfluidic device region, and wherein the reaction specific reagent is more expensive than the nonspecific reagent.

[0523] In some instances, utilization of the flow cell devices and/or fluidic systems disclosed herein may convey advantages in terms of reduced consumption of costly reagents. In some

instances, for example, utilization of the flow cell devices and/or fluidic systems disclosed herein may results in at least a 5%, at least a 7.5%, at least a 10%, at least a 12.5%, at least a 15%, at least a 17.5%, at least a 20%, at least a 22.5%, at least a 25%, at least a 30%, at least a 35%, at least a 40%, at least a 45%, or at least a 50% reduction in reagent consumption compared to the reagent consumption encountered when operating, *e.g.*, current commercially-available nucleic acid sequencing systems.

[0524] FIG. 31 illustrates a non-limiting example of a simple fluidics system comprising a single capillary flow cell connected to various fluid flow control components, where the single capillary is optically accessible and compatible with mounting on a microscope stage or in a custom imaging instrument for use in various imaging applications. A plurality of reagent reservoirs is fluidically-coupled with the inlet end of the single capillary flow cell device, where the reagent flowing through the capillary at any given point in time is controlled by a programmable rotary valve that allows the user to control the timing and duration of reagent flow. In this non-limiting example, fluid flow is controlled by a programmable syringe pump that provides precise control and timing of volumetric fluid flow and fluid flow velocity.

[0525] FIG. 32 illustrates a non-limiting example of a fluidics system that comprises a capillary flow cell cartridge having integrated diaphragm valves to reduce or minimize dead volume and conserve certain key reagents. The integration of miniature diaphragm valves into the cartridge allows the valve to be positioned in close proximity to the inlet of the capillary, thereby reducing or minimizing dead volume within the device and reducing the consumption of costly reagents. The integration of valves and other fluid control components within the capillary flow cell cartridge also allows greater fluid flow control functionality to be incorporated into the cartridge design.

[0526] FIG. 33 illustrates a non-limiting example of a capillary flow cell cartridge-based fluidics system used in combination with a microscope setup, where the cartridge incorporates or mates with a temperature control component such as a metal plate that makes contact with the capillaries within the cartridge and serves as a heat source /sink. The microscope setup may comprise an illumination system (*e.g.*, including a laser, LED, or halogen lamp, *etc.*, as a light source), an objective lens, an imaging system (*e.g.*, a CMOS or CCD camera), and a translation stage to move the cartridge relative to the optical system, which allows, *e.g.*, fluorescence and/or bright field images to be acquired for different regions of the capillary flow cells as the stage is moved.

Temperature control modules

[0527] In some implementations the disclosed systems will include temperature control functionality for the purpose of facilitating the accuracy and reproducibility of assay or analysis results. Examples of temperature control components that may be incorporated into the instrument system (or capillary flow cell cartridge) design include, but are not limited to, resistive heating elements, infrared light sources, Peltier heating or cooling devices, heat sinks, thermistors, thermocouples, and the like. In some instances, the temperature control module (or "temperature controller") may provide for a programmable temperature change at a specified, adjustable time prior to performing specific assay or analysis steps. In some instances, the temperature controller may provide for programmable changes in temperature over specified time intervals. In some embodiments, the temperature controller may further provide for cycling of temperatures between two or more set temperatures with specified frequency and ramp rates so that thermal cycling for amplification reactions may be performed.

[0528] FIG. 34 illustrates one non-limiting example for temperature control of the flow cells (*e.g.*, capillary flow cells or microfluidic device-based flow cells) through the use of a metal plate that is placed in contact with the flow cell cartridge. In some instances, the metal plate may be integrated with the cartridge chassis. In some instances, the metal plate may be temperature controlled using a Peltier or resistive heater.

[0529] FIG. 35 illustrates one non-limiting approach for temperature control of the flow cells (*e.g.*, capillary or microfluidic channel flow cells) that comprises a non-contact thermal control mechanism. In this approach, a stream of temperature-controlled air is directed through the flow cell cartridge (*e.g.*, towards a single capillary flow cell device or a microfluidic channel flow cell device) using an air temperature control system. The air temperature control system comprises a heat exchanger, *e.g.*, a resistive heater coil, fins attached to a Peltier device, *etc.*, that is capable of heating and/or cooling the air and holding it at a constant, user-specified temperature. The air temperature control system also comprises an air delivery device, such as a fan, which directs the stream of heated or cooled air to the capillary flow cell cartridge. In some instances, the air temperature control system may be set to a constant temperature T1 so that the air stream, and consequently the flow cell or cartridge (*e.g.*, capillary flow cell or microfluidic channel flow cell) is kept at a constant temperature T2, which in some cases may differ from the set temperature T1 depending on the environment temperature, air flow rate, *etc.* In some instances, two or more such air temperature control systems may be installed around the capillary flow cell device or flow cell cartridge so that the capillary or cartridge may be rapidly cycled between several different temperatures by controlling which one of the air temperature control systems is active at a given time. In another approach, the temperature setting of the air temperature

control system may be varied so the temperature of the capillary flow cell or cartridge may be changed accordingly.

Fluid dispensing robotics

[0530] In some implementations, the disclosed systems may comprise an automated, programmable fluid-dispensing (or liquid-dispensing) system for use in dispensing reagents or other solutions into, *e.g.*, microplates, capillary flow cell devices and cartridges, microfluidic devices and cartridges, *etc.* Suitable automated, programmable fluid-dispensing systems are commercially available from a number of vendors, *e.g.*, Beckman Coulter, Perkin Elmer, Tecan, Velocity 11, and many others. In a preferred aspect of the disclosed systems, the fluid-dispensing system further comprises a multichannel dispense head, *e.g.*, a 4 channel, 8 channel, 16 channel, 96 channel, or 384 channel dispense head, for simultaneous delivery of programmable volumes of liquid (*e.g.*, ranging from about 1 microliter to several milliliters) to multiple wells or locations on a flow cell cartridge or microfluidic cartridge.

Cartridge- and/or microplate-handling (pick-and-place) robotics

[0531] In some implementations, the disclosed system may comprise a cartridge- and/or microplate-handling robotic system for automated replacement and positioning of microplates, capillary flow cell cartridges, or microfluidic device cartridges in relation to the optical imaging system, or for optionally moving microplates, capillary flow cell cartridges, or microfluidic device cartridges between the optical imaging system and a fluid-dispensing system. Suitable automated, programmable microplate-handling robotic systems are commercially available from a number of vendors, including Beckman Coulter, Perkin Elemer, Tecan, Velocity 11, and many others. In a preferred aspect of the disclosed systems, an automated microplate-handling robotic system is configured to move collections of microwell plates comprising samples and/or reagents to and from, *e.g.*, refrigerated storage units.

Spectroscopy or imaging modules

[0532] As indicated above, in some implementations the disclosed analysis systems may include optical imaging capabilities and may also include other spectroscopic measurement capabilities. For example, the disclosed imaging modules may be configured to operate in any of a variety of imaging modes known to those of skill in the art including, but not limited to, bright-field, dark-field, fluorescence, luminescence, or phosphorescence imaging. In some instances, the one or more capillary flow cells or microfluidic devices of a fluidics sub-system comprise a window

that allows at least a section of one or more capillaries or one or more fluid channels in each flow cell or microfluidic device to be illuminated and imaged.

[0533] In some embodiments, single wavelength excitation and emission fluorescence imaging may be performed. In some embodiments, dual wavelength excitation and emission (or multi-wavelength excitation or emission) fluorescence imaging may be performed. In some instances, the imaging module is configured to acquire video images. The choice of imaging mode may impact the design of the flow cells devices or cartridges in that all or a portion of the capillaries or cartridge can be optically transparent over the spectral range of interest. In some instances, a plurality of capillaries within a capillary flow cell cartridge may be imaged in their entirety within a single image. In some instances, only a single capillary or a subset of capillaries within a capillary flow cell cartridge, or portions thereof, may be imaged within a single image. In some instances, a series of images may be "tiled" to create a single high-resolution image of one, two, several, or the entire plurality of capillaries within a cartridge. In some instances, a plurality of fluid channels within a microfluidic chip may be imaged in their entirety within a single image. In some instances, only a single fluid channel or a subset of fluid channels within a microfluidic chip, or portions thereof, may be imaged within a single image. In some instances, a series of images may be "tiled" to create a single high-resolution image of one, two, several, or the entire plurality of fluid channels within a cartridge.

[0534] A spectroscopy or imaging module may comprise, *e.g.*, a microscope equipped with a CMOS of CCD camera. In some instances, the spectroscopy or imaging module may comprise, *e.g.*, a custom instrument such as one of the imaging modules described herein that is configured to perform a specific spectroscopic or imaging technique of interest. In general, the hardware associated with the spectroscopy or imaging module may include light sources, detectors, and other optical components, as well as processors or computers.

Light sources

[0535] Any of a variety of light sources may be used to provide the imaging or excitation light, including but not limited to, tungsten lamps, tungsten-halogen lamps, arc lamps, lasers, light emitting diodes (LEDs), or laser diodes. In some instances, a combination of one or more light sources, and additional optical components, *e.g.*, lenses, filters, apertures, diaphragms, mirrors, and the like, may be configured as an illumination system (or sub-system).

Detectors

[0536] Any of a variety of image sensors may be used for imaging purposes, including but not limited to, photodiode arrays, charge-coupled device (CCD) cameras, or complementary metal–oxide–semiconductor (CMOS) image sensors. As used herein, "imaging sensors" may be one-dimensional (linear) or two-dimensional array sensors. In many instances, a combination of one or more image sensors, and additional optical components, *e.g.*, lenses, filters, apertures, diaphragms, mirrors, and the like, may be configured as an imaging system (or sub-system). In some instances, *e.g.*, where spectroscopic measurements are performed by the system rather than imaging, suitable detectors may include, but are not limited to, photodiodes, avalanche photodiodes, and photomultipliers.

[0537] *Other optical components:* The hardware components of the spectroscopic measurement or imaging module may also include a variety of optical components for steering, shaping, filtering, or focusing light beams through the system. Examples of suitable optical components include, but are not limited to, lenses, mirrors, prisms, apertures, diffraction gratings, colored glass filters, long-pass filters, short-pass filters, bandpass filters, narrowband interference filters, broadband interference filters, dichroic reflectors, optical fibers, optical waveguides, and the like. In some instances, as noted above, the spectroscopic measurement or imaging module may further comprise one or more translation stages or other motion control mechanisms for the purpose of moving capillary flow cell devices and cartridges relative to the illumination and/or detection/imaging sub-systems, or *vice versa*.

[0538] *Total internal reflection:* In some instances, the optical module or sub-system may be designed to use all or a portion of an optically transparent wall of the capillaries or microfluidic channels in flow cell devices and cartridges as a waveguide for delivering excitation light to the capillary or channel lumen(s) via total internal reflection. When incident excitation light strikes the surface of the capillary or channel lumen at an angle with respect to a normal to the surface that is larger than the critical angle (determined by the relative refractive indices of the capillary or channel wall material and the aqueous buffer within the capillary or channel), total internal reflection occurs at the surface and the light propagates through the capillary or channel wall along the length of the capillary or channel. Total internal reflection generates an evanescent wave at the lumen surface which penetrates the lumen interior for extremely short distances, and which may be used to selectively excite fluorophores at the surface, *e.g.*, labeled nucleotides that have been incorporated by a polymerase into a growing oligonucleotide through a solid-phase primer extension reaction.

[0539] *Light-tight housings and environmental control chambers:* In some implementations, the disclosed systems may comprise a light-tight housing to prevent stray ambient light from

creating glare and obscuring, *e.g.*, relatively faint fluorescence signals. In some implementations, the disclosed systems may comprise an environmental control chamber that enables the system to operate under a tightly controlled temperature, humidity level, *etc.*

Processors and computers

[0540] In some instances, the disclosed systems may comprise one or more processors or computers. The processor may be a hardware processor such as a central processing unit (CPU), a graphic processing unit (GPU), a general-purpose processing unit, or a computing platform. The processor may be comprised of any of a variety of suitable integrated circuits, microprocessors, logic devices, field-programmable gate arrays (FPGAs) and the like. In some instances, the processor may be a single core or multi core processor, or a plurality of processors may be configured for parallel processing. Although the disclosure is described with reference to a processor, other types of integrated circuits and logic devices are also applicable. The processor may have any suitable data operation capability. For example, the processor may perform 512 bit, 256 bit, 128 bit, 64 bit, 32 bit, or 16 bit data operations.

[0541] The processor or CPU can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location. The instructions can be directed to the CPU, which can subsequently program or otherwise configure the CPU to implement, *e.g.*, the system control methods of the present disclosure. Examples of operations performed by the CPU can include fetch, decode, execute, and write back.

[0542] Some processors may comprise a processing unit of a computer system. The computer system may enable cloud-based data storage and/or computing. In some instances, the computer system may be operatively coupled to a computer network ("network") with the aid of a communication interface. The network may be the internet, an intranet and/or extranet, an intranet and/or extranet that is in communication with the internet, or a local area network (LAN). The network in some cases is a telecommunication and/or data network. The network may include one or more computer servers, which may enable distributed computing, such as cloud-based computing.

[0543] The computer system may also include computer memory or memory locations (*e.g.*, random-access memory, read-only memory, flash memory), electronic storage units (*e.g.*, hard disk), communication interfaces (*e.g.*, network adapters) for communicating with one or more other systems, and peripheral devices, such as cache, other memory units, data storage units and/or electronic display adapters. In some instances, the communication interface may allow

the computer to be in communication with one or more additional devices. The computer may be able to receive input data from the coupled devices for analysis. Memory units, storage units, communication interfaces, and peripheral devices may be in communication with the processor or CPU through a communication bus (solid lines), such as may be incorporated into a motherboard. A memory or storage unit may be a data storage unit (or data repository) for storing data. The memory or storage units may store files, such as drivers, libraries and saved programs. The memory or storage units may store user data, *e.g.*, user preferences and user programs.

[0544] The system control, image processing, and/or data analysis methods as described herein can be implemented by way of machine-executable code stored in an electronic storage location of the computer system, such as, for example, in the memory or electronic storage unit. The machine-executable or machine-readable code can be provided in the form of software. During use, the code can be executed by the processor. In some cases, the code can be retrieved from the storage unit and stored in memory for ready access by the processor. In some situations, the electronic storage unit can be precluded, and machine-executable instructions are stored in memory.

[0545] In some instances, the code may be pre-compiled and configured for use with a machine having a processor adapted to execute the code. In some instances, the code may be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

[0546] Some aspects of the systems and methods provided herein can be embodied in software. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine-readable medium. Machine-executable code can be stored on an electronic storage unit, such as memory (*e.g.*, read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over

various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

[0547] In some instances, the system control, image processing, and/or data analysis methods of the present disclosure may be implemented by way of one or more algorithms. An algorithm may be implemented by way of software upon execution by the central processing unit.

[0548] The present disclosure provides computers, processors, or systems that are programmed to implement methods of the disclosure. FIG. 63 shows a computer or computer system 6301 that is programmed or otherwise configured to acquire images of sample support structures, e.g., multiple surface flow cell, to process, store, and communicate images to other electronic devices. The computer system 6301 can regulate various aspects of the present disclosure. The computer system 6301 can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device.

[0549] The computer system 6301 includes a central processing unit (CPU, also "processor" and "computer processor" herein) 6305, which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system 6301 also includes memory or memory location 6310 (e.g., random-access memory, read-only memory, flash memory), electronic storage unit 6315 (e.g., hard disk), communication interface 6320 (e.g., network adapter) for communicating with one or more other systems, and peripheral devices 6325, such as cache, other memory, data storage and/or electronic display adapters. The memory 6310, storage unit 6315, interface 6320 and peripheral devices 6325 are in communication with the CPU 6305 through a communication bus (solid lines), such as a motherboard. The storage unit 6315 can be a data storage unit (or data repository) for storing data. The computer system 6301 can be operatively coupled to a computer network ("network") 6330 with the aid of the communication interface 6320. The network 6330 can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network 6330 in some cases is a telecommunication and/or data network. The network 6330 can include one or more computer servers, which can enable distributed computing, such as cloud computing. The network 6330, in some cases with the aid of the computer system 6301, can implement a peer-to-peer network, which may enable devices coupled to the computer system 6301 to behave as a client or a server.

[0550] The CPU 6305 can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory 6310. The instructions can be directed to the CPU 6305, which can subsequently program or otherwise configure the CPU 6305 to implement methods of the present disclosure. Examples of operations performed by the CPU 6305 can include fetch, decode, execute, and writeback.

[0551] The CPU 6305 can be part of a circuit, such as an integrated circuit. One or more other components of the system 6301 can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

[0552] The storage unit 6315 can store files, such as drivers, libraries and saved programs. The storage unit 6315 can store user data, e.g., user preferences and user programs. The computer system 6301 in some cases can include one or more additional data storage units that are external to the computer system 6301, such as located on a remote server that is in communication with the computer system 6301 through an intranet or the Internet.

[0553] The computer system 6301 can communicate with one or more remote computer systems through the network 6330. For instance, the computer system 6301 can communicate with a remote computer system of a user. Examples of remote computer systems include personal computers (e.g., portable PC), slate or tablet PC's (e.g., Apple® iPad, Samsung® Galaxy Tab), telephones, Smart phones (e.g., Apple® iPhone, Android-enabled device, Blackberry®), or personal digital assistants. The user can access the computer system 6301 via the network 6330.

[0554] Methods as described herein can be implemented by way of machine (e.g., computer processor) executable code stored on an electronic storage location of the computer system 6301, such as, for example, on the memory 6310 or electronic storage unit 6315. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor 6305. In some cases, the code can be retrieved from the storage unit 6315 and stored on the memory 6310 for ready access by the processor 6305. In some situations, the electronic storage unit 6315 can be precluded, and machine-executable instructions are stored on memory 6310.

[0555] The code can be pre-compiled and configured for use with a machine having a processer adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

[0556] Aspects of the systems and methods provided herein, such as the computer system 6301, can be embodied in programming. Various aspects of the technology may be thought of as "products" or "articles of manufacture" typically in the form of machine (or processor)

executable code and/or associated data that is carried on or embodied in a type of machine readable medium. Machine-executable code can be stored on an electronic storage unit, such as memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

[0557] Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, etc. shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

[0558] The computer system 6301 can include or be in communication with an electronic display 6335 that comprises a user interface (UI) 6340 for providing, for example, a control interface for an optical system. Examples of UI's include, without limitation, a graphical user interface (GUI) and web-based user interface.

[0559] Methods and systems of the present disclosure can be implemented by way of one or more algorithms. An algorithm can be implemented by way of software upon execution by the central processing unit 6305. The algorithm can, for example, control an optical system.

[0560] *System control software:* In some instances, the system may comprise a computer (or processor) and a computer-readable medium that includes code for providing a user interface as well as manual, semi-automated, or fully-automated control of all system functions, *e.g.*, control of the fluid flow control module(s), the temperature control module(s), and/or the spectroscopy or imaging module(s), as well as other data analysis and display options. The system computer or processor may be an integrated component of the system (*e.g.*, a microprocessor or mother board embedded within the instrument) or may be a stand-alone module, for example, a main frame computer, a personal computer, or a laptop computer. Examples of fluid flow control functions provided by the system control software include, but are not limited to, volumetric fluid flow rates, fluid flow velocities, the timing and duration for sample and reagent addition, buffer addition, and rinse steps. Examples of temperature control functions provided by the system control software include, but are not limited to, specifying temperature set point(s) and control of the timing, duration, and ramp rates for temperature changes. Examples of spectroscopic measurement or imaging control functions provided by the system control software include, but are not limited to, autofocus capability, control of illumination or excitation light exposure times and intensities, control of image acquisition rate, exposure time, and data storage options.

[0561] *Image processing software:* In some instances, the system may further comprise a computer (or processor) and computer-readable medium that includes code for providing image processing and analysis capability. Examples of image processing and analysis capability that may be provided by the software include, but are not limited to, manual, semi-automated, or fully-automated image exposure adjustment (*e.g.* white balance, contrast adjustment, signal-averaging and other noise reduction capability, *etc.*), automated edge detection and object identification (*e.g.*, for identifying clonally-amplified clusters of fluorescently-labeled oligonucleotides on the lumen surface of capillary flow cell devices), automated statistical analysis (*e.g.*, for determining the number of clonally-amplified clusters of oligonucleotides identified per unit area of the capillary lumen surface, or for automated nucleotide base-calling

in nucleic acid sequencing applications), and manual measurement capabilities (*e.g.* for measuring distances between clusters or other objects, *etc.*). Optionally, instrument control and image processing/analysis software may be written as separate software modules. In some embodiments, instrument control and image processing/analysis software may be incorporated into an integrated package.

[0562] Any of a variety of image processing methods known to those of skill in the art may be used for image processing / pre-processing. Examples include, but are not limited to, Canny edge detection methods, Canny-Deriche edge detection methods, first-order gradient edge detection methods (*e.g.*, the Sobel operator), second order differential edge detection methods, phase congruency (phase coherence) edge detection methods, other image segmentation algorithms (*e.g.*, intensity thresholding, intensity clustering methods, intensity histogram-based methods, *etc.*), feature and pattern recognition algorithms (*e.g.*, the generalized Hough transform for detecting arbitrary shapes, the circular Hough transform, *etc.*), and mathematical analysis algorithms (*e.g.*, Fourier transform, fast Fourier transform, wavelet analysis, auto-correlation, *etc.*), or any combination thereof.

Nucleic acid sequencing systems and applications

[0563] Nucleic acid sequencing provides one non-limiting example of an application for the disclosed flow cell devices (*e.g.*, capillary flow cell devices or cartridges and microfluidic devices and cartridges) and imaging systems. Many "second generation" and "third generation" sequencing technologies utilize a massively parallel, cyclic array approach to perform sequencing-by-nucleotide incorporation, in which accurate decoding of a single-stranded template oligonucleotide sequence tethered to a solid support relies on successfully classifying signals that arise from the stepwise addition of A, G, C, and T nucleotides by a polymerase to a complementary oligonucleotide strand. These methods typically require the oligonucleotide template to be modified with a known adapter sequence of fixed length, affixed to a solid support (*e.g.*, the lumen surface(s) of the disclosed capillary flow cell devices or microfluidic chips) in a random or patterned array by hybridization to surface-tethered capture probes (also referred to herein as "adapters" or "primers" tethered to the interior flow cell surfaces) of known sequence that are complementary to that of the adapter sequence, and then probed through a cyclic series of single base addition primer extension reactions that use, *e.g.*, fluorescently-labeled nucleotides to identify the sequence of bases in the template oligonucleotides. These processes thus require the use of miniaturized fluidics systems that offer precise, reproducible control of the timing of reagent introduction to the flow cell in which the sequencing reactions are performed, and small volumes to reduce or minimize the consumption of costly reagents.

[0564] Existing commercially-available NGS flow cells are constructed from layers of glass that have been etched, lapped, and/or processed by other methods to meet the tight dimensional tolerances required for imaging, cooling, and/or other requirements. When flow cells are used as consumables, the costly manufacturing processes required for their fabrication result in costs per sequencing run that are too high to make sequencing routinely accessible to scientists and medical professionals in the research and clinical fields.

[0565] This disclosure provides an example of a low-cost flow cell architecture that includes low cost glass or polymer capillaries or microfluidic channels, fluidics adapters, and cartridge chassis. Utilizing glass or polymer capillaries that are extruded in their final cross-sectional geometry may eliminate the need for multiple high-precision and costly glass manufacturing processes. Robustly constraining the orientation of the capillaries or microfluidic channels and providing convenient fluidic connections using molded plastic and/or elastomeric components further reduces cost. Laser bonding the components of the polymer cartridge chassis provides a fast and efficient sealing of the capillary or the microfluidic channels and structurally stabilizing the capillaries or channels and flow cell cartridge without requiring the use of fasteners or adhesives.

[0566] The disclosed devices and systems may be configured to perform nucleic acid sequencing using any of a variety of "sequencing-by-nucleotide incorporation", "sequencing-by-nucleotide binding", "sequencing-by-nucleotide base-pairing", and "sequencing-by-avidity" sequencing biochemistries. The improvements in flow cell device design disclosed herein, *e.g.*, comprising hydrophilic coated surfaces that maximize foreground signals for, *e.g.*, fluorescently-labeled nucleic acid clusters disposed thereon, while minimizing background signal may give rise to improvements in CNR for images used for base-calling purposes, in combination with improvements in optical imaging system design for fast multiple-surface flow cell imaging (comprising simultaneous or near-simultaneous imaging of the interior flow cell surfaces) achieved through improved objective lens and/or tube lens designs that provide for larger depth of field and larger fields-of-view, and reduced reagent consumption (achieved through improved flow cell design) may give rise to dramatic improvements in base-calling accuracy, shortened imaging cycle times, shortened overall sequencing reaction cycle times, and higher throughput nucleic acid sequencing at reduced cost per base.

[0567] The systems disclosed herein may be configured to implement any of a variety of different sequencing methodologies using a variety of different sequencing chemistries. For example, FIG. 40 provides a non-limiting example of a flow chart for implementing a sequencing-by-avidity method. A nucleotide conjugate may be used to form a multivalent binding complex with a plurality of primed target nucleic acid sequences tethered to a support

surface, *e.g.*, one or more interior surfaces of a flow cell, such that the multivalent binding complex exhibits a significantly longer persistence time than afforded by the binding interactions between single nucleotides and single primed target nucleic acid sequences. In general, such a sequencing-by-avidity approach will comprise one or more of the following steps: hybridization of target nucleic acid sequences to adapter / primer sequences tethered to the support surface; clonal amplification to create clusters of amplified target sequences on the support surface; contacting the support surface with a nucleotide conjugate comprising a plurality of nucleotide moieties conjugated to a polymer core, wherein the nucleotide conjugate may further comprise one or more detectable labels, *e.g.*, fluorophores, to create a stable, multivalent binding complex; washing out of any excess, unbound nucleotide conjugate; detection of multivalent binding complexes, *e.g.*, by fluorescence imaging of the support surface; identification of a nucleotide in the target nucleic acid sequence (base-calling); destabilization of the multivalent binding complex, *e.g.*, by changing the ionic strength, ionic composition, and/or pH of the buffer; rinsing of the flow cell; and performing a primer extension reaction to add a nucleotide comprising the complementary base for the nucleotide that was identified. The cycle may be repeated to identify additional nucleotide bases in the sequence, followed by processing and assembly of the sequence data. In some instances, data processing may comprise calculation of sequencing performance metrics, such as a Q-score, in real-time as the sequencing run is performed or as part of a post-run data processing step.

[0568] In some instances, the disclosed hydrophilic, polymer coated flow cell devices used in combination with the optical imaging systems disclosed herein may confer one or more of the following additional advantages for a nucleic acid sequencing system: (i) decreased fluidic wash times (due to reduced non-specific binding, and thus faster sequencing cycle times), (ii) decreased imaging times (and thus faster turnaround times for assay readout and sequencing cycles), (iii) decreased overall work flow time requirements (due to decreased cycle times), (iv) decreased detection instrumentation costs (due to the improvements in CNR), (v) improved readout (base-calling) accuracy (due to improvements in CNR), (vi) improved reagent stability and decreased reagent usage requirements (and thus reduced reagents costs), and (vii) fewer run-time failures due to nucleic acid amplification failures.

[0569] The methods, devices, and systems disclosed herein for performing nucleic acid sequencing are suitable for a variety of sequencing applications and for sequencing nucleic acid molecules derived from any of a variety of samples and sources. Nucleic acids, in some instances, may be extracted from any of a variety of biological samples, *e.g.*, blood samples, saliva samples, urine samples, cell samples, tissue samples, and the like. For example, the disclosed devices and systems may be used for the analysis of nucleic acid molecules derived

from any of a variety of different cell, tissue, or sample types known to those of skill in the art. For example, nucleic acids may be extracted from cells, or tissue samples comprising one or more types of cells, derived from eukaryotes (such as animals, plants, fungi, protista), archaebacteria, or eubacteria. In some cases, nucleic acids may be extracted from prokaryotic or eukaryotic cells, such as adherent or non-adherent eukaryotic cells. Nucleic acids are variously extracted from, for example, primary or immortalized rodent, porcine, feline, canine, bovine, equine, primate, or human cell lines. Nucleic acids may be extracted from any of a variety of different cell, organ, or tissue types (*e.g.*, white blood cells, red blood cells, platelets, epithelial cells, endothelial cells, neurons, glial cells, astrocytes, fibroblasts, skeletal muscle cells, smooth muscle cells, gametes, or cells from the heart, lungs, brain, liver, kidney, spleen, pancreas, thymus, bladder, stomach, colon, or small intestine). Nucleic acids may be extracted from normal or healthy cells. Alternatively or in combination, acids are extracted from d. ased cells, such as cancerous cells, or from pathogenic cells that are infecting a host. Some nucleic acids may be extracted from a distinct subset of cell types, *e.g.*, immune cells (such as T cells, cytotoxic (killer) T cells, helper T cells, alpha beta T cells, gamma delta T cells, T cell progenitors, B cells, B-cell progenitors, lymphoid stem cells, myeloid progenitor cells, lymphocytes, granulocytes, Natural Killer cells, plasma cells, memory cells, neutrophils, eosinophils, basophils, mast cells, monocytes, dendritic cells, and/or macrophages, or any combination thereof), undifferentiated human stem cells, human stem cells that have been induced to differentiate, rare cells (*e.g.*, circulating tumor cells (CTCs), circulating epithelial cells, circulating endothelial cells, circulating endometrial cells, bone marrow cells, progenitor cells, foam cells, mesenchymal cells, or trophoblasts). Other cells are contemplated and consistent with the disclosure herein.

[0570] Nucleic acids may optionally be attached to one or more non-nucleotide moieties such as labels and other small molecules, large molecules (such as proteins, lipids, sugars, *etc.*), and solid or semi-solid supports, for example through covalent or non-covalent linkages with either the 5′ or 3′ end of the nucleic acid. Labels include any moiety that is detectable using any of a variety of detection methods known to those of skill in the art, and thus renders the attached oligonucleotide or nucleic acid similarly detectable. Some labels, *e.g.*, fluorophores, emit electromagnetic radiation that is optically detectable or visible. Alternatively or in combination, some labels comprise a mass tag that renders the labeled oligonucleotide or nucleic acid visible in mass spectral data, or a redox tag that renders the labeled oligonucleotide or nucleic acid detectable by amperometry or voltammetry. Some labels comprise a magnetic tag that facilitates separation and/or purification of the labeled oligonucleotide or nucleic acid. The

nucleotide or polynucleotide is often not attached to a label, and the presence of the oligonucleotide or nucleic acid is directly detected.

[0571] *Flow cell devices configured for sequencing:* In some instances, one or more flow cell devices according to the present disclosure may be configured for nucleic acid sequencing applications, *e.g.*, wherein two or more interior flow cell device surfaces comprise hydrophilic polymer coatings that further comprise one or more capture oligonucleotides, *e.g.*, adapter/primer oligonucleotides, or any other oligonucleotides as disclosed herein. In some instances, the hydrophilic, polymer-coated surfaces of the disclosed flow cell devices may comprise a plurality of oligonucleotides tethered thereto that have been selected for use in sequencing a eukaryotic genome. In some instances, the hydrophilic, polymer-coated surfaces of the disclosed flow cell devices may comprise a plurality of oligonucleotides tethered thereto that have been selected for use in sequencing a prokaryotic genome or portion thereof. In some instances, the hydrophilic, polymer-coated surfaces of the disclosed flow cell devices may comprise a plurality of oligonucleotides tethered thereto that have been selected for use in sequencing a viral genome or portion thereof. In some instances, the hydrophilic, polymer-coated surfaces of the disclosed flow cell devices may comprise a plurality of oligonucleotides tethered thereto that have been selected for use in sequencing a transcriptome.

[0572] In some instances, a flow cell device of the present disclosure may comprise a first surface in an orientation generally facing the interior of a first flow channel, a second surface in an orientation generally facing the interior of the first flow channel and further generally facing or parallel to the first surface, a third surface generally facing the interior of a second flow channel, and a fourth surface, generally facing the interior of the second flow channel and generally opposed to or parallel to the third surface; wherein said second and third surfaces may be located on or attached to opposite sides of a generally planar substrate, e.g., an interposer substrate, which may be a reflective, transparent, or translucent substrate. In some instances, an imaging surface or imaging surfaces within a flow cell may be located within the center of a flow cell or within or as part of a division between two subunits or subdivisions of a flow cell, wherein said flow cell may comprise a top surface and a bottom surface, one or both of which may be transparent to such detection mode as may be utilized; and wherein a surface comprising oligonucleotides adapters/primers tethered to one or more polymer coatings may be placed or interposed within the lumen of the flow cell. In some instances, the top and/or bottom surfaces do not include attached oligonucleotide adapters/primers. In some instances, said top and/or bottom surfaces do comprise attached oligonucleotide adapters/primers. In some instances, either said top or said bottom surface may comprise attached oligonucleotide adapters/primers.

A surface or surfaces placed or interposed within the lumen of a flow cell may be located on or attached to one side, to an opposite side, or to both sides of a generally planar substrate which may be a reflective, transparent, or translucent substrate.

[0573] In general, at least one layer of the one or more layers of low nonspecific binding coating on the flow cell device surfaces may comprise functional groups for covalently or non-covalently attaching oligonucleotide molecules, *e.g.*, adapter or primer sequences, or the at least one layer may already comprise covalently or non-covalently attached oligonucleotide adapter or primer sequences at the time that it is deposited on the support surface. In some instances, the oligonucleotides tethered to the polymer molecules of at least one third layer may be distributed at a plurality of depths throughout the layer.

[0574] In some instances, the oligonucleotide adapter or primer molecules are covalently coupled to the polymer in solution, *e.g.*, prior to coupling or depositing the polymer on the surface. In some instances, the oligonucleotide adapter or primer molecules are covalently coupled to the polymer after it has been coupled to or deposited on the surface. In some instances, at least one hydrophilic polymer layer comprises a plurality of covalently-attached oligonucleotide adapter or primer molecules. In some instances, at least two, at least three, at least four, or at least five layers of hydrophilic polymer comprise a plurality of covalently-attached adapter or primer molecules.

[0575] In some instances, the oligonucleotide adapter or primer molecules may be coupled to the one or more layers of hydrophilic polymer using any of a variety of suitable conjugation chemistries known to those of skill in the art. For example, the oligonucleotide adapter or primer sequences may comprise moieties that are reactive with amine groups, carboxyl groups, thiol groups, and the like. Examples of suitable amine-reactive conjugation chemistries that may be used include, but are not limited to, reactions involving isothiocyanate, isocyanate, acyl azide, NHS ester, sulfonyl chloride, aldehyde, glyoxal, epoxide, oxirane, carbonate, aryl halide, imidoester, carbodiimide, anhydride, and fluorophenyl ester groups. Examples of suitable carboxyl-reactive conjugation chemistries include, but are not limited to, reactions involving carbodiimide compounds, *e.g.*, water soluble EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCL). Examples of suitable sulfydryl-reactive conjugation chemistries include maleimides, haloacetyls and pyridyl disulfides.

[0576] One or more types of oligonucleotide molecules may be attached or tethered to the support surface. In some instances, the one or more types of oligonucleotide adapters or primers may comprise spacer sequences, adapter sequences for hybridization to adapter-ligated template library nucleic acid sequences, forward amplification primers, reverse amplification primers, sequencing primers, and/or molecular barcoding sequences, or any combination thereof. In

some instances, 1 primer or adapter sequence may be tethered to at least one layer of the surface. In some instances, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 different primer or adapter sequences may be tethered to at least one layer of the surface.

[0577] In some instances, the tethered oligonucleotide adapter and/or primer sequences may range in length from about 10 nucleotides to about 100 nucleotides. In some instances, the tethered oligonucleotide adapter and/or primer sequences may be at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides in length. In some instances, the tethered oligonucleotide adapter and/or primer sequences may be at most 100, at most 90, at most 80, at most 70, at most 60, at most 50, at most 40, at most 30, at most 20, or at most 10 nucleotides in length. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the length of the tethered oligonucleotide adapter and/or primer sequences may range from about 20 nucleotides to about 80 nucleotides. Those of skill in the art will recognize that the length of the tethered oligonucleotide adapter and/or primer sequences may have any value within this range, *e.g.*, about 24 nucleotides.

[0578] In some instances, the number of coating layers and/or the material composition of each layer is chosen so as to adjust the resultant surface density of oligonucleotide adapters/primers (or other attached molecules) on the coated interior flow cell surfaces. In some instances, the surface density of oligonucleotide adapters/primers may range from about 1,000 primer molecules per μm^2 to about 1,000,000 primer molecules per μm^2. In some instances, the surface density of oligonucleotide primers may be at least 1,000, at least 10,000, at least 100,000, or at least 1,000,000 molecules per μm^2. In some instances, the surface density of oligonucleotide primers may be at most 1,000,000, at most 100,000, at most 10,000, or at most 1,000 molecules per μm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of primers may range from about 10,000 molecules per μm^2 to about 100,000 molecules per μm^2. Those of skill in the art will recognize that the surface density of primer molecules may have any value within this range, *e.g.*, about 455,000 molecules per μm^2. In some instances, the surface properties of the capillary or channel lumen coating, including the surface density of tethered oligonucleotide primers, may be adjusted to improve or optimize, *e.g.*, solid-phase nucleic acid hybridization specificity and efficiency, and/or solid-phase nucleic acid amplification rate, specificity, and efficiency.

[0579] In some embodiments, the hydrophilic coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density of greater than 8, 000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70, 000, 80,000, or 90,000. In some embodiments, the hydrophilic

coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density of greater than 100,000, 200,000, 300,000, 400,000, 500,000, or 600,000 nucleic acid polonies/mm^2. In some embodiments, the hydrophilic coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density in a range from 10,000 to 900,000 nucleic acid polonies/mm^2.

[0580] In some instances, the tethered adapter or primer sequences may comprise modifications designed to facilitate the specificity and efficiency of nucleic acid amplification as performed on the low-binding supports. For example, in some instances the primer may comprise polymerase stop points such that the stretch of primer sequence between the surface conjugation point and the modification site is always in single-stranded form and functions as a loading site for 5' to 3' helicases in some helicase-dependent isothermal amplification methods. Other examples of primer modifications that may be used to create polymerase stop points include, but are not limited to, an insertion of a PEG chain into the backbone of the primer between two nucleotides towards the 5' end, insertion of an abasic nucleotide (*e.g.*, a nucleotide that has neither a purine nor a pyrimidine base), or a lesion site which can be bypassed by the helicase.

Nucleic acid hybridization

[0581] In some instances, the hydrophilic, polymer coated flow cell device surfaces disclosed herein may provide advantages when used alone or in combination with improved buffer formulations for performing solid-phase nucleic acid hybridization and/or solid-phase nucleic acid amplification reactions as part of genotyping or nucleic acid sequencing applications. In some instances, the polymer-coated flow cell devices disclosed herein may provide advantages in terms of improved nucleic acid hybridization rate and specificity, and improved nucleic acid amplification rates and specificity that may be achieved through one or more of the following additional aspects of the present disclosure: (i) primer design (*e.g.*, sequence and/or modifications), (ii) control of tethered primer density on the solid support, (iii) the surface composition of the solid support, (iv) the surface polymer density of the solid support, (v) the use of improved hybridization conditions before and during amplification, and/or (vi) the use of improved amplification formulations that decrease non-specific primer amplification or increase template amplification efficiency.

[0582] In some instances, it may be desirable to vary the surface density of tethered oligonucleotide adapters or primers on the coated flow cell surfaces and/or the spacing of the tethered adapters or primers away from the coated flow cell surface (*e.g.*, by varying the length of a linker molecule used to tether the adapter or primers to the surface) in order to "tune" the support for optimal performance when, *e.g.*, using a given amplification method. In some

instances, adjusting the surface density of tethered oligonucleotide adapters or primers may impact the level of specific and/or non-specific amplification observed on the surface in a manner that varies according to the amplification method selected. In some instances, the surface density of tethered oligonucleotide adapters or primers may be varied by adjusting the ratio of molecular components used to create the support surface. For example, in the case that an oligonucleotide primer – PEG conjugate is used to create the final layer of a low-binding support, the ratio of the oligonucleotide primer – PEG conjugate to a non-conjugated PEG molecule may be varied. The resulting surface density of tethered primer molecules may then be estimated or measured using any of a variety of techniques known to those of skill in the art. Examples include, but are not limited to, the use of radioisotope labeling and counting methods, covalent coupling of a cleavable molecule that comprises an optically-detectable tag (*e.g.*, a fluorescent tag) that may be cleaved from a support surface of defined area, collected in a fixed volume of an appropriate solvent, and then quantified by comparison of fluorescence signals to that for a calibration solution of known optical tag concentration, or using fluorescence imaging techniques provided that care has been taken with the labeling reaction conditions and image acquisition settings to ensure that the fluorescence signals are linearly related to the number of fluorophores on the surface (*e.g.*, that there is no significant self-quenching of the fluorophores on the surface).

[0583] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, either alone or in combination with improved or optimized buffer formulations, may yield relative hybridization rates that range from about 2x to about 20x faster than that for a conventional hybridization protocol. In some instances, the relative hybridization rate may be at least 2x, at least 3x, at least 4x, at least 5x, at least 6x, at least 7x, at least 8x, at least 9x, at least 10x, at least 12x, at least 14x, at least 16x, at least 18x, at least 20x, at least 25x, at least 30x, or at least 40x that for a conventional hybridization protocol.

[0584] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, either alone or in combination with improved or optimized buffer formulations, may yield total hybridization reaction times (*e.g.*, the time required to reach 90%, 95%, 98%, or 99% completion of the hybridization reaction) of less than 60 minutes, 50 minutes, 40 minutes, 30 minutes, 20 minutes, 15 minutes, 10 minutes, or 5 minutes for any of these completion metrics.

[0585] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, either alone or in combination with improved or optimized buffer formulations, may yield improved hybridization specificity compared to that for a conventional hybridization protocol. In some instances, the hybridization specificity that may be achieved is better than 1 base mismatch in 10 hybridization events, 1 base mismatch in20 hybridization events, 1 base

mismatch in 30 hybridization events, 1 base mismatch in 40 hybridization events, 1 base mismatch in 50 hybridization events, 1 base mismatch in 75 hybridization events, 1 base mismatch in 100 hybridization events, 1 base mismatch in 200 hybridization events, 1 base mismatch in 300 hybridization events, 1 base mismatch in 400 hybridization events, 1 base mismatch in 500 hybridization events, 1 base mismatch in 600 hybridization events, 1 base mismatch in 700 hybridization events, 1 base mismatch in 800 hybridization events, 1 base mismatch in 900 hybridization events, 1 base mismatch in 1,000 hybridization events, 1 base mismatch in 2,000 hybridization events, 1 base mismatch in 3,000 hybridization events, 1 base mismatch in 4,000 hybridization events, 1 base mismatch in 5,000 hybridization events, 1 base mismatch in 6,000 hybridization events, 1 base mismatch in 7,000 hybridization events, 1 base mismatch in 8,000 hybridization events, 1 base mismatch in 9,000 hybridization events, or 1 base mismatch in 10,000 hybridization events.

[0586] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, either alone or in combination with improved or optimized buffer formulations, may yield improved hybridization efficiency (*e.g.*, the fraction of available oligonucleotide primers on the support surface that are successfully hybridized with target oligonucleotide sequences) compared to that for a conventional hybridization protocol. In some instances, the hybridization efficiency that may be achieved is better than 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, or 99% for any of the input target oligonucleotide concentrations specified below and in any of the hybridization reaction times specified above. In some instances, *e.g.*, wherein the hybridization efficiency is less than 100%, the resulting surface density of target nucleic acid sequences hybridized to the support surface may be less than the surface density of oligonucleotide adapter or primer sequences on the surface.

[0587] In some instances, use of the disclosed hydrophilic, polymer-coated flow cell devices for nucleic acid hybridization (or nucleic acid amplification) applications using conventional hybridization (or amplification) protocols, or improved or optimized hybridization (or amplification) protocols, may lead to a reduced requirement for the input concentration of target (or sample) nucleic acid molecules contacted with the support surface. For example, in some instances, the target (or sample) nucleic acid molecules may be contacted with the support surface at a concentration ranging from about 10 pM to about 1 μM (*e.g.*, prior to annealing or amplification). In some instances, the target (or sample) nucleic acid molecules may be administered at a concentration of at least 10 pM, at least 20 pM, at least 30 pM, at least 40 pM, at least 50 pM, at least 100 pM, at least 200 pM, at least 300 pM, at least 400 pM, at least 500 pM, at least 600 pM, at least 700 pM, at least 800pM, at least 900 pM, at least 1 nM, at least 10 nM, at least 20 nM, at least 30 nM, at least 40 nM, at least 50 nM, at least 60 nM, at least 70 nM,

at least 80 nM, at least 90 nM, at least 100 nM, at least 200 nM, at least 300 nM, at least 400 nM, at least 500 nM, at least 600 nM, at least 700 nM, at least 800 nM, at least 900 nM, or at least 1 μM. In some instances, the target (or sample) nucleic acid molecules may be administered at a concentration of at most 1 μM, at most 900 nM, at most 800 nm, at most 700 nM, at most 600 nM, at most 500 nM, at most 400 nM, at most 300 nM, at most 200 nM, at most 100 nM, at most 90 nM, at most 80 nM, at most 70 nM, at most 60 nM, at most 50 nM, at most 40 nM, at most 30 nM, at most 20 nM, at most 10 nM, at most 1 nM, at most 900 pM, at most 800 pM, at most 700 pM, at most 600 pM, at most 500 pM, at most 400 pM, at most 300 pM, at most 200 pM, at most 100 pM, at most 90 pM, at most 80 pM, at most 70 pM, at most 60 pM, at most 50 pM, at most 40 pM, at most 30 pM, at most 20 pM, or at most 10 pM. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the target (or sample) nucleic acid molecules may be administered at a concentration ranging from about 90 pM to about 200 nM. Those of skill in the art will recognize that the target (or sample) nucleic acid molecules may be administered at a concentration having any value within this range, *e.g.*, about 855 nM.

[0588] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, either alone or in combination with improved or optimized hybridization buffer formulations, may result in a surface density of hybridized target (or sample) oligonucleotide molecules (*e.g.*, prior to performing any subsequent solid-phase or clonal amplification reaction) ranging from about from about 0.0001 target oligonucleotide molecules per μm^2 to about 1,000,000 target oligonucleotide molecules per μm^2. In some instances, the surface density of hybridized target oligonucleotide molecules may be at least 0.0001, at least 0.0005, at least 0.001, at least 0.005, at least 0.01, at least 0.05, at least 0.1, at least 0.5, at least 1, at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, at least 1,000, at least 1,500, at least 2,000, at least 2,500, at least 3,000, at least 3,500, at least 4,000, at least 4,500, at least 5,000, at least 5,500, at least 6,000, at least 6,500, at least 7,000, at least 7,500, at least 8,000, at least 8,500, at least 9,000, at least 9,500, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, at least 60,000, at least 65,000, at least 70,000, at least 75,000, at least 80,000, at least 85,000, at least 90,000, at least 95,000, at least 100,000, at least 150,000, at least 200,000, at least 250,000, at least 300,000, at least 350,000, at least 400,000, at least 450,000, at least 500,000, at least 550,000, at least 600,000, at least 650,000, at least 700,000, at least 750,000, at least 800,000, at least 850,000, at least

900,000, at least 950,000, or at least 1,000,000 molecules per μm^2. In some instances, the surface density of hybridized target oligonucleotide molecules may be at most 1,000,000, at most 950,000, at most 900,000, at most 850,000, at most 800,000, at most 750,000, at most 700,000, at most 650,000, at most 600,000, at most 550,000, at most 500,000, at most 450,000, at most 400,000, at most 350,000, at most 300,000, at most 250,000, at most 200,000, at most 150,000, at most 100,000, at most 95,000, at most 90,000, at most 85,000, at most 80,000, at most 75,000, at most 70,000, at most 65,000, at most 60,000, at most 55,000, at most 50,000, at most 45,000, at most 40,000, at most 35,000, at most 30,000, at most 25,000, at most 20,000, at most 15,000, at most 10,000, at most 9,500, at most 9,000, at most 8,500, at most 8,000, at most 7,500, at most 7,000, at most 6,500, at most 6,000, at most 5,500, at most 5,000, at most 4,500, at most 4,000, at most 3,500, at most 3,000, at most 2,500, at most 2,000, at most 1,500, at most 1,000, at most 900, at most 800, at most 700, at most 600, at most 500, at most 400, at most 300, at most 200, at most 100, at most 90, at most 80, at most 70, at most 60, at most 50, at most 40, at most 30, at most 20, at most 10, at most 5, at most 1, at most 0.5, at most 0.1, at most 0.05, at most 0.01, at most 0.005, at most 0.001, at most 0.0005, or at most 0.0001 molecules per μm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of hybridized target oligonucleotide molecules may range from about 3,000 molecules per μm^2 to about 20,000 molecules per μm^2. Those of skill in the art will recognize that the surface density of hybridized target oligonucleotide molecules may have any value within this range, *e.g.*, about 2,700 molecules per μm2.

[0589] Stated differently, in some instances the use of the disclosed low-binding supports alone or in combination with improved or optimized hybridization buffer formulations may result in a surface density of hybridized target (or sample) oligonucleotide molecules (*e.g.*, prior to performing any subsequent solid-phase or clonal amplification reaction) ranging from about 100 hybridized target oligonucleotide molecules per mm^2 to about 1×10^{12} hybridized target oligonucleotide molecules per mm^2. In some instances, the surface density of hybridized target oligonucleotide molecules may be at least 100, at least 500, at least 1,000, at least 4,000, at least 5,000, at least 6,000, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, at least 60,000, at least 65,000, at least 70,000, at least 75,000, at least 80,000, at least 85,000, at least 90,000, at least 95,000, at least 100,000, at least 150,000, at least 200,000, at least 250,000, at least 300,000, at least 350,000, at least 400,000, at least 450,000, at least 500,000, at least 550,000, at least 600,000, at least 650,000, at least 700,000, at least 750,000, at least 800,000, at least 850,000, at least 900,000, at least 950,000, at least 1,000,000, at least 5,000,000, at least 1

x 10^7, at least 5 x 10^7, at least 1 x 10^8, at least 5 x 10^8,at least 1 x 10^9, at least 5 x 10^9, at least 1 x 10^{10}, at least 5 x 10^{10}, at least 1 x 10^{11}, at least 5 x 10^{11}, or at least 1 x 10^{12} molecules per mm^2. In some instances, the surface density of hybridized target oligonucleotide molecules may be at most 1 x 10^{12}, at most 5 x 10^{11}, at most 1 x 10^{11}, at most 5 x 10^{10}, at most 1 x 10^{10}, at most 5 x 10^9, at most 1 x 10^9, at most 5 x 10^8, at most 1 x 10^8, at most 5 x 10^7, at most 1 x 10^7, at most 5,000,000, at most 1,000,000, at most 950,000, at most 900,000, at most 850,000, at most 800,000, at most 750,000, at most 700,000, at most 650,000, at most 600,000, at most 550,000, at most 500,000, at most 450,000, at most 400,000, at most 350,000, at most 300,000, at most 250,000, at most 200,000, at most 150,000, at most 100,000, at most 95,000, at most 90,000, at most 85,000, at most 80,000, at most 75,000, at most 70,000, at most 65,000, at most 60,000, at most 55,000, at most 50,000, at most 45,000, at most 40,000, at most 35,000, at most 30,000, at most 25,000, at most 20,000, at most 15,000, at most 10,000, at most 5,000, at most 1,000, at most 500, or at most 100 molecules per mm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of hybridized target oligonucleotide molecules may range from about 5,000 molecules per mm^2 to about 50,000 molecules per mm^2. Those of skill in the art will recognize that the surface density of hybridized target oligonucleotide molecules may have any value within this range, *e.g.*, about 50,700 molecules per mm^2.

[0590] In some instances, the target (or sample) oligonucleotide molecules (or nucleic acid molecules) hybridized to the oligonucleotide adapter or primer molecules attached to the low-binding support surface may range in length from about 0.02 kilobases (kb) to about 20 kb or from about 0.1 kilobases (kb) to about 20 kb. In some instances, the target oligonucleotide molecules may be at least 0.001kb, at least 0.005kb, at least 0.01kb, at least 0.02kb, at least 0.05kb, at least 0.1 kb in length, at least 0.2 kb in length, at least 0.3 kb in length, at least 0.4 kb in length, at least 0.5 kb in length, at least 0.6 kb in length, at least 0.7 kb in length, at least 0.8 kb in length, at least 0.9 kb in length, at least 1 kb in length, at least 2 kb in length, at least 3 kb in length, at least 4 kb in length, at least 5 kb in length, at least 6 kb in length, at least 7 kb in length, at least 8 kb in length, at least 9 kb in length, at least 10 kb in length, at least 15 kb in length, at least 20 kb in length, at least 30 kb in length, or at least 40 kb in length, or any intermediate value spanned by the range described herein, *e.g.*, at least 0.85 kb in length.

[0591] In some instances, the target (or sample) oligonucleotide molecules (or nucleic acid molecules) may comprise single-stranded or double-stranded, multimeric nucleic acid molecules (*e.g.*, concatemers) further comprising repeats of a regularly occurring monomer unit. In some instances, the single-stranded or double-stranded, multimeric nucleic acid molecules may be at least 0.001 kb, at least 0.005 kb, at least 0.01 kb, at least 0.02 kb, at least 0.05 kb, at least 0.1 kb

in length, at least 0.2 kb in length, at least 0.3 kb in length, at least 0.4 kb in length, at least 0.5 kb in length, at least 1 kb in length, at least 2 kb in length, at least 3 kb in length, at least 4 kb in length, at least 5 kb in length, at least 6 kb in length, at least 7 kb in length, at least 8 kb in length, at least 9 kb in length, at least 10 kb in length, at least 15 kb in length, or at least 20 kb in length, at least 30 kb in length, or at least 40 kb in length, or any intermediate value spanned by the range described herein, *e.g.*, about 2.45 kb in length.

[0592] In some instances, the target (or sample) oligonucleotide molecules (or nucleic acid molecules) may comprise single-stranded or double-stranded multimeric nucleic acid molecules (*e.g.*, concatemers) comprising from about 2 to about 100 copies of a regularly repeating monomer unit. In some instances, the number of copies of the regularly repeating monomer unit may be at least 2, at least 3, at least 4, at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, and at least 100. In some instances, the number of copies of the regularly repeating monomer unit may be at most 100, at most 95, at most 90, at most 85, at most 80, at most 75, at most 70, at most 65, at most 60, at most 55, at most 50, at most 45, at most 40, at most 35, at most 30, at most 25, at most 20, at most 15, at most 10, at most 5, at most 4, at most 3, or at most 2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the number of copies of the regularly repeating monomer unit may range from about 4 to about 60. Those of skill in the art will recognize that the number of copies of the regularly repeating monomer unit may have any value within this range, *e.g.*, about 17. Thus, in some instances, the surface density of hybridized target sequences in terms of the number of copies of a target sequence per unit area of the support surface may exceed the surface density of oligonucleotide primers even if the hybridization efficiency is less than 100%.

[0593] *Nucleic acid surface amplification (NASA):* As used herein, the phrase "nucleic acid surface amplification" (NASA) is used interchangeably with the phrase "solid-phase nucleic acid amplification" (or simply "solid-phase amplification"). In some aspects of the present disclosure, nucleic acid amplification formulations are described which, in combination with the disclosed hydrophilic, polymer-coated flow cell devices, provide for improved amplification rates, amplification specificity, and amplification efficiency. As used herein, specific amplification refers to amplification of template library oligonucleotide strands that have been tethered to the solid support either covalently or non-covalently. As used herein, non-specific amplification refers to amplification of primer-dimers or other non-template nucleic acids. As

used herein, amplification efficiency is a measure of the percentage of tethered oligonucleotides on the support surface that are successfully amplified during a given amplification cycle or amplification reaction. Nucleic acid amplification performed on surfaces disclosed herein may obtain amplification efficiencies of at least 50%, 60%, 70%, 80%, 90%, 95%, or greater than 95%, such as 98% or 99%.

[0594] Any of a variety of thermal cycling or isothermal nucleic acid amplification schemes may be used with the disclosed low-binding supports. Examples of nucleic acid amplification methods that may be utilized with the disclosed low-binding supports include, but are not limited to, polymerase chain reaction (PCR), multiple displacement amplification (MDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, bridge amplification, isothermal bridge amplification, rolling circle amplification, circle-to-circle amplification, helicase-dependent amplification, recombinase-dependent amplification, or single-stranded binding (SSB) protein-dependent amplification.

[0595] In some instances, improvements in amplification rate, amplification specificity, and amplification efficiency may be achieved using the disclosed hydrophilic, polymer-coated flow cell devices, either alone or in combination with formulations of the amplification reaction components. In addition to inclusion of nucleotides, one or more polymerases, helicases, single-stranded binding proteins, *etc.* (or any combination thereof), the amplification reaction mixture may be adjusted in a variety of ways to achieve improved performance including, but are not limited to, choice of buffer type, buffer pH, organic solvent mixtures, buffer viscosity, detergents and zwitterionic components, ionic strength (including adjustment of both monovalent and divalent ion concentrations), antioxidants and reducing agents, carbohydrates, BSA, polyethylene glycol, dextran sulfate, betaine, other additives, and the like.

[0596] The use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may yield increased amplification rates compared to those obtained using conventional supports and amplification protocols. In some instances, the relative amplification rates that may be achieved may be at least 2x, at least 3x, at least 4x, at least 5x, at least 6x, at least 7x, at least 8x, at least 9x, at least 10x, at least 12x, at least 14x, at least 16x, at least 18x, or at least 20x that for use of conventional supports and amplification protocols for any of the amplification methods described above.

[0597] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized buffer formulations, may yield total amplification reaction times (*e.g.*, the time required to reach 90%, 95%, 98%, or 99%

completion of the amplification reaction) of less than 180 mins, 120mins, 90min, 60 minutes, 50 minutes, 40 minutes, 30 minutes, 20 minutes, 15 minutes, 10 minutes, 5 minutes, 3 minutes, 1 minute, 50 s, 40s, 30s, 20s, or 10s for any of these completion metrics.

[0598] In some instances, the use of the disclosed low-binding supports alone or in combination with improved or optimized amplification buffer formulations may enable faster amplification reaction times (*e.g.*, the times required to reach 90%, 95%, 98%, or 99% completion of the amplification reaction) of no more than 60 minutes, 50 minutes, 40 minutes, 30 minutes, 20 minutes, or 10 minutes. Similarly, use of the disclosed low-binding supports alone or in combination with improved or optimized buffer formulations may enable amplification reactions to be completed in some cases in no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or no more than 30 cycles.

[0599] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may yield increased specific amplification and/or decreased non-specific amplification compared to that obtained using conventional supports and amplification protocols. In some instances, the resulting ratio of specific amplification-to-non-specific amplification that may be achieved is at least 4:1 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 20:1, 30:1, 40:1, 50:1, 60:1, 70:1, 80:1, 90:1, 100:1, 200:1, 300:1, 400:1, 500:1, 600:1, 700:1, 800:1, 900:1, or 1,000:1.

[0600] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may yield increased amplification efficiency compared to that obtained using conventional supports and amplification protocols. In some instances, the amplification efficiency that may be achieved is better than 50%, 60%, 70% 80%, 85%, 90%, 95%, 98%, or 99% in any of the amplification reaction times specified above.

[0601] In some instances, the clonally-amplified target (or sample) oligonucleotide molecules (or nucleic acid molecules) hybridized to the oligonucleotide adapter or primer molecules attached to the hydrophilic, polymer-coated flow cell device surfaces may range in length from about 0.02 kilobases (kb) to about 20 kb or from about 0.1 kilobases (kb) to about 20 kb. In some instances, the clonally-amplified target oligonucleotide molecules may be at least 0.001 kb, at least 0.005 kb, at least 0.01 kb, at least 0.02 kb, at least 0.05 kb, at least 0.1 kb in length, at least 0.2 kb in length, at least 0.3 kb in length, at least 0.4 kb in length, at least 0.5 kb in length, at least 1 kb in length, at least 2 kb in length, at least 3 kb in length, at least 4 kb in length, at least 5 kb in length, at least 6 kb in length, at least 7 kb in length, at least 8 kb in length, at least

9 kb in length, at least 10 kb in length, at least 15 kb in length, or at least 20 kb in length, or any intermediate value spanned by the range described herein, *e.g.*, at least 0.85 kb in length.

[0602] In some instances, the clonally-amplified target (or sample) oligonucleotide molecules (or nucleic acid molecules) may comprise single-stranded or double-stranded, multimeric nucleic acid molecules (*e.g.*, concatemers) further comprising repeats of a regularly occurring monomer unit. In some instances, the clonally-amplified single-stranded or double-stranded, multimeric nucleic acid molecules may be at least 0.1 kb in length, at least 0.2 kb in length, at least 0.3 kb in length, at least 0.4 kb in length, at least 0.5 kb in length, at least 1 kb in length, at least 2 kb in length, at least 3 kb in length, at least 4 kb in length, at least 5 kb in length, at least 6 kb in length, at least 7 kb in length, at least 8 kb in length, at least 9 kb in length, at least 10 kb in length, at least 15 kb in length, or at least 20 kb in length, or any intermediate value spanned by the range described herein, *e.g.*, about 2.45 kb in length.

[0603] In some instances, the clonally-amplified target (or sample) oligonucleotide molecules (or nucleic acid molecules) may comprise single-stranded or double-stranded multimeric nucleic acid (*e.g.*, concatemers) molecules comprising from about 2 to about 100 copies of a regularly repeating monomer unit. In some instances, the number of copies of the regularly repeating monomer unit may be at least 2, at least 3, at least 4, at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, at least 80, at least 85, at least 90, at least 95, and at least 100. In some instances, the number of copies of the regularly repeating monomer unit may be at most 100, at most 95, at most 90, at most 85, at most 80, at most 75, at most 70, at most 65, at most 60, at most 55, at most 50, at most 45, at most 40, at most 35, at most 30, at most 25, at most 20, at most 15, at most 10, at most 5, at most 4, at most 3, or at most 2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the number of copies of the regularly repeating monomer unit may range from about 4 to about 60. Those of skill in the art will recognize that the number of copies of the regularly repeating monomer unit may have any value within this range, *e.g.*, about 12. Thus, in some instances, the surface density of clonally-amplified target sequences in terms of the number of copies of a target sequence per unit area of the support surface may exceed the surface density of oligonucleotide primers even if the hybridization and/or amplification efficiencies are less than 100%.

[0604] In some instances, the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may yield increased clonal copy number compared to that obtained using conventional supports and amplification protocols. In some instances, *e.g.*, wherein the

clonally-amplified target (or sample) oligonucleotide molecules comprise concatenated, multimeric repeats of a monomeric target sequence, the clonal copy number may be substantially smaller than compared to that obtained using conventional supports and amplification protocols. Thus, in some instances, the clonal copy number may range from about 1 molecule to about 100,000 molecules (*e.g.*, target sequence molecules) per amplified colony. In some instances, the clonal copy number may be at least 1, at least 5, at least 10, at least 50, at least 100, at least 500, at least 1,000, at least 2,000, at least 3,000, at least 4,000, at least 5,000, at least 6,000, at least 7,000, at least 8,000, at least 9,000, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, at least 60,000, at least 65,000, at least 70,000, at least 75,000, at least 80,000, at least 85,000, at least 90,000, at least 95,000, or at least 100,000 molecules per amplified colony. In some instances, the clonal copy number may be at most 100,000, at most 95,000, at most 90,000, at most 85,000, at most 80,000, at most 75,000, at most 70,000, at most 65,000, at most 60,000, at most 55,000, at most 50,000, at most 45,000, at most 40,000, at most 35,000, at most 30,000, at most 25,000, at most 20,000, at most 15,000, at most 10,000, at most 9,000, at most 8,000, at most 7,000, at most 6,000, at most 5,000, at most 4,000, at most 3,000, at most 2,000, at most 1,000, at most 500, at most 100, at most 50, at most 10, at most 5, or at most 1 molecule per amplified colony. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the clonal copy number may range from about 2,000 molecules to about 9,000 molecules. Those of skill in the art will recognize that the clonal copy number may have any value within this range, *e.g.*, about 2,220 molecules in some instances, or about 2 molecules in others.

[0605] As noted above, in some instances the amplified target (or sample) oligonucleotide molecules (or nucleic acid molecules) may comprise concatenated, multimeric repeats of a monomeric target sequence. In some instances, the amplified target (or sample) oligonucleotide molecules (or nucleic acid molecules) may comprise a plurality of molecules each of which comprises a single monomeric target sequence. Thus, the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may result in a surface density of target sequence copies that ranges from about 100 target sequence copies per mm^2 to about 1×10^{12} target sequence copies per mm^2. In some instances, the surface density of target sequence copies may be at least 100, at least 500, at least 1,000, at least 5,000, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, at least 60,000, at least 65,000, at least 70,000, at least 75,000, at least 80,000, at

least 85,000, at least 90,000, at least 95,000, at least 100,000, at least 150,000, at least 200,000, at least 250,000, at least 300,000, at least 350,000, at least 400,000, at least 450,000, at least 500,000, at least 550,000, at least 600,000, at least 650,000, at least 700,000, at least 750,000, at least 800,000, at least 850,000, at least 900,000, at least 950,000, at least 1,000,000, at least 5,000,000, at least 1 x 10^7, at least 5 x 10^7, at least 1 x 10^8, at least 5 x 10^8,at least 1 x 10^9, at least 5 x 10^9, at least 1 x 10^{10}, at least 5 x 10^{10}, at least 1 x 10^{11}, at least 5 x 10^{11}, or at least 1 x 10^{12} of clonally amplified target sequence molecules per mm^2. In some instances, the surface density of target sequence copies may be at most 1 x 10^{12}, at most 5 x 10^{11}, at most 1 x 10^{11}, at most 5 x 10^{10}, at most 1 x 10^{10}, at most 5 x 10^9, at most 1 x 10^9, at most 5 x 10^8, at most 1 x 10^8, at most 5 x 10^7, at most 1 x 10^7, at most 5,000,000, at most 1,000,000, at most 950,000, at most 900,000, at most 850,000, at most 800,000, at most 750,000, at most 700,000, at most 650,000, at most 600,000, at most 550,000, at most 500,000, at most 450,000, at most 400,000, at most 350,000, at most 300,000, at most 250,000, at most 200,000, at most 150,000, at most 100,000, at most 95,000, at most 90,000, at most 85,000, at most 80,000, at most 75,000, at most 70,000, at most 65,000, at most 60,000, at most 55,000, at most 50,000, at most 45,000, at most 40,000, at most 35,000, at most 30,000, at most 25,000, at most 20,000, at most 15,000, at most 10,000, at most 5,000, at most 1,000, at most 500, or at most 100 target sequence copies per mm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of target sequence copies may range from about 1,000 target sequence copies per mm^2 to about 65,000 target sequence copies mm^2. Those of skill in the art will recognize that the surface density of target sequence copies may have any value within this range, *e.g.*, about 49,600 target sequence copies per mm^2.

[0606] In some instances, the use of the disclosed low-binding supports alone or in combination with improved or optimized amplification buffer formulations may result in a surface density of clonally-amplified target (or sample) oligonucleotide molecules (or clusters) ranging from about from about 100 molecules per mm2 to about 1 x 10^{12} colonies per mm^2. In some instances, the surface density of clonally-amplified molecules may be at least 100, at least 500, at least 1,000, at least 5,000, at least 10,000, at least 15,000, at least 20,000, at least 25,000, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, at least 60,000, at least 65,000, at least 70,000, at least 75,000, at least 80,000, at least 85,000, at least 90,000, at least 95,000, at least 100,000, at least 150,000, at least 200,000, at least 250,000, at least 300,000, at least 350,000, at least 400,000, at least 450,000, at least 500,000, at least 550,000, at least 600,000, at least 650,000, at least 700,000, at least 750,000, at least 800,000, at least 850,000, at least 900,000, at least 950,000, at least 1,000,000, at least 5,000,000, at least 1 x 10^7,

at least 5 x 10^7, at least 1 x 10^8, at least 5 x 10^8,at least 1 x 10^9, at least 5 x 10^9, at least 1 x 10^{10}, at least 5 x 10^{10}, at least 1 x 10^{11}, at least 5 x 10^{11}, or at least 1 x 10^{12} molecules per mm^2. In some instances, the surface density of clonally-amplified molecules may be at most 1 x 10^{12}, at most 5 x 10^{11}, at most 1 x 10^{11}, at most 5 x 10^{10}, at most 1 x 10^{10}, at most 5 x 10^9, at most 1 x 10^9, at most 5 x 10^8, at most 1 x 10^8, at most 5 x 10^7, at most 1 x 10^7, at most 5,000,000, at most 1,000,000, at most 950,000, at most 900,000, at most 850,000, at most 800,000, at most 750,000, at most 700,000, at most 650,000, at most 600,000, at most 550,000, at most 500,000, at most 450,000, at most 400,000, at most 350,000, at most 300,000, at most 250,000, at most 200,000, at most 150,000, at most 100,000, at most 95,000, at most 90,000, at most 85,000, at most 80,000, at most 75,000, at most 70,000, at most 65,000, at most 60,000, at most 55,000, at most 50,000, at most 45,000, at most 40,000, at most 35,000, at most 30,000, at most 25,000, at most 20,000, at most 15,000, at most 10,000, at most 5,000, at most 1,000, at most 500, or at most 100 molecules per mm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of clonally-amplified molecules may range from about 5,000 molecules per mm^2 to about 50,000 molecules per mm^2. Those of skill in the art will recognize that the surface density of clonally-amplified colonies may have any value within this range, *e.g.*, about 48,800 molecules per mm^2.

[0607] In some instances. the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may yield signal from the amplified and labeled nucleic acid populations (*e.g.*, a fluorescence signal) that has a coefficient of variance of no greater than 50%, such as 50%, 40%, 30%, 20%, 15%, 10%, 5%, or less than 5%.

[0608] Similarly, in some instances the use of the disclosed hydrophilic, polymer-coated flow cell devices, alone or in combination with improved or optimized amplification reaction formulations, may yield signal from the amplified and non-labeled nucleic acid populations that has a coefficient of variance of no greater than 50%, such as 50%, 40%, 30%, 20%, 10%, 5%, or less than 5%.

Fluorescence imaging of hydrophilic, polymer-coated flow cell device surfaces

[0609] The disclosed hydrophilic, polymer-coated flow cell devices comprising, *e.g.*, clonal clusters of labeled target nucleic acid molecules disposed thereon may be used in any of a variety of nucleic acid analysis applications, *e.g.*, nucleic acid base discrimination, nucleic acid base classification, nucleic acid base calling, nucleic acid detection applications, nucleic acid sequencing applications, and nucleic acid-based (genetic and genomic) diagnostic applications.

In many of these applications, fluorescence imaging techniques may be used to monitor hybridization, amplification, and/or sequencing reactions performed on the low-binding supports. Fluorescence imaging may be performed using any of the optical imaging modules disclosed herein, as well as a variety of fluorophores, fluorescence imaging techniques, and other fluorescence imaging instruments known to those of skill in the art.

[0610] In some instances, the performance of nucleic acid hybridization and/or amplification reactions using the disclosed hydrophilic, polymer-coated flow cell devices and reaction buffer formulations may be assessed using fluorescence imaging techniques, where the contrast-to-noise ratio (CNR) of the images provides a key metric in assessing amplification specificity and non-specific binding on the support. CNR is commonly defined as: CNR = (Signal – Background) / Noise. The background term is commonly taken to be the signal measured for the interstitial regions surrounding a particular feature (diffraction limited spot, DLS) in a specified region of interest (ROI). As noted above, while signal-to-noise ratio (SNR) is often considered to be a benchmark of overall signal quality, it can be shown that improved CNR can provide a significant advantage over SNR as a benchmark for signal quality in applications that require rapid image capture (*e.g.*, sequencing applications for which cycle times can be reduced or minimized). At high CNR, the imaging time required to reach accurate signal discrimination (and thus accurate base-calling in the case of sequencing applications) can be drastically reduced even with moderate improvements in CNR.

[0611] In most ensemble-based sequencing approaches, the background term is typically measured as the signal associated with 'interstitial' regions. In addition to "interstitial" background (B_{inter}), "intrastitial" background (B_{intra}) exists within the discrete regions occupied by amplified DNA colonies. The combination of these two background signal terms dictates the achievable CNR in the image, and subsequently directly impacts the optical instrument requirements, architecture costs, reagent costs, run- times, cost/genome, and ultimately the accuracy and data quality for cyclic array-based sequencing applications. The B_{inter} background signal arises from a variety of sources; a few examples include auto-fluorescence from consumable flow cells, non-specific adsorption of detection molecules that yield spurious fluorescence signals that may obscure the foreground signal from the ROI, and the presence of non-specific DNA amplification products (*e.g.*, those arising from primer dimers). In typical next generation sequencing (NGS) applications, this background signal in the current field-of-view (FOV) is averaged over time and subtracted. The signal arising from individual DNA colonies (*e.g.*, (S) - B_{inter} in the FOV) yields a discernable feature that can be classified. In some instances, the intrastitial background (B_{intra}) can contribute a confounding fluorescence

signal that is not specific to the target of interest but is present in the same ROI, thus making it far more difficult to average and subtract.

[0612] The implementation of nucleic acid amplification on the hydrophilic, polymer-coated substrate surfaces of the present disclosure may decrease the B_{inter} background signal by reducing non-specific binding, may lead to improvements in specific nucleic acid amplification, and may lead to a decrease in non-specific amplification that can impact the background signal arising from both the interstitial and intrastitial regions. In some instances, the disclosed low nonspecific binding support surfaces, optionally used in combination with improved hybridization and/or amplification reaction buffer formulations, may lead to improvements in CNR by a factor of 2, 5, 10, 100, 200, 500, or 1000-fold over those achieved using conventional supports and hybridization, amplification, and/or sequencing protocols. Although described here in the context of using fluorescence imaging as the read-out or detection mode, the same principles apply to the use of the disclosed low nonspecific binding supports and nucleic acid hybridization and amplification formulations for other detection modes as well, including both optical and non-optical detection modes.

[0613] *Alternative sequencing biochemistries:* In addition to the sequencing-by-nucleotide incorporation approach described above, the disclosed flow cell devices and optical imaging systems are compatible with other emerging nucleic acid sequencing biochemistries as well. Examples include the "sequencing-by-nucleotide binding" approach described in U.S. Patent No. 10, 655, 176 B2, and the "sequencing-by-avidity" approach described in U.S. Patent No. 10,768,173 B2.

[0614] In some embodiments, the "sequencing-by-nucleotide binding" approach, as currently being developed by Omniome, Inc. (San Diego, CA) is based on performing repetitive cycles of detecting a stabilized complex that forms at each position along the template (*e.g.* a ternary complex that includes the primed template (tethered to a sample support structure), a polymerase, and a cognate nucleotide for the position), under conditions that prevent covalent incorporation of the cognate nucleotide into the primer, and then extending the primer to allow detection of the next position along the template. In the sequencing-by-binding approach, detection of the nucleotide at each position of the template occurs prior to extension of the primer to the next position. Generally, the methodology is used to distinguish the four different nucleotide types that can be present at positions along a nucleic acid template by uniquely labelling each type of ternary complex (*e.g.,* Different types of ternary complexes differing in the type of nucleotide it contains) or by separately delivering the reagents to form each type of ternary complex. In some instances, the labeling may comprise fluorescence labeling of, *e.g.*,

the cognate nucleotide or the polymerase that participate in the ternary complex. The approach is thus compatible with the disclosed flow cell devices and imaging systems.

[0615] In some embodiments, the "sequencing-by-avidity" approach, as currently being developed by Element Biosciences, Inc. (San Diego, CA) relies on the increased avidity (or "functional affinity") derived from forming a complex comprising a plurality of individual non-covalent binding interactions. In some embodiments, sequencing-by-avidity comprises the detection of a multivalent binding complex formed between a fluorescently-labeled nucleotide conjugate, a polymerase, and a plurality of primed target nucleic acid molecules tethered to a sample support structure, which allows the detection / base-calling step to be separated from the nucleotide incorporation step. In some embodiments, fluorescence imaging is used to detect the bound complex and thereby determine the identity of the N+1 nucleotide in the target nucleic acid sequence (where the primer extension strand is N nucleotides in length). Following the imaging step, the multivalent binding complex is disrupted and washed away, the correct blocked nucleotide is incorporated into the primer extension strand, and the cycle is repeated, in some embodiments.

[0616] In some instances, a nucleotide conjugate of the present disclosure may comprise a plurality of nucleotide moieties or nucleotide analog moieties conjugated to a polymer core, *e.g.*, through the 5' end of the nucleotide, either directly or via a linker. By way of non-limiting example, the nucleotide moieties may include ribonucleotide moieties, ribonucleotide analog moieties, deoxyribonucleotide moieties, deoxyribonucleotide analog moieties, or any combination thereof. In some instances, the nucleotides or nucleotide analogs may comprise deoxyadenosine, deoxyguanosine, thymidine, deoxyuridine, deoxycytidine, adenosine, guanosine, 5-methyl-uridine, and/or cytidine. In some instances, the nucleotide or nucleotide analog moieties may comprise a nucleotide that has been modified to inhibit elongation during a polymerase reaction or a sequencing reaction, such as wherein the at least one nucleotide or nucleotide analog is a nucleotide that lacks a 3' hydroxyl group; a nucleotide that has been modified to contain a blocking group at the 3' position; and/or a nucleotide that has been modified with a 3'-0-azido group, a 3'-0-azidomethyl group, a 3'-0-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-0-malonyl group, or a 3'-0-benzyl group.

[0617] In some instances, the polymer core may comprise a linear or branched polymer, *e.g.*, linear or branched polyethylene glycol (PEG), polypropylene glycol, polyvinyl alcohol, polylactic acid, polyglycolic acid, poly-glycine, polyvinyl acetate, a dextran, a protein, or other such polymers, or copolymers incorporating any two or more of the foregoing, or incorporating other polymers as are known in the art. In some instances, the polymer is a PEG. In some

instances, the polymer is a branched PEG. In some instances, a branched polymer may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more branches or arms, or 2, 4, 8, 16, 32, 64, or more, branches or arms. In some instances, the branches or arms may radiate from a central moiety.

[0618] In some instances, the nucleotide conjugate may further comprise one or more detectable labels, *e.g.*, one, two, three, four, five, six, seven, eight, nine, ten, fifteen, twenty, or more than twenty detectable labels. In some instances, the one or more detectable labels may comprise one or more fluorophores (*e.g.*, cyanine dye 3 (Cy3), cyanine dye 5 (Cy5), *etc.*), one or more quantum dots, a fluorescence resonance energy transfer (FRET) donor, and/or a FRET acceptor.

[0619] In some instances, the nucleotide conjugate may further comprise a binding moiety attached to each branch of the polymer core or to a subset of branches. Examples of suitable binding moieties include, but are not limited to, biotin, avidin, streptavidin, or the like, polyhistidine domains, complementary paired nucleic acid domains, G-quartet forming nucleic acid domains, calmodulin, maltose-binding protein, cellulase, maltose, sucrose, glutathione-S-transferase, glutathione, 0-6-methylguanine-DNA methyltransferase, benzylguanine and derivatives thereof, benzylcysteine and derivatives thereof, an antibody, an epitope, a protein A, or a protein G. The binding moiety may be any interactive molecule or fragment thereof known in the art to bind to or facilitate interactions between proteins, between proteins and ligands, between proteins and nucleic acids, between nucleic acids, or between small molecule interaction domains or moieties.

[0620] As noted above, in the sequencing-by-avidity approach a multivalent binding complex is formed between, *e.g.*, a fluorescently-labeled nucleotide conjugate, a polymerase, and a plurality of primed target nucleic acid molecules tethered to a sample support structure (*e.g.*, a flow cell surface) when the nucleotide moieties of the nucleotide conjugate are complementary to a nucleotide residue of the target sequence. The stability of the multivalent binding complex thus formed allows the detection / base-calling step in a sequencing reaction cycle to be separated from the nucleotide incorporation step.

[0621] The stability of the multivalent binding complex – a ternary complex formed between two or more nucleotide moieties of the nucleotide conjugate, two or more polymerase molecules, and two or more primed target nucleic acid sequences - is evidenced by the prolonged persistence times of the complex. For example, in some instances, said multivalent binding complexes (ternary complexes) may have a persistence time of less than 0.5 seconds, less than 1 second, greater than 1 second, greater than 2 seconds, greater than 3 seconds, greater than 4 seconds, greater than 5 seconds, greater than 10 seconds, greater than 15 seconds, greater than 20 seconds, greater than 30 seconds, greater than 60 seconds, greater than 120 seconds,

greater than 360 seconds, greater than 720 seconds, greater than 1,440 seconds, greater than 3,600 seconds, or more, or for a time within a range defined by any two or more of these values.

[0622] The use of nucleotide conjugates to form a multivalent binding complex with the polymerase and primed target nucleic acid results in an effective local concentration of the nucleotide that is increased many fold over the average nucleotide concentration that can be achieved using single unconjugated or untethered nucleotides, which in turn both enhances the stability of the complex and increases signal intensity following wash steps. The high signal intensity persists throughout the binding, washing, and imaging steps, and contributes to shorter image acquisition times. Following the imaging step, the multivalent binding complex can be destabilized, *e.g.*, by changing the ionic composition, ionic strength, and/or the pH of the buffer, and washed away. A primer extension reaction may then be performed to extend the complementary strand by one base.

[0623] *Nucleic acid sequencing system performance:* In some instances, the disclosed nucleic acid sequencing systems, comprising one or more of the disclosed flow cell devices used in combination with one or more of the disclosed optical imaging systems, and optionally utilizing one of the emerging sequencing biochemistries such as the "sequencing-by-trapping" (or "sequencing-by-avidity") approach described above, may provide improved nucleic acid sequencing performance in terms of, *e.g.*, reduced sample input requirements, reduced image acquisition cycle time, reduced sequencing reaction cycle time, reduced sequencing run time, improved base-calling accuracy, reduced reagent consumption and cost, higher sequencing throughput, and reduced sequencing cost.

[0624] *Nucleic acid sample input (pM):* In some instances, the sample input requirements for the disclosed system may be significantly reduced due to the improved hybridization and amplification efficiencies that may be attained, and the high CNR images that may be acquired for base-calling, using the disclosed hydrophilic, polymer coated flow cell devices and imaging systems. In some instances, the nucleic acid sample input requirement for the disclosed systems may range from about 1 pM to about 10,000 pM. In some instances, the nucleic acid sample input requirement may be at least 1 pM, at least 2 pM, at least 5 pM, at least 10 pM, at least 20 pM, at least 50 pM, at least 100 pM, at least 200 pM, at least 500 pM, at least 1,000 pM, at least 2,000 pM, at least 5,000 pM, at least 10,000 pM. In some instances, the nucleic acid sample input requirement for the disclosed systems may be at most 10,000 pM, at most 5,000 pM, at most 2,000 pM, at most 1,000 pM, at most 500 pM, at most 200 pM, at most 100 pM, at most 50 pM, at most 20 pM, at most 10 pM, at most 5 pM, at most 2 pM, or at most 1 pM. Any of the lower and upper values described in this paragraph may be combined to form a range included

within the present disclosure, for example, in some instances the nucleic acid sample input requirement for the disclosed systems may range from about 5 pM to about 500 pM. Those of skill in the art will recognize that the nucleic acid sample input requirement may have any value within this range, *e.g.*, about 132 pM. In one example, a nucleic acid sample input of about 100 pM is sufficient to generate signals for reliable base-calling.

[0625] *Nucleic acid sample input (nanograms):* In some instances, the nucleic acid sample input requirement for the disclosed systems may range from about 0.05 nanograms to about 1,000 nanograms. In some instances, the nucleic acid sample input requirement may be at least 0.05 nanograms, at least 0.1 nanograms, at least 0.2 nanograms, at least 0.4 nanograms, at least 0.6 nanograms, at least 0.8 nanograms, at least 1.0 nanograms, at least 2 nanograms, at least 4 nanograms, at least 6 nanograms, at least 8 nanograms, at least 10 nanograms, at least 20 nanograms, at least 40 nanograms, at least 60 nanograms, at least 80 nanograms, at least 100 nanograms, at least 200 nanograms, at least 400 nanograms, at least 600 nanograms, at least 800 nanograms, or at least 1,000 nanograms. In some instances, the nucleic acid sample input requirement may be at most 1,000 nanograms, at most 800 nanograms, at most 600 nanograms, at most 400 nanograms, at most 200 nanograms, at most 100 nanograms, at most 80 nanograms, at most 60 nanograms, at most 40 nanograms, at most 20 nanograms, at most 10 nanograms, at most 8 nanograms, at most 6 nanograms, at most 4 nanograms, at most 2 nanograms, at most 1 nanograms, at most 0.8 nanograms, at most 0.6 nanograms, at most 0.4 nanograms, at most 0.2 nanograms, at most 0.1 nanograms, or at most 0.05 nanograms. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the nucleic acid sample input requirement for the disclosed systems may range from about 0.6 nanograms to about 400 nanograms. Those of skill in the art will recognize that the nucleic acid sample input requirement may have any value within this range, *e.g.*, about 2.65 nanograms.

Sequencing systems with wide FOV and methods of using

[0626] In some embodiments, disclosed herein are methods for sequencing nucleic acid molecules comprising an operation of providing a flow cell comprising one or more surfaces, wherein each surface comprises one or more samples herein immobilized thereon. The one or more samples may include a composition comprising: a plurality of clonally amplified sample nucleic acid molecules; or a cellular sample comprising a plurality of DNA amplicons. The methods herein for sequencing nucleic acid molecules may further comprise an operation of causing, by an illumination system, the one or more samples to fluoresce in different colors in a first sequencing cycle. The methods herein for sequencing nucleic acid molecules may further

comprise an operation of detecting, by at least an image sensor and in a first FOV of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in one or more color channels for each color at a first z-level of the one or more surfaces. One or more color channels may be used for the detection. The detection in different channels may occur in parallel (e.g., with an image sensor for each channel) or sequentially (e.g., sharing a single image sensor). The first z-level may be closest along z direction to a first surface than the other surfaces of the one or more surfaces. The methods herein for sequencing nucleic acid molecules may further comprise an operation of detecting, at least by an image sensor and in a second FOV of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in one or more color channels for each color at a second z-level of the one or more surfaces. The second z-level may be closest to a first surface than the other surfaces of the one or more surfaces. Within the same first sequencing cycle, the methods herein for sequencing nucleic acid molecules may further comprise an operation of moving the sample relative to the optical system and focusing a third FOV relative to the optical system. After that, the methods herein for sequencing nucleic acid molecules may further comprise an operation of detecting, by the at least one image sensor and in the third FOV of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in one or more color channels for each color at the first z-level of the one or more surfaces. The third FOV may be displaced from the first FOV in x,y, or both directions. The methods herein for sequencing nucleic acid molecules may further comprise an operation of detecting, at least by the image sensor and in the fourth FOV of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in one or more color channels for each color at the second z-level of the one or more surfaces. The fourth FOV may be displaced from the third FOV only in the z direction but not in the x or y direction. After completion of the first sequencing cycle, the methods herein for sequencing nucleic acid molecules may further comprise sequencing the one or more samples for a second sequencing cycle which can an operation of causing, by the illumination system, the one or more samples to fluoresce in on and off events in different colors in a second sequencing cycle, after administering regent(s) in the second sequencing cycle. The sequencing in a second sequencing cycle may include detecting, by at least one image sensor and in the first FOV of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in the one or more color channels for each color at the first z-level of the one or more surfaces. The methods herein for sequencing nucleic acid molecules may further comprise an operation of detecting, at least by an image sensor and in the second FOV of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in the one or more color channels for each color at the second z-levels of the one or more surfaces. The same detection operation can be repeated in the second sequencing cycle for the third and fourth FOVs.

[0627] In some embodiments, the one or more samples may also include a third z level and subsequent z-levels, and a fifth FOV can be imaged at the third z-levels, and a sixth FOV may be imaged at a subsequent z level (e.g., 4th or 5th z level). The third z-level or subsequent z-levels may be closest along z direction to a first surface than the other surfaces of the one or more surfaces. A seventh FOV may be imaged at the third z-level but with offset in x, y, or both direction from the fifth FOV.

[0628] After completion of the first and second sequencing cycles, the methods herein for sequencing nucleic acid molecules may further comprise an operation of uniquely identifying a first number of morphological features, a second number of RNA features, and a third number of protein features of the one or more samples based on the detection in the first and second sequencing cycles. For example, a first polony in the first FOV of the first z-level may correspond to the base calling of AT in the first and second sequencing cycles, and AT at the first z-level uniquely corresponds to a first morphological target of a first cell type, while a second polony in the second FOV of the second z-level may correspond to the same base calling of AT in the first and second sequencing cycles, and AT at the second z-level uniquely corresponds to a protein target of the first cell type.

[0629] In some embodiments, disclosed herein are optical systems, comprising: a stage configured to hold a solid support; a light source configured to illuminate a FOV on said solid support; and an optical assembly disposed at least partly within an optical path from said stage to said light source, wherein said optical assembly is configured to provide an illumination over the FOV said solid support.

[0630] In some embodiments, disclosed herein sequencing systems (e.g., as shown in FIG. 75) comprising: an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2 wherein the one or more surfaces are axially displaced from each other, the optical system comprising: a light source; an objective lens; at least one image sensor; and wherein the light source comprises a center wavelength λ with a root-mean-square (RMS) wavefront error of less than 0.09λ, 0.08λ, 0.07λ, 0.06λ, or 0.02λ.

[0631] In some embodiments, disclosed herein sequencing systems (e.g., as shown in FIG. 75) comprising: an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 1 mm^2 or 10 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising: a light source; an objective lens; and at least one image sensor, wherein the optical resolution of the optical system is at least 0.2 um, 0.3 um, 0.4 um, 0.5 um, 0.8 um, or 1 um in a plane orthogonal to an axial axis.

[0632] In some embodiments, disclosed herein sequencing systems (e.g., as shown in FIG. 75) comprising: an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2, 20 mm^2, or 50 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising: a light source; and an objective lens; and at least one image sensor, wherein the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV.

[0633] In some embodiments, disclosed herein sequencing systems (e.g., as shown in FIG. 75) comprising: an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2, 20 mm^2, or 50 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising: a light source; and an imaging module that lacks an objective lens; and at least one image sensor, wherein the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV.

[0634] In some embodiments, disclosed herein sequencing systems (e.g., as shown in FIG. 75) comprising: an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2 or 50 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising: a light source; an objective lens; and at least one image sensor, wherein the optical resolution of the optical system is at least 0.2 um, 0.3 um, 0.4 um, 0.5 um, 0.8 um, or 1 um in a plane orthogonal to an axial axis, and wherein the system is configured to complete a sequencing cycle in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds.

[0635] In some embodiments, disclosed herein sequencing systems (e.g., as shown in FIG. 75) comprising: an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm2 or 50 mm2 wherein the one or more surfaces are axially displaced from each other, the optical system comprising: a light source; an imaging module that lacks an objective lens; and at least one image sensor, wherein the optical resolution of the optical system is at least 0.2 um, 0.3um, 0.5 um, 0.8 um, or 1 um in a plane orthogonal to an axial axis, and wherein the system is configured to complete a sequencing cycle in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds.

[0636] In some embodiments, the FOV is greater than 10 square millimeters (mm2), 20 mm2, 30 mm2, 40 mm2, 50 mm2, 60 mm2, 70 mm2, 80 mm2, 100 mm2, 150 mm2, 180 mm2, 200 mm2, 300 mm2, 400 mm2, or 500 mm2 with a peak-to-valley variation of at most about 5% in

the illumination energy at the FOV. In some embodiments, the FOV is greater than 30 mm2. In some embodiments, the FOV is greater than 50 mm2 or 60 mm2. In some embodiments, the optical assembly comprises an imaging module disclosed herein (e.g., as shown in FIGS. 90-92). In some embodiments, the optical assembly lacks an objective lens. In some embodiments, the optical assembly lacks a tube lens. In some embodiments, the stage does not adjust or move along an optical axis of said optical system. In some embodiments, illumination generated by the light source has an irradiance of at least about 40 milliwatts per square millimeter (mW/mm^2). In some embodiments, illumination generated by the light source has an irradiance of at least about 20, 30, 40, 50, 60, 70, 80, 90, or 100 mW/mm^2. In some embodiments, illumination generated by the light source has an irradiance of no more than 20, 30, 40, 50, 60, 70, 80, 90, or 100 mW/mm^2. In some embodiments, the optical assembly is configured to receive an emission light from the one or more samples immobilized on said solid support. In some embodiments, the emission light has a wavelength of about 500 nanometers to about 750 nanometers. In some embodiments, the emission light has a wavelength of 500 nanometers to 750 nanometers. In some embodiments, the emission light has a wavelength of about 400 nanometers to about 1200 nanometers. In some embodiments, the emission light has a wavelength of 400 nanometers to 1200 nanometers. In some embodiments, the optical assembly has a working distance of at least about 1mm to 25 mm. In some embodiments, the optical system comprises a focusing element within said optical path of said optical system, and wherein the focusing element is movable by an actuator, e.g., a motion coil housed within said optical assembly. In some embodiments, the optical system comprises a motor external to the optical system is configured to move the focusing element along the optical axis in one or both directions. The motor may be coupled directly with a piece of a first, second, or third housing of the optical assembly, and the piece of the first, second, or third housing of the optical assembly is coupled directly with the focusing element. In some embodiments, the light source is a pulsed light source. In some embodiments, the optical system has a composite root mean square error of less than about 0.05. In some embodiments, the optical assembly has an illumination efficiency of at least 70%, 80%, 90%, 92%, or 95%.

[0637] In some embodiments, the one or more samples comprise a probe configured to bind a nucleic acid molecule. In some embodiments, said probe is bound to a surface of said solid support (e.g., a surface of a flow cell device). In some embodiments, the probe comprises a nucleic acid probe. In some embodiments, the probe comprises a nucleic acid probe for detecting RNA or DNA target(s). In some embodiments, the probe comprises an analyte detection complex herein. In some embodiments, the analyte detection complex is configured to

detect protein targets. In some embodiments, the probe herein may comprise one or more antibodies and/or a barcoded oligonucleotide.

[0638] In some embodiments, the light source comprises one or more laser lights. In some embodiments, the light source comprises a single laser light. In some embodiments, the optical assembly comprises a dichroic filter configured to transmit said illumination, e.g., from the light source to the one or more samples. In some embodiments, optical assembly comprises a first segment comprising a first housing comprising a first plurality of lenses, a second segment comprising a second housing, and a third segment comprising a third housing comprising a second plurality of lenses. In some embodiments, the first segment and said third segment are optically aligned. In some embodiments, the first segment is positioned between said third segment and said stage. In some embodiments, said third segment is positioned between said first segment and an image sensor of the optical system. In some embodiments, the first plurality of lenses are movable along said optical path with a range of about 0 to about 2 millimeters. In some embodiments, each of the first plurality of lenses is an asymmetric convex-convex lens. In some embodiments, the first plurality of lenses comprises one or more asymmetric convex-convex lenses. In some embodiments, each of the second plurality of lenses is an asymmetric convex-convex lens. In some embodiments, the second plurality of lenses comprises one or more asymmetric convex-convex lenses. In some embodiments, the asymmetric concave-concave lens is an aspheric asymmetric concave-concave lens. In some embodiments, optical system is configured to acquire images of the solid support without moving an optical compensator into or out from the optical path between the solid support and a detector of the optical system.

[0639] In some embodiments, the optical assembly is configured to generate one or more spatial constrictions lateral to said optical path of light which travels therethrough.

[0640] In some embodiments, the optical assembly is configured to generate one or more field curvature corrections lateral to said optical path of light which travels therethrough. In some embodiments, the optical assembly is configured to generate at least one field curvature correction lateral to the optical path of light travels therethrough in a first segment, second segment, or third segment, e.g., a waist. In some embodiments, the optical assembly is configured to generate at least two field curvature corrections lateral to the optical path of light travels therethrough in a first segment, second segment, or third segment, e.g., double waist.

[0641] In some embodiments, the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV within a wavelength range from 420 nm to 1000 nm. In some embodiments, the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV within a wavelength range from 500 nm to 750 nm. In some

embodiments, the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV with at least two different colors. In some embodiments, the system is configured to complete a sequencing cycle by acquiring flow cell images from at least two different color channels in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds. In some embodiments, the system is configured to complete a sequencing cycle by acquiring flow cell images from at least two different color channels and from at least two different z levels in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds. In some embodiments, the light source comprises one or more lasers. In some embodiments, the light source comprises a single laser. In some embodiments, the energy variation across the FOV comprises a root mean square of energy differences. In some embodiments, the energy variation across the FOV comprises a ratio of the root mean square of energy differences to an average energy level, and wherein the ratio is less than 5%, 6%, 7%, 8%, 9%, 10% or 15%. In some embodiments, the RMS wavefront error is for a field of view (FOV) of 1.5 mm, 2 mm, 2.5 mm, 3 mm, 3.5 mm, 4 mm, 5 mm, 8 mm, 10mm, or 15 mm in x or y direction that is orthogonal to an axial axis. In some embodiments, the one or more surfaces comprises four surfaces, and the RMS wavefront error is greater for a fourth surface than a first, second, or third surface of the one or more surfaces. In some embodiments, the one or more surfaces comprises three or more surfaces. In some embodiments, the RMS wavefront error of the optical system is less than a diffraction limit of the optical system. In some embodiments, the RMS wavefront error is less than 2%, 5%, 7%, 9%, 10%, 12% or 15% of a predetermined wavelength. In some embodiments, the predetermined wavelength is a central wavelength of the light source. In some embodiments, the predetermined wavelength is a wavelength of the light source used to measure the RMS wavefront error of the light source. In some embodiments, the optical system further comprising a numerical aperture (NA) of less than 0.4, 0.5, 0.6, or 0.7. In some embodiments, the optical system further comprising a numerical aperture (NA) of greater than 0.25. In some embodiments, the optical system further comprising a numerical aperture (NA) in a range from 0.25 to 0.5. In some embodiments, the resolution of the optical system is less than 1000, 800, or 500 nm. In some embodiments, the optical system is configured to complete a sequencing cycle within less than 10 minutes and wherein the FOV of the flow cell images is greater than 10 mm^2, 20 mm^2, 40 mm^2, 60 mm^2, or 100 mm^2. In some embodiments, the optical system is configured to complete a sequencing cycle within less than 60 seconds, 30 seconds, or 10 seconds per mm^2 per cycle. In some embodiments, the flow cell images are from two or more different color channels.

[0642] In some embodiments, the flow cell images are from 4 different color channels. In some embodiments, the one or more color channels comprises 1, 2, 3, or 4 color channels. In some

embodiments, the flow cell images comprises a FOV that is greater than 10 mm^2, 20 mm^2, 40 mm^2, 60 mm^2, or 100 mm^2. In some embodiments, the flow cell images are of the one or more samples of at least two z-levels. In some embodiments, the optical system is configured to enable a sequencing cycle time of less than 2 mins, 3 mins, or 6 mins. In some embodiments, the optical system is configured to enable an imaging cycle time of less than 2 mins, 3 mins, or 6 mins. In some embodiments, the flow cell images comprises optical signals emitting from a sample immobilized on a support. In some embodiments, the sample comprises a volumetric sample. In some embodiments, the volumetric sample comprises a thickness that is greater than 0.2 um, 0.3 um, 0.4 um, 0.5 um, 1 um, 1.5 um, 2 um, 2.5 um, or 5 um. In some embodiments, the sample comprises an in situ sample of a cell, tissue, or both. In some embodiments, the light source is configured to uniformly illuminate an area of a sample that is greater than 1 mm^2 , 10 mm^2, 50 mm^2 or 100 mm^2 with less than 10% variance in illumination power across the illuminated area. In some embodiments, the system further comprising: a processor configured to process the flow cell images to correct for optical aberration. In some embodiments, the optical system further comprising: a processor configured to process the flow cell images to generate an optical resolution that is about identical in the flow cell images of the one or more surfaces. In some embodiments, the objective lens comprises an optical aperture stop with an adjustable size configured to change the NA of the optical system. In some embodiments, changing the adjustable size of the optical aperture is configured to change the NA of the optical system in the range from 0.4 to 0.6. In some embodiments, changing the adjustable size of the optical aperture is configured to change the NA of the optical system in the range from 0.25 to 0.85. In some embodiments, the processor is configured to detect a fluorescent-labeled composition comprising nucleic acids and disposed on the one or more surfaces to determine an identity of a nucleotide. In some embodiments, the at least one image sensor comprising pixels having a pixel size such that a spatial sampling frequency for the optical system is at least twice an optical resolution of the optical system. In some embodiments, the flow cell images of the one or more surfaces are acquired without moving an optical compensator into an optical path between the objective lens and the at least one image sensor. In some embodiments, the optical system is configured to acquire the flow cell images of the one or more surfaces with the optical solution without moving an optical compensator into an optical path between the objective lens and the at least one image sensor. In some embodiments, the flow cell images of one or more surfaces are acquired without moving an optical compensator out from an optical path between the objective lens and the at least one image sensor. In some embodiments, the optical system is configured to acquire the flow cell images of the one or more surfaces with the optical solution without moving an optical compensator out from an optical path between the objective lens and

the at least one image sensor. In some embodiments, the flow cell images are acquired after refocusing the optical system for each of the one or more surfaces. In some embodiments, the one or more surfaces comprise at least three surfaces that are interior surfaces of two fluidic channels of the flow cell. In some embodiments, the two fluidic channels are displaced from each other along an axial direction. In some embodiments, the hydrophilic coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density of greater than 10,000 nucleic acid polonies/mm^2. In some embodiments, the hydrophilic coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density of greater than 100,000 nucleic acid polonies/mm^2. In some embodiments, the flow cell has a total thickness of about 0.33, 0.335, 0.34, 0.345, 0.35, 0.355, or 0.36 mm along a direction orthogonal to the image plane. In some embodiments, the flow cell has a top or bottom wall thickness of at least 700 um along an axial direction orthogonal to the image plane. In some embodiments, the flow cell has a gap of at least 50 um along an axial direction orthogonal to the image plane in a first or second fluidic channel. In some embodiments, the flow cell has an interposer of at least 500 um along an axial direction orthogonal to the image plane between a first and second fluidic channel. In some embodiments, the flow cell has a top wall thickness of about 0.9 mm, a gap defined by a first fluidic channel of about 0.1 mm, an interposer wall thickness of about 0.145mm, a second gap defined by a second fluidic channel of about 0.1 mm, and a bottom wall thickness of about 0.9 mm. In some embodiments, the flow cell has a total thickness of about 0.22, 0.225, 0.23, 0.235, 0.24, 0.245, 0.25, or 0.255 mm along a direction orthogonal to the one or more surfaces. In some embodiments, the flow cell has a top wall thickness of about 0.93 mm, a gap defined by a first fluidic channel of about 0.07 mm, an interposer wall thickness of about 0.1 mm, a second gap defined by a second fluidic channel of about 0.07 mm, and a bottom wall thickness of about 0.93 mm. In some embodiments, the flow cell has a total thickness of about 0.2 to 0.39 mm along an axial direction orthogonal to the image plane and wherein each of a first and second fluidic channel has a gap of about 50 um to 100 um. In some embodiments, the optical system comprises 1, 2, 3, or 4 channels configured to detect nucleic acid ponies disposed on at least one of the one or more surfaces and have been labeled with 1, 2, 3, or 4 distinct detectable labels. In some embodiments, the optical system further comprises a focusing mechanism configured to refocus the optical system between acquiring the flow images of two different surfaces of the one or more surfaces. In some embodiments, the focusing mechanism comprises an autofocus laser and an autofocus sensor. In some embodiments, the optical system is configured to image two or more FOVs on at least one surface of the one or more surfaces. In some embodiments, the optical resolution of the flow cell images is diffraction-limited across the entire FOV. In some embodiments, the at least one image sensor comprises an active area

with a diagonal of greater than or equal to about 15 mm. In some embodiments, the objective lens has a magnifying power sufficient to image the two or more FOVs. In some embodiments, the optical system further comprises a dichroic mirror and bandpass filter set. In some embodiments, the determining of the nucleotide in the nucleic acid molecule comprises performing sequencing-by-avidity, sequencing-by-nucleotide base-pairing, sequencing-by-nucleotide binding, or sequencing-by-nucleotide incorporation reaction on at least one of the one or more surfaces. In some embodiments, a first surface of the one or more surfaces is disposed in an optical path between the objective lens and a second surface of the one or more surfaces, and wherein the second surface is disposed in the optical path between the first surface and a third surface of the one or more surfaces. In some embodiments, the system further comprises: a fluid flow controller configured to control sequential and iterative delivery of a reagent to the one or more surfaces. In some embodiments, the light source comprises a illumination system. In some embodiments, the processor is programed to instruct the system to iteratively perform a sequencing method comprising: contacting the plurality of primed target nucleic acid sequences coupled to the one or more surfaces with a nucleotide composition to form a transient binding complex between the plurality of primed target nucleic acid sequences and a plurality of nucleotide moieties when a nucleotide moiety of the nucleotide composition is complementary to a nucleotide of the primed target nucleic acid sequence; and imaging the one or more surfaces of the flow cell to detect the transient binding complex and thereby determine an identity of the nucleotide of the primed target nucleic acid sequence. In some embodiments, the illumination system comprises an optical system designed to project periodic patterns of light on each of the one or more surfaces of the flow cell, and wherein a relative orientation or phase shift of a plurality of the periodic patterns of light is adjustable. In some embodiments, the illumination system comprises a first optical arm comprising a first light emitter to emit light and a first beam splitter to split light emitted by the first light emitter to project a first plurality of fringes on the one or more surfaces. In some embodiments, the illumination system further comprises a second optical arm comprising a second light emitter to emit light and a second beam splitter to split light emitted by the second light emitter to project a second plurality of fringes on the one or more surfaces. In some embodiments, the illumination system further comprises an optical element to combine a first optical path of the first arm and a second path of the second arm.

[0643] In some embodiments, the first beam splitter comprises a first transmissive diffraction grating and the second beam splitter comprises a second transmissive diffraction grating. In some embodiments, the first and second light emitters emit unpolarized light, and wherein the first and second transmissive diffraction gratings are to diffract unpolarized light emitted by a

respective one of the first and second light emitters. In some embodiments, the optical element to combine an optical path of the first plurality of fringes and the second plurality of fringes comprises a mirror with holes, with the mirror arranged to reflect light diffracted by the first transmissive diffraction grating and with the holes arranged to pass through at least first orders of light diffracted by the second transmissive diffraction grating. In some embodiments, the system further comprises: one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes. In some embodiments, the one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes comprise a first rotating optical window to phase shift the first plurality of fringes and a second rotating optical window to phase shift the second plurality of optical fringes. In some embodiments, the one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes comprise a first linear motion stage to translate the first diffraction grating and a second linear motion stage to translate the second diffraction grating. In some embodiments, the one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes comprise a single rotating optical window, wherein the single rotating optical window is positioned after the mirror with holes in an optical path to the sample. In some embodiments, an axis of rotation of the single rotating optical window is offset by about 45 degrees from an optical axis of each of the gratings. In some embodiments, the first plurality of fringes is angularly offset from the second plurality of fringes on the sample plane by about 90 degrees. In some embodiments, the sample comprises a plurality of features regularly patterned in a rectangular array or hexagonal array. In some embodiments, the objective lens is configured to project each of the first plurality of fringes and the second plurality of fringes on the sample. In some embodiments, the system further comprises: one or more optical beam blockers for blocking zero orders of light emitted by each of the first and second diffraction gratings. In some embodiments, the optical element to combine an optical path of the first arm and the second arm comprises a polarizing beam splitter, wherein the first diffraction grating diffracts vertically polarized light and wherein the second diffraction grating diffracts horizontally polarized light. In some embodiments, the first and second beam splitters each comprise a beam splitter cube or plate. In some embodiments, the first beam splitter comprises a first reflective diffraction grating and the second beam splitter comprises a second reflective diffraction grating.

[0644] In some embodiments, the illumination system comprises a multiple beam splitter slide comprising a plurality of beam splitters mounted on a linear translation stage. In some embodiments, the plurality of beam splitters has fixed orientations with respect to the system's optical axis. In some embodiments, the plurality of beam splitters comprises a plurality of diffraction gratings. In some embodiments, the plurality of diffraction gratings comprises two

diffraction gratings. In some embodiments, the illumination system comprises a fixed two-dimensional diffraction grating used in combination with a spatial filter wheel to project one-dimensional diffraction patterns on the one or more surfaces of the flow cell. In some embodiments, the one or more samples are at least uni-plex, 4-plex, 8-plex, 16-plex, 32-plex, 64-plex, 128-plex, 256-plex, or 512-plex. In some embodiments, the system is configured to sequence the one or more samples to uniquely identify a first number of morphological features, a second number of RNA features, and a third number of protein features within no more than two sequencing cycles and at two different z levels. In some embodiments, a sum of the first, second, and third number is greater than 10, 20, 32, 64, or 128. In some embodiments, each of the first, second, and third number is a non-zero integer. In some embodiments, the one or more samples comprises more than 2, 4, 8, 16, 32 or 64 types of cells. . In some embodiments, the one or more samples comprises more than 10, 20, 40, 60, 100, 200, 300, 400, 500 or more targets.

[0645] FIG. 103A-103D shows identification of various morphological features using the methods and systems disclosed herein. FIG. 103A shows location and identification of nucleus of cells identified using the systems and methods herein. FIG. 103B-103C shows location and identification of cell membranes and mitochondria using the systems and methods herein. FIG. 103D shows merged location and identification of all three morphological targets in FIGS.103A-103C which confirms that the mitochondrial targets and the nucleus are within the cell membrane.

[0646] FIG. 104 shows identification of 4 different RNA targets using the methods and systems disclosed herein. In some embodiments, more targets can be uniquely identified by separating the targets into different "batches." For example, using the systems and methods, 100 probes can allow unique identification of 100 different mRNA targets within a single batch that turns on for emitting fluorescent signals while the other batches are turned off from emitting fluorescent signals. Using 6 batches may allow unique identification of 600 different targets in the sample with the systems and methods herein. Details about turning different batches on and off for increasing throughput in imaging and sequencing analysis are disclosed in PCT Application No. PCT/US2023/074933 and PCT/US2023/65972, each of which is incorporated herein by reference in its entirety.

[0647] FIG. 105A-105G show identification of various protein targets within the same in situ sample(s) using the methods and systems disclosed herein, e.g., Histone-H3, p53, Jun N-terminal Kinase (JNK1/2/3), a-Tubulin, phosphorylated-p53, and phosphorylated-JNK1/2/3, respectively. The while contours are cell contours that can be estimated using various image processing algorithms.

[0648] In some embodiments, the system and method disclosed herein is used to image in situ cell samples immobilized on at one surface of a flow cell device. The surface may be positioned on a sample stage of the system to undergo a sequencing run that includes 300 sequence cycles. Each sequencing cycle may be completed within a sequencing cycle time of less than 3 minutes for a predetermined FOV. In each sequencing cycle, sequencing reagent may be administered to the surface, and possibility other surfaces. Five to ten different z levels of the samples may be imaged, each after autofocusing on the corresponding z level. Each z level has multiple FOVs that tiles up to cover the samples on the surface (e.g., 10-40 FOVs). Each FOV may be greater than 10 mm^2 with an image resolution of 0.45 um. Each z level may be separated from its immediately adjacent z level for about 0.45 um. At each z-level, the illumination system may illuminate the FOV with less than 5% energy variation across the FOV. Four flow cell images of the same FOV may be obtained simultaneously, each from a different color channel. After imaging of the sample region, which includes multiple FOVs and 7 z levels for each FOV, the sequencing run may continue to the next sequencing cycle. The same FOVs may be imaged in the next sequencing cycle. After two sequencing cycles are completed, base calling of the two sequencing cycles may be performed. In some embodiments, base calling of the a sequencing cycle (e.g., the first sequencing cycle) may be performed in parallel while the next sequencing cycle is in progress to reduce sequencing analysis time needed for base calling. The 16 different probes at each z level may be: AA, AC, AT, AG, CA, CC, CT, CG, TT, TA, TC, TG, GA, GG, CG, and CT, which can be used to uniquely identify 16 targets that are either morphological targets, RNA targets, mRNA targets, and/or protein targets at a different z-level. A total of n targets (i.e., n =16 * number of z levels) can be identified at 5-10 different z levels with an imaging time of less than 20 minutes for a FOV of 10 mm^2. When the targets are separated into different batches (e.g., 10 batches), a total of m (i.e., m = 16* number of z levels * 10 batches) targets can be identified with an imaging time of 5x, 10x, 20x, or 50x less than the time to sequencing m or n targets due to time savings in the non-cycle time, imaging cycle time, and/or sequencing-cycle time.

[0649] For RNA or mRNA detections, the systems and methods herein are compared with gold standard FISH methods for identifying same various types of RNAs. For example, for the cell types of HUVEC, HeLa, HCT119, and A549, the value of correlation coefficient (r) of number of transcripts per cell (e.g., BRCA2, TP53, NRAS, etc.) is greater than 0.91 for each different cell types.

[0650] In some embodiments, the methods and systems herein advantageously reduces the hands-on time that requires human manipulation during a sequencing run (e.g., 300 cycles) to be less than 10 hours, 8 hours, 6 hours, 2 hours, 1 hour, or 30 minutes and allows automatic

performance of sequencing runs. In some embodiments, the methods and systems herein advantageously sequence the cells in situ without the need to reconstruct the spatial relationship of targets or features of the cells. In other words, the morphological targes, RNA or protein targets are detected herein while they are located inside cells or tissue. In some embodiments, the methods and systems herein advantageously allow unique identification of morphological features, RNA, and protein targets within a single sequencing run or even within identical sequencing cycles, which greatly facilitate detection and analysis of biologically or pathologically-significant markers inside cells or tissues relative to its surroundings.

[0651] For protein targets, locations and identities of protein targets (FIG. 106B) are determined using the methods and systems herein and are compared with those determined using traditional immunofluorescence (FIG. 106A). In this particular embodiment, the protein target is tubulin in cell samples. FIG. 106C shows a merged image of FIGS. 106A-106B which indicates significant similarity in determination of protein locations and identities.

[0652] In some embodiments, the sequencing cycle time herein can be further reduced by adjusting a focusing element of the image module disclosed herein without the need of using an objective lens, and without the need to move the sample stage or objective lens to focus the sample(s) relative to the optical system.

[0653] *FOV images required to tile flow cell:* In some instances, the field-of-view (FOV) of the disclosed optical imaging module is sufficiently large that a multi-channel (or multi-lane) flow cell (*e.g.*, the fluid channel portions thereof) of the present disclosure may be imaged by tiling from about 10 FOV images (or "frames") to about 1,000 FOV images (or "frames"). In some embodiments, one or more samples immobilized on a single surface (e.g., the first, second, third, or fourth surface) may be imaged by tiling from 2 FOVs to 50 FOVs. "). In some embodiments, one or more samples immobilized on a single surface (e.g., the first, second, third, or fourth surface) may be imaged by tiling no more than 50, 40, 30, 25, 20, 15, or 10 FOVs. In some embodiments, the images herein are flow cell images covering a FOV that covers at least part of the active area or ROI. In some instances, an image of the entire multi-channel flow cell may require tiling at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, at least 650, at least 700, at least 750, at least 800, at least 850, at least 900, at least 950, or at least 1,000 FOV images (or "frames"). In some instances, an image of the entire multi-channel flow cell may require tiling at most 1,000, at most 950, at most 900, at most 850, at most 800, at most 750, at most 700, at most 650, at most

600, at most 550, at most 500, at most 450, at most 400, at most 350, at most 300, at most 250, at most 200, at most 150, at most 100, at most 90, at most 80, at most 80, at most 70, at most 60, at most 50, at most 40, at most 30, at most 20, at most 10, at most 9, at most 8, at most 7, at most 6, at most 5, at most 4, at most 3, or at most 2 FOV images (or "frames"). Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances an image of the entire multi-channel flow cell may require tiling from about 30 to about 100 FOV images. Those of skill in the art will recognize that in some instances the number of required FOV images may have any value within this range, *e.g.*, about 54 FOV images.

Imaging cycle time

[0654] In some instances, the combination of wide FOV, image sensor response sensitivity, and/or fast FOV translation times enables shortened imaging cycle times (*e.g.*, the time required to acquire a sufficient number of FOV images to tile the entire multichannel flow cell (or the fluid channel portions thereof).

[0655] A sufficient number of FOV images to tile the entire multichannel flow cell (or the fluid channel portions thereof) can include one or more FOVs. Each FOV can cover at least part of a tile of the flow cell device. Each FOV can be in the range from 1 mm^2 to 200 mm^2. Each FOV can be in the range from 4 mm^2 to 80 mm^2. Each FOV can be in the range from 5 mm^2 to 70 mm^2.

[0656] In some embodiments, the total sequencing time to complete a sequencing run can include the number of cycles multiplied by the sequencing cycle time plus the non-cycle dependent time in the sequencing run.

[0657] In some embodiments, the total sequencing time to complete a sequencing run may include non-cycle dependent time that is outside a sequencing cycle and cycle-dependent time which is needed to complete individual sequencing cycles. The cycle-dependent time may include the sequencing cycle time for each cycle of a plurality of cycles in the sequencing run. The sequencing cycle time may include imaging cycle time and non-imaging cycle time.

[0658] For example, the total sequencing time to complete 150 sequencing cycles may include 8 hours of non-sequencing time and a cycle-dependent time of 12.5 hours, which makes the total sequencing time of 20.5 hours. For the 12.5 hours of cycle-dependent time, it includes a sequencing cycle time of 5 minutes for each cycle of the 150 cycles. And among the sequencing cycle time, 3 minutes is the imaging cycle time, and the rest 2 minutes in each cycle is the non-

imaging cycle time. The imaging cycle time of 3 minutes is used to image 10 FOVs at 2 different z-levels so that imaging time for each FOV is 9 seconds.

[0659] In some embodiments, imaging cycle time can include the time required to acquire flow cell images from all the color channels and from one or more z levels within a single sequencing cycle of a sequence run. In other words, imaging cycle time can be the portion of time in a sequencing cycle that is used for imaging. Imaging cycle time may include focusing of the optical system, moving of the sample stage, objective lens, or other elements of the optical system before flow cell images are acquired, illumination of the FOV(s) and imaging data acquisition time. In some embodiments, imaging cycle time may include a total time in a cycle for imaging multiple FOVs in a sequence, including illumination time of a corresponding FOV (which may overlap with data acquisition in time), focusing of the optical system with respect to the corresponding FOV, moving of the sample stage, objective lens, or other elements of the optical system before flow cell images are acquired. The multiple FOVs may cover a total area of no less than 100 mm^2, 200 mm^2, 300 mm^2, 400 mm^2, 500 mm^2, or more. The total area may include two or more FOVs titled together.

[0660] In some embodiments, the non-cycle dependent time and time duration for completing each sequencing cycle can vary based on different sequencing chemistry and/or different sequencing applications. The non-cycle dependent time and time duration for completing each sequencing cycle can vary based on the type of samples, e.g., traditional 2D samples or in situ samples. The time duration for completing each sequencing cycle can vary based on the number of FOVs of the sample being imaged.

[0661] In some embodiments, the non-cycle dependent time in a sequencing run, for example, for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, or their combinations can be in a range from one hour to a couple of hours. In some embodiments, the non-cycle dependent time in a sequencing run, for example, for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, or their combinations can be in a range from 30 minutes to 1 hour. In some embodiments, the non-cycle dependent time in a sequencing run, for example, for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, or their combinations can be in a range from 40 minutes to 1.5 hours. In some embodiments, the non-cycle dependent time in a sequencing run, for example, for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, or their combinations can be in a range

from 50 minutes to 2 hours. In some embodiments, the non-cycle dependent time in a sequencing run, for example, for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, or their combinations can be in a range from 50 minutes to 3 hours. In some embodiments, the non-cycle dependent time in a sequencing run, for example, for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, or their combinations can be in a range from 50 minutes to 4 hours.

[0662] In each cycle, the total time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from a couple of seconds to a plurality of minutes. In each cycle, the total time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from 1 second to 1 minute. In each cycle, the total time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from 5 seconds to 1 minute. In each cycle, the total time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from 10 seconds to 4 minutes. In each cycle, time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from 2 seconds to 5 minutes. In each cycle, time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from 2 seconds to 5 minutes.

[0663] In some embodiments, the sequencing cycle time for each cycle includes both the imaging cycle time within the cycle and the rest of the time duration within each cycle. In some embodiments, the imaging cycle time can include: time for positioning the sample along z and in the x-y plane for imaging, time for autofocusing the sample relative to the optical system (e.g., the objective lens), time for excitation of the samples, and time for collection of the emitted optical signal by the image sensor.

[0664] In some embodiments where more than one z-levels are sequenced in a single sequencing cycle, the imaging cycle time for each cycle includes: time duration for positioning the sample relative to the optical system (e.g., objective of the optical system); time duration for autofocusing of the sample at a first z-level, time duration for light excitation of the sample(s) and acquiring flow cell images from different color channels in parallel or sequentially, time for autofocusing of the sample at a second z level, time duration for light excitation of the sample(s)

and acquiring flow cell images from different color channels in parallel or sequentially. In some embodiments, the imaging cycle time for each cycle at two different z-levels is less than 10 seconds, 20 seconds, 30 seconds, 40 seconds, 50 seconds, or 1 minute. In some embodiments, the imaging cycle time for each cycle at two different z-levels is less than 1 minute, 1.5 minutes, 2 minutes, 3 minutes, 5 minutes, or 10 minutes.

[0665] In some embodiments, the imaging cycle time in each cycle is for imaging one or more FOVs. In some embodiments, the imaging cycle time in each cycle is for imaging some or all tiles of a flow cell device at one or more surfaces. In some embodiments, the imaging cycle time in each cycle is for imaging some or all tiles of a flow cell device at multiple z-levels (e.g., of in situ sample(s) immobilized on a same surface or of two different surfaces). The one or more FOVs can be at different z-levels or at different x,y positions. Each FOV can be greater than 4 mm^2, $5mm^2$, $8mm^2$, $10mm^2$, $15mm^2$, $20mm^2$, $25mm^2$, $30mm^2$, or 40 mm^2 and an image resolution can be greater than 1 um (e.g., 0.8 um or 0.3um). In some embodiments, the imaging time in each cycle is for a FOV of greater than 10 mm^2, $20mm^2$, $30mm^2$, $40mm^2$, $50mm^2$, $60mm^2$, $70mm^2$, $80mm^2$, $90mm^2$, $100mm^2$, $120mm^2$, 150 mm^2, or 200 mm^2 and the image resolution can be greater than 1 um (e.g., 0.8 um).

[0666] In some embodiments, the imaging cycle time in each cycle is for a FOV of greater than 4 mm^2, $5mm^2$, $8mm^2$, $10mm^2$, $15mm^2$, $20mm^2$, $25mm^2$, $30mm^2$ and an image resolution of greater than 1 um. In some embodiments, the imaging cycle time in each cycle is for a FOV of greater than 10 mm^2, $20mm^2$, $30mm^2$, $40mm^2$, $50mm^2$, $60mm^2$, $70mm^2$, $80mm^2$, $90mm^2$, $100mm^2$, $120mm^2$, 150 mm^2, or 200 mm^2 and an image resolution of greater than 0.8 um, 0.7 um, 0.6 um 0.5 um, 0.4 um, 0.3 um, or 0.2 um.

[0667] In some instances, the imaging cycle time may range from about 10 seconds to about 10 minutes. In some instances, the imaging cycle time may be at least 10 seconds at least 20 seconds, at least 30 seconds, at least 40 seconds, at least 50 seconds, at least 1 minute, at least 2 minutes, at least 3 minutes, at least 4 minutes, at least 5 minutes, at least 6 minutes, at least 7 minutes, at least 8 minutes, at least 9 minutes, or at least 10 minutes. In some instances, the imaging cycle time may be at most 10 minutes, at most 9 minutes, at most 8 minutes, at most 7 minutes, at most 6 minutes, at most 5 minutes, at most 4 minutes, at most 3 minutes, at most 2 minutes, at most 1 minute, at most 50 second, at most 40 second, at most 30 seconds, at most 20 seconds, or at most 10 seconds. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the imaging cycle time may range from about 20 seconds to about 1 minute. Those of skill in the art will recognize that in some instances the imaging cycle time may have any value within this range, *e.g.*, about 57 seconds.

Sequencing cycle time

[0668] In some instances, shortened sequencing reaction steps, *e.g.*, due to reduced wash time requirements for the disclosed hydrophilic, polymer-coated flow cells, may result in shortened overall sequencing cycle times. In some instances, the sequencing cycle times for the disclosed systems may range from about 1 minute to about 60 minutes. In some instances, the sequencing cycle time may be at least 1 minute, at least 2 minutes, at least 3 minutes, at least 4 minutes, at least 5 minutes, at least 6 minutes, at least 7 minutes, at least 8 minutes, at least 9 minutes, at least 10 minutes, at least 15 minutes, at least 20 minutes, at least 25 minutes, at least 30 minutes, at least 35 minutes, at least 40 minutes, at least 45 minutes, at least 50 minutes, at least 55 minutes, or at least 60 minutes. In some instances, the sequencing reaction cycle time may be at most 60 minutes, at most 55 minutes, at most 50 minutes, at most 45 minutes, at most 40 minutes, at most 35 minutes, at most 30 minutes, at most 25 minutes, at most 20 minutes, at most 15 minutes, at most 10 minutes, at most 9 minutes, at most 8 minutes, at most 7 minutes, at most 6 minutes, at most 5 minutes, at most 4 minutes, at most 3 minutes, at most 2 minutes, or at most 1 minutes. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the sequencing cycle time may range from about 2 minutes to about 15 minutes. Those of skill in the art will recognize that in some instances the sequencing cycle time may have any value within this range, *e.g.*, about 1 minute, 12 seconds.

[0669] In some embodiments, the sequencing cycle time can be the total time required to complete a sequencing cycle, which can include imaging cycle time and non-imaging cycle time. The non-imaging cycle time can include time for preparing the sample for imaging (e.g., after a previous cycle is completed) specific for a current cycle and other possible processing time after imaging within a sequencing cycle (e.g., during a current cycle) of a sequence run. In other words, non-imaging cycle time can be the total time duration of a sequencing cycle excluding the imaging cycle time within the cycle. In some embodiments, sequencing cycle time can be the entire time for completing a cycle which can include imaging cycle time within the cycle. Sequencing cycle time may include time for introduction of reagents to the sample, allowing the sample to react with the reagents, positioning the sample for imaging (e.g., moving the sample relative to the imager from focusing on a first tile in a previous cycle to focusing on a second tile in a current cycle), imaging, introduction of different reagents to the sample, washing of the sample, etc., within a single cycle.

[0670] In some embodiments, the sequencing cycle time can be the total time required to sequence multiplex or uniplex samples within the sequencing cycle. For example, the multiplex sample can be 50-plex, and can be imaged with a time that is 300x, 200x, 100x, 50x, 25x, or

10x, or even less than the time required to sequence a uniplex sample of similar sample density on the flow cell device.

[0671] In some embodiments, the sequencing cycle time can be the total time required to sequence samples at multiple z-levels within the sequencing cycle.

[0672] In some embodiments, the sequencing cycle time can be the total time required to sequence in situ sample(s) with different cell types or tissue types within the sequencing cycle. For example, the in situ sample can include 10, 20, 30, 40 50, 80, 100, 200, 300, or more different types of cells/tissues, and can be imaged with a time that is 300x, 200x, 100x, 50x, 25x, or 10x, or even less than the time required to sequence a sample with a single type of cells of similar sample density on the flow cell device.

[0673] In some embodiments, the systems and methods herein are configured for uniquely identifying various types of targets or features in the sample(s) (e.g., inside cells or tissue) in a single sequencing run. The various types of targes can include a first number of morphological features, a second number of RNA targets, and/or a third number of protein targets. The first, second, and third numbers can be identical or different non-zero integers. The sum of the first, second and third number can be greater than 10, 20, 30, 40, 50, 80, or 100. The sum of the first, second and third number can be greater than 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 800, or 1000. For example, the one or more samples herein can include 3 different cell types. As a nonlimiting example, the cell types can be PC3, HeLa, and MCF7 cells. In some embodiments, identification of cell membrane feature(s) of the different cells may be used to determine the different cell types.

[0674] *Sequencing read length:* In some instances, the enhanced CNR images that may be achieved using the disclosed hydrophilic, polymer-coated flow cell devices in combination with the disclosed imaging systems, and in some cases, the use of milder sequencing biochemistries, may enable longer sequencing read lengths for the disclosed systems. In some instances, the maximum (single read) read length may range from about 50 bp to about 500 bp. In some instances, the maximum (single read) read length may be at least 50 bp, at least 100 bp, at least 150 bp, at least 200 bp, at least 250 bp, at least 300 bp, at least 350 bp, at least 400 bp, at least 450 bp, or at least 500 bp. In some instances, the maximum (single read) read length is at most 500 bp, at most 450 bp, at most 400 bp, at most 350 bp, at most 300 bp, at most 250 bp, at most 200 bp, at most 150 bp, at most 100 bp, or at most 50 bp. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the maximum (single read) read length may range from about 100 bp to about 450 bp. Those of skill in the art will recognize that in some

instances the maximum (single read) read length may have any value within this range, *e.g.*, about 380 bp.

[0675] *Sequencing run time:* In some instances, the sequencing run time for the disclosed nucleic acid sequencing systems may range from about 8 hours to about 20 hours. In some instances, the sequencing run time for the disclosed nucleic acid sequencing systems may range from 8 hours to 30 hours. In some instances, the sequencing run time is at least 8 hours, at least 9 hours, at least 10 hours, at least 12 hours, at least 14 hours, at least 16 hours, at least 18 hours, at least 20 hours, at least 24 hours, or at least 30 hours. In some instances, the sequencing run time is at most 30 hours, 25 hours, 20 hours, at most 18 hours, at most 16 hours, at most 14 hours, at most 12 hours, at most 10 hours, at most 9 hours, or at most 8 hours. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the sequencing run time may range from about 10 hours to about 16 hours. Those of skill in the art will recognize that in some instances the sequencing run time may have any value within this range, *e.g.*, about 7 hours, 35 minutes.

[0676] *Average base-calling accuracy:* In some instances, the disclosed nucleic acid sequencing systems may provide an average base-calling accuracy of at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 96%, at least 98%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% correct over the course of a sequencing run. In some instances, the disclosed nucleic acid sequencing systems may provide an average base-calling accuracy of at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 96%, at least 98%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% correct per every 1,000 bases, 10,0000 bases, 25,000 bases, 50,000 bases, 75,000 bases, or 100,000 bases called.

[0677] *Average Q-score:* In some instances, the quality or accuracy of a sequencing run may be assessed by calculating a Phred quality score (also referred to as a quality score or "Q-score"), which indicates the probability that a given base is called incorrectly by the sequencing system. For example, in some instances base calling accuracy for a specific sequencing chemistry and/or sequencing system may be assessed for a large empirical data set derived from performing sequencing runs on a library of known nucleic acid sequences. The Q-score may then be calculated according to the equation:

$$Q = -10\, log_{10}\, P$$

where P is the base calling error probability. A Q-score of 30, for example, indicates a probability of making a base calling error of 1 in every 1000 bases called (or a base calling accuracy of 99.9%).

[0678] In some instances, the disclosed nucleic acid sequencing systems may provide a more accurate base readout. In some instances, for example, the disclosed nucleic acid sequencing systems may provide a Q-score for base-calling accuracy over a sequencing run that ranges from about 20 to about 50. In some instances, the average Q-score for the run may be at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50. Those of skill in the art will recognize that the average Q-score may have any value within this range, *e.g.*, about 32.

[0679] *Q-score vs. % nucleotides identified:* In some instances, the disclosed nucleic acid sequencing systems may provide a Q-score of greater than 20 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed nucleic acid sequencing systems may provide a Q-score of greater than 25 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed nucleic acid sequencing systems may provide a Q-score of greater than 30 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed nucleic acid sequencing systems may provide a Q-score of greater than 35 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed nucleic acid sequencing systems may provide a Q-score of greater than 40 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed nucleic acid sequencing systems may provide a Q-score of greater than 45 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed compositions and methods for nucleic acid sequencing may provide a Q-score of greater than 50 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. The Q-score may be for an in situ sample as disclosed elsewhere herein. For example, the Q-score of a sequencing operation in an in situ sample can be one of the aforementioned values.

[0680] *Reagent consumption:* In some instances, the disclosed nucleic acid sequencing systems may have lower reagent consumption rates and costs due to, *e.g.*, the use of the disclosed flow cell devices and fluidic systems that minimize fluid channel volumes and dead volumes. For example, a quad surface flow cell may have fluid channels with a channel height of about 70

um, which reduces the channel volume than existing flow cells. As another example, a quad surface flow cell may have a same common line thus a same dead volume as a traditional single surface flow cell, so that the dead volume per channel volume can be significantly reduced. As yet another example, a quad surface flow cell may have a cleaning out as shown in FIG. 64F so that residuals of reagent can be pulled out and less washing reagent is required to achieve a same desired contamination threshold as existing flow cell devices. As yet another example, air gaps can be introduced into the flow cell devices between liquid reagents without damaging the surface coating or samples tethered thereon to efficiently facilitate cleaning of the fluidic channels and reduce reagent consumption accordingly. In some instances, the disclosed nucleic acid sequencing systems may thus require an average of at least 5% less, at least 10% less, at least 15% less, at least 20% less, at least 25% less, at least 30% less, at least 35% less, at least 40% less, at least 45% less, or at least 50% less reagent by volume per Gbase sequenced that that consumed by an Illumina MiSeq sequencer.

[0681] *Sequencing throughput:* In some instances, the disclosed nucleic acid sequencing systems may provide a sequencing throughput ranging from about 50 Gbase/run to about 200 Gbase/run. In some instances, the sequencing throughput may be at least 50 Gbase/run, at least 75 Gbase/run, at least 100 Gbase/run, at least 125 Gbase/run, at least 150 Gbase/run, at least 175 Gbase/run, or at least 200 Gbase/run. In some instances, the sequencing throughput may be at most 200 Gbase/run, at most 175 Gbase/run, at most 150 Gbase/run, at most 125 Gbase/run, at most 100 Gbase/run, at most 75 Gbase/run, or at most 50 Gbase/run. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the sequencing throughput may range from about 75 Gbase/run to about 150 Gbase/run. Those of skill in the art will recognize that in some instances the sequencing throughput may have any value within this range, *e.g.*, about 119 Gbase/run.

[0682] *Sequencing cost:* In some instances, the disclosed nucleic acid sequencing systems may provide nucleic acid sequencing at a cost ranging from about $5 per Gbase to about $30 per Gbase. In some instances, the sequencing cost may be at least $5 per Gbase, at least $10 per Gbase, at least $15 per Gbase, at least $20 per Gbase, at least $25 per Gbase, or at least $30 per Gbase. In some instances, the sequencing cost may be at most $30 per Gbase, at most $25 per Gbase, at most $20 per Gbase, at most $15 per Gbase, at most $10 per Gbase, or at most $30 per Gbase. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the sequencing cost may range from about $10 per Gbase to about $15 per Gbase. Those of skill in the art will

recognize that in some instances the sequencing cost may have any value within this range, *e.g.*, about $7.25 per Gbase.

Further embodiments

[0683] Disclosed herein are imaging systems configured to image multiple surfaces of a flow cell, e.g., a first interior surface, a second interior surface, a third interior surface, and a fourth interior surface, the imaging systems comprising: a) an objective lens; b) at least one image sensor; and c) at least one tube lens disposed in an optical path between the objective lens and the at least one image sensor; wherein said optical system has a numerical aperture (NA) of less than 0.6 and a field-of-view (FOV) of greater than 1.0 mm^2; and wherein the at least one tube lens is configured to correct imaging performance such that images of the first interior surface of the flow cell and the second, third, and fourth interior surfaces of the flow cell have substantially the same optical resolution.

[0684] In some embodiments, the flow cell has a wall thickness of at least 700 μm and a fluid-fillable gap between the first interior surface and the second interior surface of at least 50 μm. In some embodiments, a second fluid-fillable gap between the third interior surface and the fourth interior surface is at least 50 μm. In some embodiments, the images of the surfaces are acquired without moving an optical compensator into an optical path between said objective lens and said at least one image sensor. In some embodiments, the imaging system has a numerical aperture (NA) of less than 0.6. In some embodiments, the imaging system has a numerical aperture (NA) of greater than 0.3. In some embodiments, the imaging system has a field-of-view (FOV) of greater than 1.5 mm^2. In some embodiments, the optical resolution of images of the surfaces are diffraction-limited across the entire field-of-view (FOV). In some embodiments, the at least one tube lens comprises, in order, an asymmetric convex-convex lens, a convex-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens. In some embodiments, the imaging system comprises two or more tube lenses which are designed to provide optimal imaging performance for the surfaces at two or more fluorescence wavelengths. In some embodiments, the imaging system further comprises a focusing mechanism configured to refocus the optical system between acquiring images of two different surfaces of the multiple surfaces. In some embodiments, the imaging system is configured to image two or more fields-of-view on at least one of the surfaces. In some embodiments, the surfaces of the flow cell are coated with a hydrophilic coating layer, and wherein said hydrophilic coating layer further comprises labeled nucleic acid colonies disposed thereon at a surface density of > 10,000 nucleic acid colonies / mm^2. In some embodiments, an image of the surface acquired using the imaging system shows a contrast to noise ratio (CNR) of at least 5 when the nucleic acid colonies are labeled with cyanine dye 3 (Cy3), the imaging system

comprises a dichroic mirror and bandpass filter set optimized for Cy3 emission, and the image is acquired under non-signal saturating conditions while the surface is immersed in 25 mM ACES, pH 7.4 buffer. In some embodiments, said imaging system comprises 1, 2, 3, or 4 imaging channels configured to detect nucleic acid colonies disposed on at least one of the surfaces that have been labeled with 1, 2, 3, or 4 distinct detectable labels. In some embodiments, the imaging system is used to monitor a sequencing-by-avidity, sequencing-by-nucleotide base-pairing, sequencing-by-nucleotide binding, or sequencing-by-nucleotide incorporation reaction on at least one of the surfaces and detect a bound or incorporated nucleotide base. In some embodiments, the imaging system is used to perform nucleic acid sequencing. In some embodiments, the imaging system is used to determine a genotype of a sample, wherein determining the genotype of the sample comprises preparing a nucleic acid molecule extracted from the sample for sequencing, and then sequencing the nucleic acid molecule. In some embodiments, the at least one image sensor comprises pixels having a pixel dimension chosen such that a spatial sampling frequency for the imaging system is at least twice an optical resolution of the imaging system. In some embodiments, a combination of the objective lens and the at least one tube lens is configured to optimize a modulation transfer function in the spatial frequency range from 700 cycles per mm to 1100 cycles per mm in the sample plane. In some embodiments, the at least one tube lens is designed to correct modulation transfer function (MTF) at one or more specified spatial frequencies, defocus, spherical aberration, chromatic aberration, coma, astigmatism, field curvature, image distortion, image contrast-to-noise ratio (CNR), or any combination thereof, for a combination of the objective lens and the at least one tube lens.

[0685] Also disclosed herein are methods of sequencing a nucleic acid molecule, the methods comprising: a) imaging a first surface and two or more axially-displaced surfaces using an optical system which comprises an objective lens and at least one image sensor, wherein said optical system has a numerical aperture (NA) of less than 0.6 and a field-of-view (FOV) of greater than 1.0 mm^2, and wherein images of the first surface and the axially-displaced surfaces having substantially the same optical resolution are acquired without moving an optical compensator into an optical path between said objective lens and said at least one image sensor; and b) detecting a fluorescently-labeled composition comprising the nucleic acid molecule, or a complement thereof, disposed on the first surface or the axially-displaced second surface to determine an identity of a nucleotide in the nucleic acid molecule.

[0686] In some embodiments, a focusing mechanism is utilized to refocus the optical system between acquiring images of the first surface and the axially-displaced surfaces. In some embodiments, the method further comprises imaging two or more fields-of-view on at least one

of the first surface or the axially-displaced surfaces. In some embodiments, the first surface and the axially-displaced surfaces comprise one or more surfaces of a flow cell. In some embodiments, the surfaces of the flow cell are coated with a hydrophilic coating layer. In some embodiments, said hydrophilic coating layer further comprises labeled nucleic acid colonies disposed thereon at a surface density of > 10,000 nucleic acid colonies / mm^2. In some embodiments, an image of a surface of the surfaces acquired using said optical system shows a contrast to noise ratio (CNR) of at least 5 when the nucleic acid colonies are labeled with cyanine dye 3 (Cy3), the optical system comprises a dichroic mirror and bandpass filter set optimized for Cy3 emission, and the image is acquired under non-signal saturating conditions while the surface is immersed in 25 mM ACES, pH 7.4 buffer. In some embodiments, said optical system comprises 1, 2, 3, or 4 imaging channels configured to detect nucleic acid colonies disposed on at least one of the surfaces that have been labeled with 1, 2, 3, or 4 distinct detectable labels. In some embodiments, the at least one image sensor comprises pixels having a pixel dimension chosen such that a spatial sampling frequency for the optical system is at least twice an optical resolution of the optical system. In some embodiments, the optical system comprises at least one tube lens positioned between the objective lens and the at least one image sensor, and wherein the at least one tube lens is configured to correct an imaging performance metric for imaging a first interior surface of a flow cell, a second interior surface of the flow cell, a third interior surface, and a fourth interior surface of the flow cell. In some embodiments, the flow cell has a wall thickness of at least 700 μm and a gap between the first interior surface and the second interior surface of at least 50 μm. In some embodiments, the flow cell has a gap between the third interior surface and the fourth interior surface of at least 50 μm. In some embodiments, the flow cell has a distance between the second interior surface and the third interior surface of at least 50 μm. In some embodiments, the at least one tube lens comprises, in order, an asymmetric convex-convex lens, a convex-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens. In some embodiments, the optical system comprises two or more tube lenses which are designed to provide optimal imaging performance at two or more fluorescence wavelengths. In some embodiments, a combination of objective lens and tube lens is configured to optimize a modulation transfer function in the mid to high spatial frequency range. In some embodiments, the imaging performance metric comprises a measurement of modulation transfer function (MTF) at one or more specified spatial frequencies, defocus, spherical aberration, chromatic aberration, coma, astigmatism, field curvature, image distortion, image contrast-to-noise ratio (CNR), or any combination thereof. In some embodiments, the optical resolution of images of the first, second, third, and/or fourth surface are diffraction-limited across the entire field-of-view (FOV). In some embodiments, the

sequencing of the nucleic acid molecule further comprises performing a sequencing-by-avidity, sequencing-by-nucleotide base-pairing, sequencing-by-nucleotide binding, or sequencing-by-nucleotide incorporation reaction on at least one of the surfaces and detecting a bound or incorporated nucleotide base. In some embodiments, the method further comprises determining a genotype of a sample, wherein determining the genotype of the sample comprises preparing said nucleic acid molecule for sequencing, and then sequencing said nucleic acid molecule.

[0687] Disclosed herein are imaging systems configured to image two or more distinct, axially-displaced surfaces, e.g., 4 surfaces, the imaging systems comprising an objective lens and at least one image sensor, wherein said imaging system has a numerical aperture (NA) of less than 0.6 and a field-of-view (FOV) of greater than 1.0 mm2, and wherein said imaging system is capable of acquiring images of the distinct, axially-displaced surfaces that have substantially the same optical resolution without moving an optical compensator into an optical path between said objective lens and said at least one image sensor.

[0688] In some embodiments, the imaging system has a numerical aperture of greater than 0.3. In some embodiments, the imaging system further comprises a focusing mechanism used to refocus the optical system between acquiring images of the two or more distinct, axially-displaced surfaces. In some embodiments, the imaging system is configured to image two or more fields-of-view on at least one of the two or more distinct, axially-displaced surfaces. In some embodiments, the two or more distinct, axially-displaced surfaces comprise two surfaces of a flow cell. In some embodiments, the two or more distinct surfaces of the flow cell are coated with a hydrophilic coating layer, and wherein said hydrophilic coating layer further comprises labeled nucleic acid colonies disposed thereon at a surface density of > 10,000 nucleic acid colonies / mm^2. In some embodiments, said imaging system comprises 1, 2, 3, or 4 imaging channels configured to detect nucleic acid colonies disposed on at least one of the two or more distinct surfaces that have been labeled with 1, 2, 3, or 4 distinct detectable labels. In some embodiments, the at least one image sensor comprises pixels having a pixel dimension chosen such that a spatial sampling frequency for the imaging system is at least twice an optical resolution of the imaging system. In some embodiments, the imaging system comprises at least one tube lens positioned between the objective lens and the at least one image sensor, and wherein the at least one tube lens is configured to correct an imaging performance metric for imaging a first, second, third, and/or fourth interior surface of a flow cell. In some embodiments, the flow cell has a wall thickness of at least 700 μm and a gap between the first interior surface and the second interior surface of at least 50 μm. In some embodiments, the flow cell has a gap between the third interior surface and the fourth interior surface of at least 50 μm. In some embodiments, the flow cell has a gap between the second interior surface and the third

interior surface of at least 50 μm. In some embodiments, the imaging system comprises two or more tube lenses which are designed to provide optimal imaging performance at two or more fluorescence wavelengths. In some embodiments, the optical resolution of images of the distinct, axially-displaced surfaces are diffraction-limited across the entire field-of-view (FOV).

[0689] Disclosed herein are methods of sequencing a nucleic acid molecule, the method comprising: a) imaging a first surface and two or more axially-displaced second surfaces using a compensation-free optical system which comprises an objective lens and at least one image sensor, wherein said optical system has a numerical aperture (NA) of less than 0.6 and a field-of-view (FOV) of greater than 1.0 mm^2; b) processing the images of the first surface and the two or more axially-displaced surfaces to correct for optical aberration such that the images of the first surface and the two or more axially-displaced surfaces have substantially the same optical resolution; and c) detecting a fluorescently-labeled composition comprising the nucleic acid molecule, or a complement thereof, disposed on the first surface or one of the two or more axially-displaced surfaces to determine an identity of a nucleotide in the nucleic acid molecule.

[0690] In some embodiments, the images of the first surface and the two or more axially-displaced surfaces are acquired without moving an optical compensator into an optical path between said objective lens and said at least one image sensor. In some embodiments, the images of the first surface and the two or more axially-displaced surfaces are acquired by just refocusing the optical system. In some embodiments, the method further comprises imaging two or more fields-of-view on at least one of the first surface and the two or more axially-displaced surfaces. In some embodiments, the first surface and the two or more axially-displaced surfaces are surfaces of a flow cell. In some embodiments, the surfaces of the flow cell are coated with a hydrophilic coating layer. In some embodiments, said hydrophilic coating layer further comprises labeled nucleic acid colonies disposed thereon at a surface density of > 10,000 nucleic acid colonies / mm^2. In some embodiments, an image of a surface of the flow cell acquired using said optical system shows a contrast to noise ratio (CNR) of at least 5 when the nucleic acid colonies are labeled with cyanine dye 3 (Cy3), the optical system comprises a dichroic mirror and bandpass filter set optimized for Cy3 emission, and the image is acquired under non-signal saturating conditions while the surface is immersed in 25 mM ACES, pH 7.4 buffer. In some embodiments, said optical system comprises 1, 2, 3, or 4 imaging channels configured to detect nucleic acid colonies disposed on at least one of the first surface and the two or more axially-displaced surfaces that have been labeled with 1, 2, 3, or 4 distinct detectable labels. In some embodiments, at least one image sensor comprises pixels having a pixel dimension chosen such that a spatial sampling frequency for the optical system is at least twice an optical resolution of the optical system. In some embodiments, the optical system comprises at least

one tube lens positioned between the objective lens and the at least one image sensor, and wherein the at least one tube lens is configured to correct an imaging performance metric for imaging at least one of the surfaces of the flow cell. In some embodiments, the flow cell has a wall thickness of at least 700 μm and a gap between the first interior surface and the second interior surface of at least 50 μm. In some embodiments, the flow has a gap between the third interior surface and the fourth interior surface of at least 50 μm. In some embodiments, the flow has a gap between the third interior surface and the second interior surface of at least 50 μm. In some embodiments, the at least one tube lens comprises, in order, an asymmetric convex-convex lens, a convex-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens. In some embodiments, the optical system comprises two or more tube lenses which are designed to provide optimal imaging performance at two or more fluorescence wavelengths. In some embodiments, a combination of objective lens and tube lens is configured to optimize a modulation transfer function in the mid to high spatial frequency range. In some embodiments, the imaging performance metric comprises a measurement of modulation transfer function (MTF) at one or more specified spatial frequencies, defocus, spherical aberration, chromatic aberration, coma, astigmatism, field curvature, image distortion, image contrast-to-noise ratio (CNR), or any combination thereof. In some embodiments, the optical resolution of images of the first surface and the two or more axially-displaced surfaces are diffraction-limited across the entire field-of-view (FOV). In some embodiments, the sequencing of the nucleic acid molecule further comprises performing a sequencing-by-avidity, sequencing-by-nucleotide binding, or sequencing-by-nucleotide incorporation reaction on at least one of the first surface and the two or more axially-displaced surfaces and detecting a bound or incorporated nucleotide base. In some embodiments, the method further comprises determining a genotype of a sample, wherein determining the genotype of the sample comprises preparing said nucleic acid molecule for sequencing, and then sequencing said nucleic acid molecule.

[0691] Disclosed herein are systems for sequencing a nucleic acid molecule comprising: a) an optical system comprising an objective lens and at least one image sensor, wherein said optical system has a numerical aperture (NA) of less than 0.6 and a field-of-view (FOV) of greater than 1.0 mm^2, and is configured to acquire images of a first surface and two or more axially-displaced surfaces; and b) a processor programmed to: i) process images of the first surface and the two or more axially-displaced surfaces to correct for optical aberration such that the images of the first surface and the two or more axially-displaced surfaces have substantially the same optical resolution; and ii) detect a fluorescently-labeled composition comprising the nucleic acid molecule, or a complement thereof, disposed on the first surface or the two or more axially-displaced surfaces to determine an identity of a nucleotide in the nucleic acid molecule.

[0692] In some embodiments, the images of the first surface and the two or more axially-displaced surfaces are acquired without moving an optical compensator into an optical path between said objective lens and said at least one image sensor. In some embodiments, the images of the first surface and the two or more axially-displaced surfaces are acquired by just refocusing the optical system. In some embodiments, the imaging system has a numerical aperture of greater than 0.3. In some embodiments, the first surface and two or more axially-displaced surfaces comprise three, four, or more surfaces of a flow cell. In some embodiments, the surfaces of the flow cell are coated with a hydrophilic coating layer, and wherein said hydrophilic coating layer further comprises labeled nucleic acid colonies disposed thereon at a surface density of > 10,000 nucleic acid colonies / mm^2. In some embodiments, said optical system comprises 1, 2, 3, or 4 imaging channels configured to detect nucleic acid colonies disposed on at least one of the first surface or two or more axially-displaced surfaces that have been labeled with 1, 2, 3, or 4 distinct detectable labels. In some embodiments, the at least one image sensor comprises pixels having a pixel dimension chosen such that a spatial sampling frequency for the optical system is at least twice an optical resolution of the optical system. In some embodiments, the system comprises at least one tube lens positioned between the objective lens and the at least one image sensor, and wherein the at least one tube lens is configured to correct an imaging performance metric for imaging a first, second, third, and/or fourth interior surface of a flow cell. In some embodiments, the flow cell has a wall thickness of at least 700 μm and a gap between the first interior surface and the second interior surface of at least 50 μm. In some embodiments, the flow cell has gap between the third interior surface and the second interior surface of at least 50 μm. In some embodiments, the flow cell has gap between the third interior surface and the fourth interior surface of at least 50 μm. In some embodiments, the optical system comprises two or more tube lenses which are designed to provide optimal imaging performance at two or more fluorescence wavelengths.

[0693] Disclosed herein are fluorescence imaging systems comprising: a) at least one light source configured to provide excitation light within one or more specified wavelength ranges; b) an objective lens configured to collect fluorescence arising from within a specified field-of-view of a sample plane upon exposure of the sample plane to the excitation light, wherein a numerical aperture of the objective lens is at least 0.3, wherein a working distance of the objective lens is at least 700 μm, and wherein the field-of-view has an area of at least 2 mm^2; and c) at least one image sensor, wherein the fluorescence collected by the objective lens is imaged onto the image sensor, and wherein a pixel dimension for the image sensor is chosen such that a spatial sampling frequency for the fluorescence imaging system is at least twice an optical resolution of the fluorescence imaging system.

[0694] In some embodiments, the numerical aperture is at least 0.75. In some embodiments, the numerical aperture is at least 1.0. In some embodiments, the working distance is at least 850 μm. In some embodiments, the working distance is at least 1,000 μm. In some embodiments, the field-of-view has an area of at least 2.5 mm^2. In some embodiments, the field-of-view has an area of at least 3 mm^2. In some embodiments, the spatial sampling frequency is at least 2.5 times the optical resolution of the fluorescence imaging system. In some embodiments, the spatial sampling frequency is at least 3 times the optical resolution of the fluorescence imaging system. In some embodiments, the system further comprises an X-Y-Z translation stage such that the system is configured to acquire a series of two or more fluorescence images in an automated fashion, wherein each image of the series is acquired for a different field-of-view. In some embodiments, a position of the sample plane is simultaneously adjusted in an X direction, a Y direction, and a Z direction to match the position of an objective lens focal plane in between acquiring images for different fields-of-view. In some embodiments, the time required for the simultaneous adjustments in the X direction, Y direction, and Z direction is less than 0.4 seconds. In some embodiments, the system further comprises an autofocus mechanism configured to adjust the focal plane position prior to acquiring an image of a different field-of-view if an error signal indicates that a difference in the position of the focal plane and the sample plane in the Z direction is greater than a specified error threshold. In some embodiments, the specified error threshold is 100 nm. In some embodiments, the specified error threshold is 50 nm. In some embodiments, the system comprises three or more image sensors, and wherein the system is configured to image fluorescence in each of three or more wavelength ranges onto a different image sensor. In some embodiments, a difference in the position of a focal plane for each of the three or more image sensors and the sample plane is less than 100 nm. In some embodiments, a difference in the position of a focal plane for each of the three or more image sensors and the sample plane is less than 50 nm. In some embodiments, the total time required to reposition the sample plane, adjust focus, and acquire an image is less than 0.4 seconds per field-of-view. In some embodiments, the total time required to reposition the sample plane, adjust focus, and acquire an image is less than 0.3 seconds per field-of-view.

[0695] Also discloser herein are fluorescence imaging systems for multiple-side imaging of a flow cell comprising: a) an objective lens configured to collect fluorescence arising from within a specified field-of-view of a sample plane within the flow cell; b) at least one tube lens positioned between the objective lens and at least one image sensor, wherein the at least one tube lens is configured to correct an imaging performance metric for a combination of the objective lens, the at least one tube lens, and the at least one image sensor when imaging an interior surface of the flow cell, and wherein the flow cell has a wall thickness of at least 700 μm

and a gap between two adjacent interior surfaces of at least 50 μm; wherein the imaging performance metric is substantially the same for imaging two or more surfaces of the flow cell without moving an optical compensator into or out of an optical path between the flow cell and the at least one image sensor, without moving one or more optical elements of the tube lens along the optical path, and without moving one or more optical elements of the tube lens into or out of the optical path.

[0696] In some embodiments, the objective lens is a commercially-available microscope objective. In some embodiments, the commercially-available microscope objective has a numerical aperture of at least 0.3. In some embodiments, the objective lens has a working distance of at least 700 μm. In some embodiments, the objective lens is corrected to compensate for a cover slip thickness (or flow cell wall thickness) of 0.17 mm. In some embodiments, the fluorescence imaging system further comprising an electro-optical phase plate positioned adjacent to the objective lens and between the objective lens and the tube lens, wherein the electro-optical phase plate provides correction for optical aberrations caused by a fluid filling the gap between two adjacent interior surfaces, e.g., the first and second surfaces or the third and fourth surfaces of the flow cell. In some embodiments, the at least one tube lens is a compound lens comprising three or more optical components. In some embodiments, the at least one tube lens is a compound lens comprising four optical components. In some embodiments, the four optical components comprise, in order, a first asymmetric convex-convex lens, a second convex-plano lens, a third asymmetric concave-concave lens, and a fourth asymmetric convex-concave lens. In some embodiments, the at least one tube lens is configured to correct an imaging performance metric for a combination of the objective lens, the at least one tube lens, and the at least one image sensor when imaging an interior surface of a flow cell having a wall thickness of at least 1 mm. In some embodiments, the at least one tube lens is configured to correct an imaging performance metric for a combination of the objective lens, the at least one tube lens, and the at least one image sensor when imaging an interior surface of a flow cell having a gap of at least 100 μm. In some embodiments, the at least one tube lens is configured to correct an imaging performance metric for a combination of the objective lens, the at least one tube lens, and the at least one image sensor when imaging an interior surface of a flow cell having a gap of at least 200 μm. In some embodiments, the system comprises a single objective lens, two tube lenses, and two image sensors, and each of the two tube lenses is designed to provide optimal imaging performance at a different fluorescence wavelength. In some embodiments, the system comprises a single objective lens, three tube lenses, and three image sensors, and each of the three tube lenses is designed to provide optimal imaging performance at a different fluorescence wavelength. In some embodiments, the system comprises a single objective lens, four tube

lenses, and four image sensors, and each of the four tube lenses is designed to provide optimal imaging performance at a different fluorescence wavelength. In some embodiments, the design of the objective lens or the at least one tube lens is configured to optimize the modulation transfer function in the mid to high spatial frequency range. In some embodiments, the imaging performance metric comprises a measurement of modulation transfer function (MTF) at one or more specified spatial frequencies, defocus, spherical aberration, chromatic aberration, coma, astigmatism, field curvature, image distortion, contrast-to-noise ratio (CNR), or any combination thereof. In some embodiments, the difference in the imaging performance metric for imaging the surfaces, e.g., the first and second surface or the third and fourth surfaces of the flow cell is less than 10%. In some embodiments, the difference in imaging performance metric for imaging the surfaces is less than 5%. In some embodiments, the use of the at least one tube lens provides for an at least equivalent or better improvement in the imaging performance metric for multiple-side imaging compared to that for a conventional system comprising an objective lens, a motion-actuated compensator, and an image sensor. In some embodiments, the use of the at least one tube lens provides for an at least 10% improvement in the imaging performance metric for multiple-side imaging compared to that for a conventional system comprising an objective lens, a motion-actuated compensator, and an image sensor.

[0697] Disclosed herein are illumination systems for use in imaging-based solid-phase genotyping and sequencing applications, the illumination system comprising: a) a light source; and b) a liquid light-guide configured to collect light emitted by the light source and deliver it to a specified field-of-illumination on a support surface comprising tethered biological macromolecules.

[0698] In some embodiments, the illumination system further comprises a condenser lens. In some embodiments, the specified field-of-illumination has an area of at least 2 mm^2. In some embodiments, the light delivered to the specified field-of-illumination is of uniform intensity across a specified field-of-view for an imaging system used to acquire images of the support surface. In some embodiments, the specified field-of-view has an area of at least 2 mm^2. In some embodiments, the light delivered to the specified field-of-illumination is of uniform intensity across the specified field-of-view when a coefficient of variation (CV) for light intensity is less than 10%. In some embodiments, the light delivered to the specified field-of-illumination is of uniform intensity across the specified field-of-view when a coefficient of variation (CV) for light intensity is less than 5%. In some embodiments, the light delivered to the specified field-of-illumination has a speckle contrast value of less than 0.1. In some embodiments, the light delivered to the specified field-of-illumination has a speckle contrast value of less than 0.05.

[0699] In another aspect, the present disclosure provides a system. The system may comprise a curved substrate. The curved substrate may comprise at least one binding moiety configured to bind to an analyte. The system may comprise an optical system comprising a light source. The light source may be configured to direct light from the light source to the curved substrate. The light may be configured to probe a presence or absence of the analyte bound to the curved surface.

[0700] The analyte may comprise an analyte as described elsewhere herein. For example, the analyte may comprise a nucleic acid. In another example, the analyte can comprise a polypeptide. The analyte may comprise a plurality of analytes. For example, the analyte can comprise a plurality of nucleic acids. The binding moiety may be selected based on the analyte. For example, the binding moiety can be selected to have binding affinity for the predetermined analyte. For example, an at least partially complementary nucleic acid can be used as a binding moiety for a nucleic acid analyte.

[0701] The curved substrate may be at least a portion of a flow cell. The curved substrate may be a component of a flow cell. For example, the curved substrate can be a portion of a curved wall of the flow cell. In another example, the curved substrate can be disposed within a flow cell. For example, the curved substrate can be disposed on a removable chip placed in the flow cell. The system may comprise a flow cell, and the flow cell may comprise the curved substrate. The curved substrate may comprise a capillary of a flow cell. For example, the curved substrate can be an interior wall of the capillary. The curved substrate may be disposed on a side of the capillary closest to the optical system. For example, for an optical system disposed above the capillary, the curved substrate can be on a top side of the capillary. The curved substrate may be disposed on an opposite side of the capillary from the optical system. For example, for an optical system disposed above the capillary, the curved substrate can be disposed on the bottom of the capillary.

[0702] In some cases, the curved substrate can comprise a glass. The glass may be an oxide (e.g., a silicon oxide) with at least partial transparency to the wavelength of the light. The curved substrate may comprise a polymer. Examples of polymers include, but are not limited to, alkyl polymers (e.g., polyethylene, polypropylene, etc.), fluoropolymers (e.g., Teflon-AF (Dupont), Cytop® (Asahi Glass, Japan)), aromatic polymers (e.g., polyxylenes (Parylene, Kisco, Calif.), polystyrene, polymethmethylacrytate), another polymer disclosed elsewhere herein, or the like, or any combination thereof. In some cases, the curved substrate comprises a glass and a polymer. For example, a glass pane can be inset into a polymer flow cell. In another example, the curved substrate can comprise a polymer coated glass.

[0703] The light source may be a light source as described elsewhere herein. The light source may comprise a laser (e.g., a diode laser, a gas laser, etc.). The light source may comprise a light emitting diode (LED). The light source may comprise an incandescent light source (e.g., a halogen lamp, a filament lamp, etc.). The light source may be configured to provide a light such as an excitation light described elsewhere herein. For example, the light source can be configured to provide light with a wavelength of about 500 nanometers (nm) to about 540 nm, about 620 nm to about 650 nm, about 460 nm to about 500 nm, or any combination thereof. The light source may be a broadband light source (e.g., a light source configured to produce light with a plurality of wavelengths). The light source may be a narrow band light source (e.g., a light source configured to provide a single or a few narrow wavelength bands).

[0704] In some cases, the system can comprise a second curved substrate. For example, the system can comprise a plurality of curved substrates in optical communication with at least one light source. The second curved substrate may not be attached to the curved substrate. For example, the second curved substrate can be a curved portion of a different flow cell from the flow cell of the first substrate. The second curved substrate may be a part of a same element of the system. For example, the second curved substrate and the curved substrate can be different parts of a substantially cylindrical component of a flow cell (e.g., a capillary). For example, the curved substrate and the second curved substrate can be opposite sides of a substantially cylindrical flow cell. In this example, the curved substrate and the second curved substrate can be shown in the dotted ovals of FIGS. 54A – 54B. The second curved substrate may comprise at least one second binding moiety configured to bind to a second analyte. The second binding moiety may be of a same type as the binding moiety. For example, nucleic acids can be used as both the binding moiety and the second binding moiety. The second binding moiety may be of a different type from the binding moiety. For example, the binding moiety can be a nucleic acid while the second binding moiety can be an antigen.

[0705] In some cases, the system is configured to probe the curved substrate in an epifluorescent configuration. The epifluorescent configuration may comprise probing light on a same side of a substrate as the light is introduced on. For example, the system can be configured to use a same objective to collect the light after interaction with the substrate as to deliver the light to the substrate. In some cases, the system is configured to probe the curved substrate in a transmissive configuration. The transmissive configuration may comprise probing light on an opposite side of a substrate as the light is introduced on. For example, a transmissive configuration can probe the transmission and/or absorption of a sample by detecting the light transmitted through the sample. FIG. 54A shows an example of a transmissive configuration, where an imaging sensor is disposed opposite from a light source. The light source and the imaging sensor may be disposed

directly opposite from one another. The light source and the imaging sensor can be disposed at an angle from one another.

[0706] To address the curved substrate 5301 and the second curved substrate 5302, the system may comprise a focal shifting assembly configured to move a focal field between the curved substrate and the second curved substrate. An example of this shifting can be seen in FIGS. 53A – 53B, where an imaging volume 4915 (e.g., a focal field) can be translated between curved substrates through use of a focal shifting assembly. In some cases, the focal field can be shifted horizontally (e.g., in a plane perpendicular to the optical axis).

[0707] The focal shifting assembly may comprise at least one movable lens. For example, a lens can be movable with respect to the reset of the system to adjust a focal field of the system. In this example, the movement of the lens can redirect the light moving through the lens to translate the focal field. The lens may be movable along the optical (e.g., z) axis. The lens may be movable out of the optical axis (e.g., in an x-y plane). The lens may be movable in three dimensions. The lens may be disposed within a lens barrel. For example, the lens can be placed in a lens barrel such as that of FIG. 51.

[0708] In some cases, the focal shifting assembly can comprise at least one movable prism. The movable prism may be configured to shift a light beam traveling through the movable prism by refraction. For example, a movable prism can refract an incident light beam, and by moving the prism with respect to other optical elements (e.g., prisms, lenses, gratings), the overall path of the light beam can be moved, thereby shifting a focal field. In some cases, the focal shifting assembly can comprise a plurality of prisms. At least one prism of the plurality of prisms can be movable. Each prism of the plurality of prisms may be movable. For example, two movable prisms can be used to achieve a fine control over the movement of the light through the prisms, which can result in fine control over the movement of the focal field.

[0709] The optical system may be movable with respect to the curved substrate. For example, the optical system can be translated with respect to the curved substrate. The optical system may be translatable in three dimensions with respect to the curved substrate. For example, the optical system can be scanned across the curved substrate. The optical system may be rotatable around the curved substrate. The optical system may be rotatable with a same curvature as the curved substrate. For example, the optical system may be rotatable such that the curved substrate is kept at a same distance throughout the rotation. An example of a rotatable optical system can be seen in FIGS. 52A – 52B.

[0710] The optical system may be configured to image a plurality of binding moieties. The optical system may be configured to image an area of the curved substrate comprising a plurality of binding moieties. For example, a plurality of binding moieties can be arranged on the

substrate such that they are present in a field of view of the optical system. The optical system may be configured to image at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 500, 1,000, or more binding moieties at a same or substantially same time. The optical system may be configured to image at most about 1,000, 500, 250, 200, 150, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, or fewer binding moieties at a same or substantially same time. The plurality of binding moieties may comprise binding moieties of a same type. For example, each binding moiety of the plurality of binding moieties may be a nucleic acid binding moiety. The plurality of binding moieties may comprise a plurality of different types of binding moieties. For example, the plurality of binding moieties can comprise nucleic acids and proteins.

[0711] The curved substrate may have a deviation from flatness. The deviation from flatness may be a measure of the extent of curvature of the curved substrate. For example, a curved substrate with a deviation from flatness of 1 millimeter can have a deviation of 1 millimeter from a flat plane of the substrate. The curved substrate can have a deviation from flatness of at least about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1,000, 1,500, 2,000, 2,500, 5,000, 10,000, or more micrometers. The curved substrate can have a deviation from flatness of at most about 10,000, 5,000, 2,500, 2,000, 1,500, 1,000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, 350, 300, 250, 200, 150, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 1, or fewer micrometers. The curved substrate may have a deviation from flatness as defined by any two of the proceeding values. For example, the curved substrate can have a deviation from flatness of about 100 to about 500 micrometers. The curved substrate may have a deviation from flatness greater than a focal depth of the optical system. For example, for an optical system with a focal depth of 10 micrometers, the curved substrate may have a deviation from flatness of 500 micrometers. Having a curvature larger than the focal depth of the optical system may permit selective imaging of portions of the curved substrate. For example, light originating outside of the focal depth can be discarded by the optical system. Thus, a plurality of analytes can be imaged from different regions of the curved substrate without moving the substrate or the optical system. Further, by moving the optical system with respect to the curved substrate, a larger number of analytes can be analyzed.

[0712] The system may comprise a plurality of sub-optical systems. The plurality of sub-optical systems may or may not be parallel to one another, adjacent to one another, or a combination thereof. The plurality of sub-optical systems may be configured to image at least partially different regions of the curved substrate. For example, the plurality of sub-optical system can be disposed radially around the curved substrate (e.g., as shown in FIG. 52). The plurality of sub-

optical systems can be configured with a focal shifting assembly as described elsewhere herein. The use of the sub-optical systems with the focal shifting assembly can enable detection over an entire cylindrical curved substrate without encircling the cylindrical substrate in sub-optical assemblies. For example, sub-optical assemblies can be disposed on one half of the cylindrical curved substrate, and the other side of the cylindrical curved substrate can be addressable using a focal shifting assembly within the sub-optical assembly. Each sub-optical system of the plurality of sub optical systems can be individually disposed perpendicular to a plurality of tangents of the curved substrate. For example, the three sub-optical assemblies of FIG. 52 can be disposed perpendicular to three different tangents of the curved substrate.

[0713] The system may comprise a stage. The curved substrate may be disposed on the stage. For example, the curved substrate can be attached to the stage. The stage may be configured to support and/or move the curved substrate within the system. The stage may comprise one or more of a tilt stage (e.g., a stage configured to tilt the curved substrate in one, two, or three dimensions), a rotation stage (e.g., a stage configured to rotate the curved substrate in one, two, or three dimensions), a translation stage (e.g., a stage configured to translate the curved substrate in one, two, or three dimensions), or the like.

[0714] The curved substrate may comprise a hydrophilic polymer coupled thereto. The hydrophilic polymer may be as described elsewhere herein. The hydrophilic polymer may reduce a surface tension of a sample in contact with the curved substrate. For example, coupling the hydrophilic polymer to the curved substrate may permit analytes to move closer to the curved substrate than in an absence of the hydrophilic polymer. The at least one binding moiety may be coupled to the hydrophilic polymer. For example, the at least one binding moiety may be bound to the hydrophilic polymer. In this example, a reactive moiety in the hydrophilic polymer can be reacted with a reactive moiety in the binding moiety.

[0715] The system can have a numerical aperture as described elsewhere herein (e.g., the numerical aperture of an objective lens and/or an optical imaging module). The system may comprise an imaging sensor as described elsewhere herein. For example, the imaging sensor can be configured to collect the light subsequent to the directing of the light to the curved substrate. In some cases, the system comprises a heater configured to heat the substrate. The heater may be a heater as described elsewhere herein. For example, the heater can be an integrated heater. In another example, the heater can be an infrared heater.

[0716] In another embodiment, the present disclosure provides a system. The system may comprise a curved substrate. The system may comprise an optical system comprising a light source. The light source may be configured to direct light from the light source to the curved substrate.

[0717] In another embodiment, the present disclosure provides a system. The system may comprise a substrate. The system may comprise an optical system. The optical system may be configured to image an area of the substrate of at least about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or more square millimeters (mm^2). The optical system may be configured to image an area of the substrate of at most about 50, 45, 40, 35, 30, 25, 20, 15, 10, 9.5, 9, 8.5, 8, 7.5, 7, 6.5, 6, 5.5, 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5, or fewer mm^2. The optical system may be configured to image an area of the substrate in a range as defined by any two of the proceeding values. For example, the optical system can be configured to image an area of the substrate of about 4.5 to about 5.5 mm^2. The substrate may be as described elsewhere herein. The optical system may be as described elsewhere herein.

[0718] The optical system may be configured to simultaneously image the area. For example, the optical system can be configured such that the entire area of the substrate within a field of view of the optical system. In this example, the entire field of view can be imaged at a same time. The optical system may be configured to image the area substantially simultaneously. For example, the optical system can be configured to image a first region and a second region of the area at a substantially same time.

[0719] The optical system may comprise a plurality of sub-optical systems. The plurality of sub-optical systems may be as described elsewhere herein. For example, the plurality of sub-optical systems can be parallel and adjacent to one another. The plurality of sub-optical systems may be configured to image the area of the substrate in parallel. For example, the plurality of sub-optical systems can be oriented adjacent to one another and configured to each image at least a portion of the area of the substrate. In this example, the plurality of sub-optical systems can each generate a sub-image, and the plurality of sub-images can be combined to form an image of the entire area.

[0720] The optical system may comprise a light source configured to provide a light beam and a lens. The lens may be configured to focus the light beam from the light source onto a focal region of the substrate comprising the area. The light source may be as described elsewhere herein. The lens may comprise a lens as described elsewhere herein. The lens may have an area of at least about 10, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,500, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500, 5,000, 10,000, or more mm^2. The lens may have an area of at most about 10,000, 5,000, 4,500, 4,000, 3,500, 3,000, 2,500, 2,000, 1,500, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 50, 10, or fewer mm^2. The lens may be a large-format lens (e.g., a lens configured for use over a large area). A homogeneity of the light beam over the focal regions can be at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, or more percent. A homogeneity of the light beam over the focal regions can be

at most about 99.9, 98, 97, 96, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, or less percent. A homogeneity of the light beam over the focal regions can be in a range as defined by any two of the proceeding values. The homogeneity may be a measure of the consistency of one or more properties of the light beam (e.g., power, wavelength, flux, etc.) over the focal region. The homogeneity may be in two dimensions or three dimensions. The homogeneity may be affected by the elements of the optical system. For example, inhomogeneity can be increased when defects are present in the lens.

[0721] The substrate may be as described elsewhere herein. For example, the substrate may be a curved substrate. For example, the substrate can be disposed as a cylinder. The substrate may be at least a portion of a capillary flow cell as described elsewhere herein. The substrate may have a deviation from flatness as described elsewhere herein.

[0722] The system may comprise a stage as described elsewhere herein, and the substrate may be disposed on the stage. For example, the stage can comprise a tilt stage, a rotation stage, a translation stage, or the like, or any combination thereof. The system may have a numerical aperture as described elsewhere herein. For example, the system can have a numerical aperture of at most about 0.6. The system may comprise a heater as described elsewhere herein. For example, the heater can be an integrated heater. The substrate may comprise a hydrophilic polymer coupled thereto as described elsewhere herein.

[0723] The optical system may be configured to image the area of the substrate with a resolution of at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 500, or more micrometers. The optical system may be configured to image the area of the substrate with a resolution of at most about 500, 250, 200, 150, 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9.5, 9, 8.5, 8, 7.5, 7, 6.5, 6, 5.5, 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, or less micrometers. The optical system may be configured to image the area of the substrate with a resolution in a range as defined by any two of the proceeding values.

[0724] The system may comprise an imaging sensor configured to collect the light subsequent to the directing to the substrate. The imaging sensor may be as described elsewhere herein. For example, the imaging sensor can be a camera. The imaging sensor may be configured to provide a color image of the light. For example, the imaging sensor can be configured to record information on the color of the light subsequent to the directing to the substrate. The imaging sensor may be configured to not record a color image of the light. For example, the imaging sensor can record the intensity of the light, but not the wavelength.

Samples

[0725] In some embodiments, the sequencing system including optical system advantageously enable sequencing and imaging of target analyte(s) or features while they remain intact inside the cell or tissue. In some embodiments, the cell or tissue and the targets (e.g., target analytes, structure elements, organelles, etc.) therewithin remain intact during sequencing and/or imaging. In some embodiments, the one or more samples being imaged using the optical systems herein can be 2D or 3D samples. The 3D samples can include in situ samples such as cells, tissues, or the like. In some embodiments, the cells or tissue samples are immobilized on the flow cell device or otherwise substrate for sequencing and/or imaging without modifying the spatial locations of targets within the cells or tissue. In some embodiments, the cells or tissue samples are immobilized on the flow cell device or otherwise substrate for sequencing or imaging without modifying the spatial relationship of targets or target analytes within the cells or tissue. In some embodiments, the cells and/or tissue are immobilized with the morphological features, RNA, mRNA, and protein targets of the samples intact inside the cell(s) or tissue during sequencing and/or imaging. In some embodiments, the spatial locations or relationships of the target analytes or targets remain intact during sequencing and/or imaging. In some embodiments, the spatial locations or relationships of the target analytes or targets during sequencing and/or imaging are not manually reconstructed using artificially added structure or features in the sample. For example, the nucleus, cell membrane, mitochondria, and extracellular matrix can retain their relative spatial relationship to each other in the sample(s), e.g., as shown in FIGS. 103A-103D, during imaging and/or sequencing.

[0726] In some embodiments, the one or more samples include target analyte(s) that are located inside the sample(s) or on the membrane of the sample(s). In some embodiments, the one or more samples include target analyte(s) that are on the exterior or interior surface of the cell. In some embodiments, the one or more samples include target analyte(s) that are on the exterior or interior surface of the cell membrane. In some embodiments, In some embodiments, the one or more samples include target analyte(s) that are part of the extracellular matrix. In some embodiments, the one or more samples include target analyte(s) that are part of and/or located on one or more organelles within the cell or tissue. In some embodiments, the one or more samples include target analytes that are on or in the glycocalyx or belong to part of the glycocalyx.

[0727] In some embodiments, the target analyte(s) comprise at least one polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the target analyte(s) comprise at least one polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the target analyte(s) comprise at least one polypeptide, enzyme or lipid located in or on a cellular structure including without limits any

cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

[0728] Depending on the sample(s) immobilized on the support (e.g., a flow cell), the flow cell images may include single or multiple z locations along an z axis orthogonal to the image plane of the flow cell images. In particular, for three dimensional samples, e.g., cells, tissues, or other *in situ* samples, the flow cell images can include multiple z-levels (i.e., axial locations) in order to cover the whole sample(s) in 3D. The z axis can extend from the objective lens of the optical system disclosed herein to the support, e.g., flow cell. The z axis can be orthogonal to the image plane of the flow cell images. Each z location of flow cell images may be separated from the adjacent z location(s) for a predetermined distance, for example, for about 0.1 um to about 15 ums. Each z location of flow cell images may be separated from the adjacent level(s) for 0.5 um to 10 ums. Each z location of flow cell images may be separated from the adjacent level(s) for 0.2 um to 2 ums. At each z location, flow cell images can be acquired from one or more sequencing cycles and/or one or more channels. Each flow cell image may include in its field of view at least part of one or more tiles or subtiles of the flow cell. FIG. 64A shows an exemplary a flow cell. The image plane is defined by the x and y axis. And the axial axis (i.e., z axis) is orthogonal to the x-y plane. Preparation of the 3D sample(s) and immobilization on flow cells for sequencing reactions and imaging using the optical systems herein are disclosed in U.S. Patent Application No. 18/078,820 filed on December 9, 2022, and is herein incorporated by reference in its entirety. In some cases, the sample can have a dimension (e.g., length, width, height, etc.) of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 2,000, 3,000, 4,000, 5,000, 10,000, or more micrometers.

[0729] In some embodiments, the one or more samples being imaged using the optical systems can be 2D or 3D samples. In some embodiments, the one or more samples herein include analytes (e.g., nucleic acids, DNA, RNA, mRNA, and/or proteins) obtained from cell or tissue with preserved spatial information to undergo sequencing and/or imaging outside the cell or tissue. In some embodiments, the one or more samples herein include analytes (e.g., nucleic acids, DNA, RNA, and/or proteins) removed from cell or tissue so that the analytes are not inside the cell or tissue anymore to undergo sequencing and/or imaging outside the cell or tissue, while keeping the rest of the cell or tissue, e.g., the structure of the cell or tissue, intact while the analytes are outside. In some embodiments, the one or more samples include analytes (e.g., nucleic acids, DNA, RNA, and/or proteins) transferred to the outside of the cell or tissue with artificially reconstructed spatial information to undergo sequencing and/or imaging outside the cell or tissue.

[0730] Although the flow cell images, samples, and the axial axis are described in a Cartesian coordinate system, any other coordinate systems can be used to define spatial locations and relationships herein. Other coordinate systems can include but are not limited to the polar coordinate system, cylindrical, or spherical coordinate systems.

[0731] In some embodiments, imaging at multiple different z-levels can be approximated by a multiplication of imaging time at each z-level. For example, imaging time at two z-levels with identical imaging parameters except the z-location can be twice the imaging time at a single z-level. In some embodiments, imaging time at two z-levels with identical imaging parameters except the z-location can be twice the imaging time at a single z-level plus focusing time for each z-level, which can be neglectable when the imaging time is in the range of several minutes. The focusing time may range from several milliseconds to several seconds for a single FOV using focusing methods disclosed in International Patent Application Ser. No. PCT/US2024/015602, filed February 12, 2024, which is incorporated herein by reference in its entirety.

[0732] In some embodiments, in any of the compositions or methods described herein, the one or more sample(s) comprises a cell, a plurality of cells, a section of a cell, an intact tissue, an organ, a tissue section, an intact tumor, or a tumor section. In some embodiments, the sample(s) comprises a fresh cellular sample, a freshly-frozen cellular sample, a sectioned cellular sample, or an FFPE cellular sample. In some embodiments, the sample(s) comprises one or more living cells or non-living cells. In some embodiments, the sample(s) can be obtained from a virus, fungus, prokaryote or eukaryote. In some embodiments, the sample(s) can be obtained from an animal, fungus, plant or bacterium. In some embodiments, the animal is a mammal or an insect. In some embodiments, the sample(s) comprises one or more virally-infected cells. In some embodiments, the sample(s) comprises a biofilm, i.e. a consortium of microorganisms that adhere together. In some embodiments, the sample(s) can be obtained from any organism including human, simian, ape, canine, feline, bovine, equine, murine, porcine, caprine, lupine, ranine, piscine, plant, insect, or bacterium. In some embodiments, the sample(s) can be obtained from any organ including head, neck, brain, breast, ovary, cervix, colon, rectum, endometrium, gallbladder, intestines, bladder, prostate, testicles, liver, lung, kidney, esophagus, pancreas, thyroid, pituitary, thymus, skin, heart, larynx, or other organs.

[0733] In some embodiments, the sample(s) harbors a plurality of target analytes including polypeptides, lipids, nucleic acids and polysaccharides, or a mixture thereof.

[0734] In some embodiments, the sample(s) harbors 2-10,000 different target analytes. In some embodiments, the target analytes comprise a plurality of target polypeptides. In some embodiments, the plurality of target polypeptides have different sequences. In some

embodiments, the sample(s) harbors 1-25 different target polypeptide, or harbors 25-50 different target polypeptides, or harbors 50-75 different target polypeptides, or harbors 75-100 different target polypeptides, or harbors any range therebetween of different target polypeptides. In some embodiments, the sample(s) harbors more than 100 different target polypeptides, or more than 250 different target polypeptides, or more than 500 different target polypeptides, or more than 1000 different target polypeptides. In some embodiments, the sample(s) harbors more than 10,000 different target polypeptides.

[0735] In some embodiments, the sample(s) can be deposited (e.g., seeded) onto a support. In some embodiments, the support comprises a planar or non-planar support. In some embodiments, the support comprises a solid or semi-solid support. In some embodiments, the support comprises a porous, semi-porous or non-porous support. The support can be made of any material such as glass, plastic or a polymer material. In some embodiments, the surface of the support can be coated with one or more compounds to produce a passivated layer on the support. In some embodiments, the passivated layer forms a porous or semi-porous layer.

[0736] In some embodiments, the sample(s) can be deposited (e.g., seeded) onto a support which is passivated with a coating that promotes cell adhesion. In some embodiments, the sample(s) can be deposited on a support that lacks immobilized capture primers which can bind target polynucleotide analytes from the sample(s). In some embodiments, the support can be coated with one or more compounds that generate a charged coated surface. In some embodiments, the support is coated with a lysine compound, poly-lysine compound, arginine compound, poly-arginine compound, or an amino-terminated compound (e.g., including amino-terminated PEG). The support can be coated with an unbranched compound, a branched compound, or a mixture of unbranched and branched compounds. In some embodiments, the support can be coated with modified peptides, including, for example and without limitation, cationic anti-microbial peptides or dual surface anti-microbial peptides. In some embodiments, the support can be coated with polycyclic peptide antibiotics comprising thioether amino acids lanthionine or methyllanthionine and/or unsaturated amino acids dehydroalaine and 2-aminoisobutryic acid. In some embodiments, the support can be coated with at least one small peptide such as melittin. In some embodiments, the support can be coated with a compound that promotes integrin-mediated cell adhesion. For example, and without limitation, the support can be coated with tripeptide arginyl-glycyl-aspartic acid (Arg-Gly-Asp; also known as RGD). In some embodiments, the support can be coated with amines or polymers having -NH2 groups which promote cell adhesion, including for example polyethyleneimine (PEI) or polydopamine (PDA).

Culturing cellular samples on a support

[0737] In some embodiments, in any of the compositions or methods described herein, the one or more sample(s) can be cultured on a support (e.g., a flow cell). In some embodiments, the methods comprise culturing the sample(s) on the support under condition suitable for expanding the sample(s) for 2-10 generations (e.g., 2-10 cellular passages), or more. The cultured cellular sample can generate a colony of cells. In some embodiments, the methods comprise culturing cells of the sample(s) to confluence or to non-confluence. In some embodiments, the methods comprise culturing the cells of the sample(s), and then inducing terminal differentiation of the cells using methods known in the art. In some embodiments, the methods comprise culturing the sample(s) on the support in a simple or complex cell culture media. Suitable cell culture media will be known to persons of ordinary skill in the art, who will be able to select a medium based on cell type and culture conditions. Exemplary cell culture media include, but are not limited to, D-MEM high glucose (e.g., from Thermo Fisher Scientific™, catalog No. 11965118), fetal bovine serum (e.g., 10% FBS; for example from Thermo Fisher Scientific™, catalog No. A3160402), MEM non-essential amino acids (e.g., 0.1 mM MEM, for example from Thermo Fisher Scientific, catalog No. 11140050), L-glutamine (e.g., 6 mM L-glutamine, for example from Thermo Fisher Scientific™, catalog No. A2916801), MEM sodium pyruvate (e.g., 1 mM sodium pyruvate, for example from Thermo Fisher Scientific™, catalog No. 11360070), and an antibiotic (e.g., 1% penicillin-streptomycin-glutamine, for example from Thermo Fisher™, catalog No. 10378016). In some embodiments, the methods comprise culturing the sample(s) at a humidity and temperature that is suitable for culturing the cell(s) on the support. Exemplary, non-limiting suitable conditions comprise approximately 37 °C with a humidified atmosphere of approximately 5-10% carbon dioxide in air. The sample(s) can be cultured with suitable aeration, e.g., with oxygen and/or nitrogen. In some embodiments, the sample comprises one or more isolated cells. The isolated cells can be isolated from, for example, one or more tissues or other samples. For example, a plurality of cells can be isolated from a tissue prior to being introduced into a flow cell and sequenced.

[0738] In some embodiments, a "simple cell medium" or related terms refer to a cell medium that typically lacks ingredients to support cell growth and/or proliferation in culture. Simple cell media can be used, for example, to wash, suspend, or dilute the sample(s). Simple cell media can be mixed with certain ingredients to prepare a cell media that can support cell growth and/or proliferation in culture. A simple cell medium comprises any one or any combination of two or more of a buffer, a phosphate compound, a sodium compound, a potassium compound, a calcium compound, a magnesium compound and/or glucose. In some embodiments, the simple

cell media comprises PBS (phosphate buffered saline), DPBS (Dulbecco's phosphate-buffered saline), HBSS (Hank's balanced salt solution), DMEM (Dulbecco's Modified Eagle's Medium), EMEM (Eagle's Minimum Essential Medium), and/or EBSS. In some embodiments, the sample(s) can be placed in a simple cell medium prior to or during the step of conducting any of the nucleic acid methods described herein.

[0739] In some embodiments, a "complex cell medium" or related terms refer to a cell media that can be used to support cell growth and/or proliferation in culture without supplementation or additives. Complex cell media can include any combination of two or more of a buffering system (e.g., HEPES), inorganic salt(s), amino acid(s), protein(s), polypeptide(s), carbohydrate(s), fatty acid(s), lipid(s), purine(s) and their derivatives (e.g., hypoxanthine), pyrimidine(s) and their derivatives, and/or trace element(s). Complex cell media includes fluids obtained from a fluid or tissue extract. Complex cell media includes artificial cell media. In some embodiments, complex cell media can be a serum-containing media, for example complex cell media includes fluids such as fetal bovine serum, blood plasma, blood serum, lymph fluid, human placental cord serum and amniotic fluid. In some embodiments, complex cell media can be a serum-free media, which are typically (but not necessarily) defined cell culture media. In some embodiments, complex cell media can be a chemically-defined media which typically (but not necessarily) include recombinant polypeptides, and ultra-pure inorganic and/or organic compounds. In some embodiments, complex cell media can be a protein-free media which include for example MEM (minimal essential media) and RPMI-1640 (Roswell Park Memorial Institute). In some embodiments, the complex cell media comprises IMDM (Iscove's Modified Dulbecco's Medium. In some embodiments, the complex cell media comprises DMEM (Dulbecco's Modified Eagle's Medium). In some embodiments, the sample(s) can be placed in a complex cell medium prior to, or during, the step of conducting any of the nucleic acid methods described herein.

Cell Fixation

[0740] In some embodiments, in any of the compositions or methods described herein, the sample(s) comprises a fixed cellular sample. In some embodiments, the sample(s) can be treated with a fixation reagent (e.g., a fixing reagent) that preserves the cell and its contents to inhibit degradation and can inhibit cell lysis. For example, and without limitation, the fixation reagent can preserve RNA harbored by the sample(s). In some embodiments, the fixation reagent inhibits loss of nucleic acids from the sample(s).

[0741] In some embodiments, the fixation reagent can cross-link the RNA to prevent the RNA from escaping the sample(s). In some embodiments, a cross-linking fixation reagent comprises

any combination of an aldehyde, formaldehyde, paraformaldehyde, formalin, glutaraldehyde, imidoesters, N-hydroxysuccinimide esters (NHS) and/or glyoxal (a bifunctional aldehyde).

[0742] In some embodiments, the fixation reagent comprises at least one alcohol, including methanol or ethanol. In some embodiments, the fixation reagent comprises at least one ketone, including acetone. In some embodiments, the fixation reagent comprises acetic acid, glacial acetic acid and/or picric acid. In some embodiments, the fixation reagent comprises mercuric chloride. In some embodiments, the fixation reagent comprises a zinc salt comprising zinc sulphate or zinc chloride. In some embodiments, the fixation reagent can denature polypeptides.

[0743] In some embodiments, the fixation reagent comprises 4% w/v of paraformaldehyde to water/PBS. In some embodiments, the fixation reagent comprises 10% of 35% formaldehyde at a neutral pH. In some embodiments, the fixation reagent comprises 2% v/v of glutaraldehyde to water/PBS. In some embodiments, the fixation reagent comprises 25% of 37% formaldehyde solution, 70% picric acid and 5% acetic acid.

[0744] In some embodiments, the sample(s) can be fixed on the support with 4% paraformaldehyde for about 30-60 minutes, or any range therebetween, and washed with PBS.

[0745] In some embodiments, the sample(s) can be stained, de-stained (e.g., with stain removed), or un-stained.

Cell Permeabilization

[0746] In some embodiments, in any of the compositions or methods described herein, the sample(s) comprises a permeabilized cellular sample. In some embodiments, the methods comprise treating the sample(s) with a permeabilization reagent that alters the cell membrane to permit penetration of reagents, such as the RCA reagents and sequencing reagents described herein, into the cells. For example, and without limitation, the permeabilization reagent removes membrane lipids from the cell membrane. In some embodiments, the sample(s) can be treated with a permeabilization reagent which comprises any combination of an organic solvent, detergent, chemical compound, cross-linking agent and/or enzyme. In some embodiments, the organic solvents comprise acetone, ethanol, and methanol. In some embodiments, the detergents comprise saponin, Triton X-100, Tween-20, sodium dodecyl sulfate (SDS), an N-lauroylsarcosine sodium salt solution, or a nonionic polyoxyethylene surfactant (*e.g.*, NP40). In some embodiments, the cross-linking agent comprises paraformaldehyde. In some embodiments, the enzyme comprises trypsin, pepsin or protease (*e.g.*, proteinase K). In some embodiments, the cells can be permeabilized using an alkaline condition, or an acidic condition with a protease enzyme. In some embodiments, the permeabilization reagent comprises water and/or PBS.

[0747] For example, and without limitation, the fixed cells can be permeabilized with 70% ethanol for about 30-60 minutes, and the permeabilizing reagent can be exchanged with PBS-T

(*e.g.*, PBS with 0.05% Tween-20). In some embodiments, the cells can be post-fixed with 3% paraformaldehyde and 0.1% glutaraldehyde for about 30-60 minutes, or any range therebetween, and washed with PBS-T, *e.g.*, washed multiple times.

[0748] In some cases, analysis of a sample (e.g., an in situ sample) using the methods and systems of the present disclosure can be performed without altering a spatial relationship of analytes within the sample. For example, a sample can be analyzed without destruction of the three-dimensional structure of the sample, thereby enabling localization of the analytes withing the sample. In this example, the morphological characteristics of the sample can be correlated with the identity of the analytes in the sample, thereby providing complimentary information regarding the sample. In some cases, the analysis of the sample may not comprise destruction of the sample (e.g., removal of supporting structures of the sample, homogenization of the sample, etc.). In some cases, a cell wall in the sample may be permeabilized, but the other portions of the sample may not be damaged. In some cases, a sample may be fixed (e.g., exposed to a fixative).

Optical systems with wide FOVs

[0749] Described herein are optical systems comprising imaging modules or optical assemblies for sequencing DNA samples nucleic acids. The optical systems and methods described herein are capable of illuminating multiple surfaces of the flow cells that are axially-displaced from each other with an illumination field with relatively uniform illumination power density. Each surface may include traditional 2D sample(s) and/or 3D samples (e.g., in situ) immobilized thereon. The illumination field (e.g., >10 mm^2) can be much wider than what traditional illumination systems are capable of providing with great homogeneity (e.g., with excitation energy variance of less than 10%), therefore advantageously achieving improved sequencing throughput within a set system run time. The speckle noise of the illumination system can be advantageously reduced using cost-effective and easy-to-implement despecklers. As such, the illumination systems and methods herein can increase effectiveness and efficiency of sequencing analysis including next generation sequencing (NGS).

[0750] In some cases, the optical systems and optical assemblies of the present disclosure can provide time sequential color imaging of large areas. Such imaging can enable enhanced sequencing or other imaging performance, where high resolution imaging of a wide area can improve throughputs and reduce the time needed to image a surface. The optical systems and optical assemblies of the present disclosure can provide for reduced volumes of the optical systems and assemblies, which can reduce footprints and enable new system architectures. The number of optical components (e.g., lenses, etc.) can be reduced using the optical systems and assemblies of the present disclosure, reducing the number of elements to be aligned and the number of failure points of the system, enhancing uptime and reducing manufacturing burden.

The optical systems and assemblies of the present disclosure may eliminate the movement of a stage in the z direction (e.g., along the optical axis, relative to the optical assembly) of the system, which can provide simpler setups as well as enhanced reliability of the optical system.

[0751] There is a need for increased throughput and flexibility in next generation sequencing (NGS) analysis systems. Disclosed herein are optical systems, designs, and methods of using thereof that may provide any one or more of the following advantages: wide field-of-view with uniformity in illumination power, reduction of speckle noise with cost-effective and easy-to-implement methods, higher system throughput for fluorescence imaging-based genomics applications, compatibility with traditional flow cell devices and/or optical systems, flexibility in analysis or comparison of samples (e.g., larger sample volume and/or increased sample variety), reduction in system volume, reduced complexity and other requirements in optical element (e.g., simpler optical setups), larger field-of-view, and improved uniformity in illumination power.

[0752] Disclosed herein are imaging modules, each imaging module is configured for multi-channel florescence imaging. The optical system herein may include multiple imaging modules or equivalently, multiple optical assemblies, e.g., an imaging module for each color channel, and one or more imaging modules may include its corresponding illumination system or a shared illumination system disclosed herein and an image acquisition system configured for acquiring flow cell images of a sample or samples immobilized on the sample stage and positioned at a sample plane. The illumination system and/or the image acquisition system may either work individually for each corresponding imaging module or may be shared among multiple imaging modules.

[0753] As disclosed herein, the imaging module is used interchangeably as optical assembly, according to some embodiments.

Image acquisition systems and methods

[0754] In some embodiments, the image acquisition system comprises one or more image sensors and one or more objective lenses. In some embodiments, the optical system for imaging next-generation sequencing (NGS) reactions, e.g. imager 7516 in FIG. 75, may include one or more multi-channel fluorescence imaging modules, each imaging module corresponding to a different color channel. Each imaging module may include an image acquisition system having a corresponding image sensor for such color channel and an objective lens. The objective lens may be shared between more than one imaging module. In some embodiments, each imaging module may include its own sensor and may lack any objective lens. In some embodiments, each imaging module is configured to generate the flow cell images without using any objective lenses.

[0755] In some embodiments, the imaging module includes 3 different segments. The segments can be optically aligned independently from one another, and can be coupled together to form the imaging module. Having multiple segments may advantageously allow each segment to be independently manufactured and optically aligned. Each segment may include its individual housing. Alternatively, the imaging module can have a housing that houses all three different segments. In some embodiments, the first segment houses a first group of lens elements therein. Some of the lens elements may be movable relative to the housing. For example, 1-3 and 5-8 in FIG. 92 are lens elements housed in the first segment. In some embodiments, the third segment section houses a second group of lens elements, G2. For example, 9-13 in FIG. 92 are in the third segment. Various methods can be used to control centration and angular alignment among multiple segments or of optical elements within a single segment, e.g., an alignment turning technology may be used for the control and angular alignment can be sub-cell based, utilizing alignment turning technology to control centration and angular alignment. The second segment may house the excitation dichroic beam splitter, e.g., 2770 as shown in FIGS. 90-91. In some embodiments, the two orthogonal lens groups may be actively aligned at a nominal 45° angle, which may control the pointing differences between the first lens element G1 and the second lens element Group G2. In some embodiments, active alignment of the two orthogonal lens groups may reduce alignment error(s) to be within a satisfactory range. The satisfactory range can be customized based on different applications. For example, the alignment error can include one or more of: decenter, tilt, lens interval error, and defocus is required to fulfill the error budget.

[0756] As disclosed herein, the focusing of the imaging module is advantageously internalized. Instead of moving multiple pieces of the lens element, e.g., the entire objective lens, one or more optical compensators, relative to the sample for focusing, the imaging module enables movement of an individual lens element or elements therewithin relative to the housing of the imaging module in order to achieve focusing at least along the z -axis. In some embodiments, a single lens element may be moved relative to the housing to achieve focusing along the z-axis. In some embodiments, two lens elements may be moved together or separately relative to the housing to achieve focusing along the z-axis. As shown in FIGS. 93A-93B a lens element may ride on linear bearings driven by an external actuator so that the lens element can move a predetermined distance automatically in a controlled fashion. The lens element may be movable along the optical axis of the optical assembly. The optical axis, e.g., 2790 in FIG. 90A, of the optical assembly between the detector and the stage may be along the z axis for the segment, e.g., first segment, that is closest to the sample stage. The optical axis of the optical assembly may be along an axis orthogonal to the z axis for the segment that is closest to the image sensor,

e.g., the third segment. The optical axis, e.g., 2791 in FIG. 90A, of the optical assembly between the light source and the stage may be along the z axis for the segment, e.g., first segment, that is closest to the sample stage. The optical axis of the optical assembly may be along an axis orthogonal to the z axis for the segment that is closest to the image sensor, e.g., the third segment.

[0757] In some embodiments, motion range of the lens element to achieve focusing along the z-axis may be customized based on size and dimension of the flow cell(s). For example, the z-motion range to image the top surface of the flow cell to the bottom surface of the flow cell can be about 810 um, with a lens element moving toward or away from the imaging sensor. In some embodiments, the housing may include hard travel stops which limit the travel range of the lens element during focusing. For example, 8 in FIG. 92 is an element that can be moved for focusing the imaging module. As another example, 5 in FIG. 92 may be an element that can be moved for focusing the imaging module. The travel range may be sufficient to allow focusing of multiple surfaces without interferences with other lens elements, e.g., touching other lens elements. For example, the lens element for focusing may move about 0.1 to 5 mm toward the sensor and about 0.1 to 4.0 mm away from the sensor. In some embodiments, the travel range may be included to help cope with deconjugation or placement error of the surfaces, e.g., the top surface, of the flow cell relative to the vertex of the lens element(s). In some embodiments, the lens element actively aligns E7 to E1-E6.

Sample stages and methods of use

[0758] In some embodiments, the optical systems herein (e.g., those used for imaging a sample or sequencing reactions) may include a sample stage configured for holding sample(s) and/or their corresponding support, e.g., a flow cell device with solid support(s), in a prespecified position relative to the optical system. In some embodiments, the sample stage may include a base stage and one or more top stages positioned thereon. FIG. 102 shows an exemplary sample stage 3800 with a base stage 3810 and top stages 3820.

[0759] The base stage, e.g., 3810 in FIG. 102, may include a thickness along z axis and a top surface. The thickness of the base stage can be customized to various numbers, e.g.. in a range from 1mm to 5cm. The top stage(s), e.g., 3820, may be positioned on the top surface 3811 of the base stage. The top surface may be planar. The top surface may be of various geometrical shapes. In some embodiments, the top surface of the base stage may be, but is not limited to, a circular shape, a donut shape, an oval, a square, a rectangle, or a diamond shape. The top surface of the base stage may include a size sufficient to position one or more top stages thereon for sequencing purposes. For example, the top surface may be sufficient to position 5, 10, 20, 30 or even more top stages on it.

[0760] The base stage may be configured to move relative to the optical system/imager (7516), e.g., relative to the focal plane of the objective lens or the focal plane of the optical system herein to allow focusing of the sample(s) positioned on the base stage for imaging. The base stage may be configured to move in one or more directions in the 3D space. For example, the base stage may be configured to move along x, y, and/or z axis, relative to the focal plane of the optical system. As another example, the base stage may be configured to rotate about an axis, e.g., the z axis, in order to focus different areas of the top surface of the base stage, thus the sample(s) positioned thereon, relative to the focal plane of the optical assembly.

[0761] In some embodiments, the sample stage may be of various geometrical shapes. In some embodiments, the base stage may be movable relative to the optical axis of the optical system. In some embodiments, the base stage may be rotatable about the optical axis or z-axis of the optical system.

[0762] The one or more top stages can be of various geometrical shapes. For example, as shown in FIG. 102, the 5 top stages are rectangular. In some embodiments, the top stage may be, but is not limited to, a circular shape, a donut shape, an oval, a square, a rectangle, or a diamond shape. In some embodiments, each top stage may have a shape and size that is sufficient to hold one or more flow cell devices thereon.

[0763] In some embodiments, the one or more top stages are movable along a radius of the top surface (of the base stage relative to the base stage, e.g., along the y axis as shown in FIG. 102. In some embodiments, the one or more top stages are movable orthogonal to a radius of the top surface of the base stage relative to the base stage, e.g., along the x axis shown in FIG. 102. In some embodiments, the one or more top stages are movable in various directions in the x-y plane.

[0764] In some embodiments, a first top stage of the one or more top stages is movable independently relative to at least a second top stage of the one or more top stages. In some embodiments, a first top stage of the one or more top stages is movable simultaneously with at least a second top stage of the one or more top stages relative to the base stage.

[0765] In some embodiments, each top stage may have one or more flow cell devices (not shown) immobilized thereon. In some embodiments, the flow cell devices may be removably secured to the corresponding top stage. In some embodiments, movement of the top stage may cause identical movement in the one or more flow cell devices immobilized thereon. The flow cell devices may be secured relative to the top stage so that there is no relative movement between the flow cell device and the corresponding top stage when the top stage moves. The flow cell device may be secured via various secure or fastening means including but not limited to mechanical clamping the flow cell device down, fastening with a magnetic or electro-

magnetic force, positioning the flow cell device into a fitted housing which is fastened to the top stage, coupling a pin or post of the top stage to a hole or a grove of the flow device or vice versa.

[0766] In some embodiments, each flow cell device may have sample(s) immobilized thereon. The sample(s) can be 2D DNA sample(s). The sample(s) can be 3D volumetric samples of in situ cell(s) and/or tissue. In some embodiments, the sample(s) may be multiplexed samples. In some embodiments, the sample(s) may be of balanced or unbalanced nucleotide diversity.

[0767] FIG. 102 shows a non-limiting example of the sample stage 3800 for holding samples that are imaged by the optical system 7516 of the sequencing system 7510 disclosed herein. In this particular embodiment, the base stage 3810 of the sample stage has a top surface 3811 that is of a circular shape. The top surface may include one or more top stages 3820 coupled thereon. In this embodiment in FIG. 102, there are 5 top stages spaced evenly on the top surface of the base stage. In some embodiments, there can be 1-30 top stages positioned on the sample stage. In some embodiments, the sample stage, e.g., the base stage and the top stages may be rotatable about the optical axis, e.g., z axis. When the base stage rotates, the top stage secured thereon may also rotate together with the base stage in the identical rotatory motion.

[0768] In some embodiments, the top stages may be movable relative to the base stage. Such movement may occur separately or simultaneously as the rotating motion of the base stage and the top stages. For example, the rotation of the base stage relative to the optical system, and linear movement of the top stage(s) relative to the base stage can occur simultaneously to position the predetermined sample area of a flow cell device relative to the optical system for imaging efficiently. As another example, the rotation of the base stage relative to the optical system, and linear movement of the top stage(s) relative to the base stage can occur sequentially and it can be controlled by the same motor. The movement of the top stage relative to the base stage may occur in the sample plane, e.g., the x-y plane, that is orthogonal to the z-axis. For each top stage, the x-axis may extend axially from the center of the top surface of the base stage, and the y-axis may be orthogonal to the x and z axis. axes. For example, the top stage at the top of FIG. 102 may move along the y and/or x-axis relative to the sample stage, so that different areas of the top stage may be moved to a specified location relative to the optical system, e.g.. the objective lens, for imaging. In some embodiments, the y axis and x axis corresponding to different top stages may change direction within the x-y plane so that the y axis is along the longest dimension of flow cell devices (e.g., along a radius of the top surface of the base stage) and the x axis is along the lateral direction of the flow cell devices (e.g., along tangential direction of the top surface of the base stage).

[0769] Each top stage may be configured to hold sample(s) and their corresponding support(s) thereon. The sample(s) and their corresponding support(s) may be immobilized on the stage to

move along with the top stage. By having various samples immobilized on different top stages, different samples may be imaged in a time-sequential fashion by rotating the particular sample region via rotation of the base stage, and/or by linearly moving the particular sample region via linear movement of the top stage so that the sample region can be placed into position relative to the optical system for imaging.

[0770] In some embodiments, the top stage may include a motion range in the x-y plane sufficient for imaging a pre-determined area of the sample(s). In some embodiments, the motion range along x-axis may be 0 to 50 mm, 0 to 40 mm, 0 to 30 mm, or 0 to 20 mm. In some embodiments, the motion range along y-axis may be 0 to 50 mm, 0 to 40 mm, 0 to 30 mm, 0 to 20 mm, 0 to 16 mm, or 0 to 10 mm. The resolution of movement along x or y axis can be customized based on different sample(s) or sequencing applications. In some embodiments, resolution of movement along x or y axis can be from 1 um to 40 um, from 1um to 30 um, from 1 um to 20 um, or from 1 um to 10 um.

[0771] In some embodiments, each of the one or more top stages comprises a motion range of greater than 15 mm and less than 80 mm, along a radius or orthogonal to the radius of the top surface of the base stage. In some embodiments, each of the one or more top stages comprises a motion range of greater than 25 mm and less than 100 mm, along a radius or orthogonal to the radius of the top surface of the base stage.

[0772] The optical system may include one or more imaging head(s), e.g., one or more optical assemblies disclosed herein. Two imaging heads are shown in FIG. 102. Having one imaging head may advantageously decrease the cost of the optical systems, volume of the system, and system complexity, while having more imaging heads may advantageously increase imaging throughput and reduce total imaging time for imaging a certain number of samples, with the trade-off of increased system hardware cost, complexity, etc. In some embodiments, the sample stage further comprises a first motor configured to actuate the sample stage, e.g., the base stage and the top stages, to rotate with a first resolution. The rotation of the sample stage can be relative to the optical system, e.g., the optical axis of the optical system. The first resolution may be an angular resolution that is less than 0.1 degrees, 0.2 degrees 0.5 degrees, 1 degree, 2 degrees, 3 degrees, 4 degrees, 5 degrees, 10 degrees, 20 degrees, 30 degrees, or 50 degrees. The first resolution may be an angular resolution that is greater than 0.1 degrees, 0.2 degrees 0.5 degrees, 1 degree, 2 degrees, 3 degrees, 4 degrees, 5 degrees, 10 degrees, 20 degrees, 30 degrees, or 50 degrees. In some embodiments, various actuation mechanisms may be used to enable rotating motion of the sample stage. For example, a geared mechanism or an induction-based motor may be used to actuate the motion of the sample stage. In some embodiments, the resolution of rotating motion can be customized. For example, the resolution may be 0.1, 0.2, 0.5

degrees. In some embodiments, the sample stage may rotate a minimum of 10 to 360 degrees. In some embodiments, the sample stage may rotate in any number of full circles. In some embodiments, the sample stage may rotate in one or both directions.

[0773] In some embodiments, the same mechanism for the base stage or a different actuation mechanism may be used to acuate the top stage(s) for their movements. In some embodiments, the sample stage further comprises one or more second motors configured to acuate some of the one or more top stages relative to the base stage at a second resolution independently without moving the rest of the top stages. In some embodiments, the sample stage further comprises a second motor configured to acuate the one or more top stages relative to the base stage at a second resolution simultaneously. In some embodiments, the second resolution is less than 0.01 mm, 0.015 mm, 0.02 mm, 0.025 mm, 0.03 mm, 0.05 mm, 0.08 mm, 0.1 mm, 0.15 mm, 0.2 mm, or 0.5 mm. In some embodiments, the second resolution is less than 0.01 mm, 0.02 mm, 0.05 mm, 0.1 mm, 0.2 mm, 0.4 mm, 0.8 mm, 1 mm, 2 mm, or 5 mm. In some embodiments, the second resolution is greater than 0.01 mm, 0.02 mm, 0.05 mm, 0.1 mm, 0.2 mm, 0.4 mm, 0.8 mm, 1 mm, 2 mm, or 5 mm. In some embodiments, the second resolution is greater than 0.01 mm, 0.015 mm, 0.02 mm, 0.025 mm, 0.03 mm, 0.05 mm, 0.08 mm, 0.1 mm, 0.15 mm, 0.2 mm, or 0.5 mm.

[0774] In some embodiments, the sample stage is coupled with one or more fluidic control devices e.g., 3830 in FIG. 102. The fluidic control device may be in fluidic communication with the sample stage (e.g., the flow cell devices) and may be configured to hold, dispense, and collect various fluids that are used in sequencing reactions in the flow cell devices in a sequencing run. The fluidic control device can be individually connected fluidically with the sample(s) on its corresponding top stage. FIG. 102 shows 5 different fluidic control devices, each in fluidic connection with sample(s) of a corresponding top stage. In some embodiments, the fluidic control devices can be immobilized relative to the base stage. In some embodiments, the fluidic control devices can be immobilized relative to the corresponding top stage. In some embodiments, each fluidic control device can include a dispenser that is configured to dispense one or more reagents to the samples. For example, the dispenser may dispense openly the reagents to a corresponding inlet of a flow cell. As another example, the dispenser may be connected to the inlet of the flow cell devices via tubing, and the reagents can travel through the tubing to contact the sample(s) in the flow cell device. In some embodiments, the fluidic control device may include one or more pumps to facilitate dispensing of fluids to the samples and/or collection of fluids from the samples.

[0775] In some embodiments, flow cell devices on multiple top stages may share a single flow control device for simplicity of the system, lower system cost, and less waste of sequencing

reagents in comparison to sequencing systems using multiple flow control devices. In such embodiments, different tubing may be used to enable fluidic communication to different flow cell devices on different top stages. Such different tubing may be for different reagents or identical reagents. Alternatively, different dispensing tips may be used to allow fluidic administration to the different flow cell devices on different top stages. In some embodiments, same dispensing tips may be shared among different top stages, and reagent dispensing can be done in a sequential manner over time.

[0776] In some embodiments, the sample(s) may be immobilized on a solid support, e.g., a flow cell, to be imaged using the optical system. The flow cell may include one or more lanes, each lane corresponding to a microfluidic channel that allows sequencing reagents or other fluids, e.g., washing buffers, in a sequencing run to flow therethrough. In some flow cells with two lanes, the lanes are positioned parallel to each other. In some embodiments, the sample stage herein may utilize flow cells with a lane orientation that is different from these flow cells. In some embodiments, the flow cells herein may include multiple lanes, and each pair of lanes may be positioned with an acute angle between their longitudinal directions so that they are not parallel to each other. For example, multiple lanes may be positioned axially along different radii of the sample stage, e.g., top stage with a predetermined angle between each adjacent pair of lanes. In such embodiments, the motion of the top stage along the y-axis of the top stage relative to the base stage can be eliminated. Instead, the base stage can be rotated at a predetermined angle to move to a next lane of the sample flow cell. Such embodiments with non-parallel lanes may advantageously remove the need for moving the top stage along its corresponding x-axis, thereby simplifying the motion in the x-y plane of the top stage relative to the base stage.

[0777] In some embodiments, the top stage here may include a manifold that can securely holds one or more flow cell devices here. The manifold may include an open state in which the flow cell device(s) can be removed or installed in the manifold. The manifold may also include a closed state in which the flow cell device is secured therewithin, and the sealed fluidic communication between the flow cell device (e.g., the cleaning outlet) and the manifold is formed. Further, in the closed state, the relative position of the flow cell device to the manifold is fixed. In some embodiments, the manifold includes a sealed fluidic communication with the fluidic control device.

[0778] In some embodiments, the fluidic control device includes one or more sealed fluidic pathways to the manifold and the flow cell devices. In some embodiments, some of the sealed fluidic pathways are configured for sealed fluidic administration to the flow cell devices. In some embodiments, some or all of the sealed fluidic pathways are configured for sealed fluidic

collection from the flow cell devices (e.g., cleaning fluidic residuals from the inlet of the flow cell devices).

[0779] In some embodiments, the sample stage and optical systems and optical assemblies described herein advantageously remove the need for movement of the sample stage along the z-axis relative to the optical assembly or optical system herein. As such, the possible problems and complexity of moving the sample stage and the sample in z-direction are also eliminated. Z movement to achieve focusing of the sample(s) can be performed by moving individual lens element(s), e.g., a single lens element, of the imaging module relative to the housing thereof, which can be more simple, convenient, and accurate compared to some optical systems.

[0780] In some embodiments, disclosed herein are methods of sequencing multiple DNA samples positioned on a rotary sample stage for DNA sequencing using various sequencing methods including but not limited to sequencing by synthesis, sequencing by avidite, sequencing by binding. Such methods may be repeated in one or more sequencing cycles in a sequencing run.

[0781] In some embodiments, the methods of sequencing multiple DNA samples positioned on a rotary sample stage for DNA sequencing comprises an operation of obtaining a sample stage comprising the base stage and the one or more top stages positioned on the top surface of the base stage, wherein the base stage is rotatable about a z-axis relative to the optical system or the imaging module of a sequencing system.

[0782] In some embodiments, the methods comprises an operation of positioning and securing a first flow cell device relative to a first top stage of the one or more top stages.

[0783] The flow cell device can have 2D or 3D samples embolized thereon. The flow cell device can have various number of microfluidic channels with channel surfaces that the sample(s) can be immobilized on. The flow cell device herein may have 2, 3, 4, or more channel surfaces. The multiple channel surfaces may be displaced from each other along the z axis so that at least 2, 3, or more channel surfaces are at 2, 3,or more different z locations relative to the optical system. For example, the flow cell device may have 2 channels along the z direction so that it has 4 surfaces at different z locations.

[0784] The flow cell devices may be secured relative to the top stage so that there is no relative movement between the flow cell device and the corresponding top stage when the top stage moves. The flow cell device may be secured via various securing or fastening means including but not limited to mechanical clamping the flow cell device down, fastening with a magnetic or electro-magnetic force, positioning the flow cell device into a fitted housing (e.g., the manifold) which is fastened to the top stage, coupling a pin or post of the stage to a hole or a grove of the flow device. For example, the flow cell device can be secured in its corresponding manifold in a

closed state, and sealed fluidic communication between the flow cell device and the manifold can be established in the closed state.

[0785] In some embodiments, the methods further comprise an operation of positioning and securing a second flow cell device relative to a second top stage of the one or more top stages. The second top stage can have identical or different securing or fastening means as the first top stage.

[0786] In some embodiment, the methods further comprise an operation of dispensing, by a first fluidic control device, one or more sequencing reagents to the first flow cell device positioned on a first top stage so that the samples can undergo sequencing reactions. Such operation of dispensing sequencing reagents can be performed openly, e.g., via a dispensing tip to an open area that leads to the channel(s) of the flow cell device. Alternatively, such operation of dispensing sequencing reagents can be performed via closed tubing.

[0787] In some embodiments, the method further comprises imaging a first sample region of the first flow cell device using the optical system of the sequencing system. The first sample region can include at least part of a first tile of the flow cell device. Such operation of imaging may include collect emitted optical signals from the sample(s) by an image sensor of the imaging module. Such operation may also include autofocusing the imaging module on the samples using various autofocusing methods. Such operation of imaging may also include generate excitation light that travels to the sample(s).

[0788] After completion of the imaging operation of the first sample region of the first flow cell device, the methods may include an operation of moving the first top stage within the x-y plane relative to the optical system while preventing the second flow cell device from moving relative to the optical system. Such operation may enable the second sample region, e.g., at least part of a second tile, to be positioned properly for imaging. Such movement can be along the x, y, or any other direction within the sample plane, e.g., the x-y plane. The base stage may remain still relative to the optical system during such movement of the first top stage.

[0789] In some embodiments, the methods further include an operation of moving the first fluidic control device so that the first fluidic device, e.g., its dispensing tip(s), stay still relative to the first flow cell device while the first flow cell device moves relative to the optical system. In some embodiments, the first fluidic control device does not need to be moved, e.g., when closed tubing connects the first fluidic control device to the first flow cell device while the first flow cell device moves relative to the optical system. In some embodiments, the first top stage is actuated by the first motor that is configured to actuate the base stage. In some embodiments, the first top stage is actuated by a second motor that is configured to actuate one or more top stages independent of actuation of the base stage.

[0790] In some embodiments, the operation of moving the first top stage within the x-y plane relative to the optical system while preventing the second flow cell device from moving relative to the optical system comprises: moving the first top stage along a radius of the top surface of the base stage with a predetermined distance relative to the optical system independently while preventing the second flow cell device from moving relative to the optical system.

[0791] In some embodiments, the operation of moving the first top stage within the x-y plane relative to the optical system while preventing the second flow cell device from moving relative to the optical system comprises: moving the first top stage along a direction orthogonal to a radius of the top surface of the base stage with a predetermined distance relative to the optical system independently while preventing the second flow cell device from moving relative to the optical system.

[0792] In some embodiments, the methods further include an operation of imaging the second sample region of the first flow cell device using the optical system of the sequencing system.

[0793] After completion of imaging the second sample region of the first flow cell device, the methods can further include an operation of moving the first top stage and imaging other sample regions of the first flow cell device till all the desired sample regions of the first flow cell device have been imaged.

[0794] In some embodiments, the methods may further include an operation of rotating the sample stage with a predetermined angular resolution to position the second flow cell device in a predetermined position relative to the optical system. This operation may occur in a same sequencing cycle with the operations of imaging the sample regions of the first flow cell device. Alternatively, this operation may occur in a different sequencing cycle with the operations of imaging the sample regions of the first flow cell device. The angular resolution can be the first resolution disclosed herein.

[0795] After rotating the sample stage with the predetermined angular resolution to position the second flow cell device in the predetermined position relative to the optical system, in some embodiments, the methods may further include an operation of dispensing by a first fluidic control device or a second fluidic control device, one or more sequencing reagents to the second flow cell device so that the samples immobilized on the second flow cell device can undergo sequencing reactions. The one or more sequencing reagents dispensed to the second flow cell device can be different from those dispensed to the first flow cell device, e.g., for different sequencing chemistry or applications. This operation of dispensing sequencing reagents to the second flow cell device may be optional, for example, if the same sequencing reagents are used and are dispensed simultaneously to the first flow cell device and the second flow cell device before imaging the first flow cell device.

[0796] In some embodiments, the method further comprises: moving the first fluidic control device or the second fluidic control device to position the second fluidic cell device in a predetermined position relative to the first fluidic control device or the second fluidic control device.

[0797] After rotating the sample stage with the predetermined angular resolution to position the second flow cell device in the predetermined position relative to the optical system, in some embodiments, the methods may further include an operation of imaging a first sample region of the second flow cell device using the optical system of the sequencing system, e.g., at least part of a first tile of the second flow cell device. The imaging operation of the first sample region of the second flow cell device can be identical to the imaging operation of the sample regions in the first flow cell device. The imaging operation can be different from the imaging operation of the first sample region of the second flow cell device, for example, when 2D samples are included on the first flow cell device while 3D sample(s) are included on the second flow cell device so that the imaging operation may including imaging different z levels for 3D sample(s) while imaging at a single z level for 2D sample(s).

[0798] In some embodiments, the rotary sample stage and methods of sequencing the samples using the rotary stage can advantageously improve sequencing capability and system throughput by allowing multiple flow cell devices to be sequenced and imaged using a single sample stage, and such multiple flow cell devices may contain different samples that can undergo different sequencing reactions. Further, in some embodiments, the rotary sample stage and methods of sequencing using the rotary stage can advantageously improve sequencing efficiency by allowing imaging of a first flow cell device to be performed simultaneously while dispensing and flowing sequencing reagents to a second flow cell device.

[0799] In some cases, an optical system can comprise a stage. The stage can be as described elsewhere herein (e.g., can be configured to hold a flow cell device, a slide, or similar otherwise solid support with samples thereon). The stage may lack movement in an optical axis of the system. Such movement may be relative to a non-movable housing of the optical assembly, e.g., the housing of the first or third element. For example, for a system configured to illuminate and image a sample at multiple different z levels along the z-axis of the sample, e.g., an in situ sample of cells or tissue, the stage may lack any movement along the z-axis relative to the non-movable housing of the optical system, e.g., the housing of the first or third element of the optical assembly during imaging of the sample. In another example, the stage and the optical assembly may not move along a z axis relative to each other but can still enable focusing of the sample so that it is within the focal plane of the optical assembly in the z-axis to focus prior to or during imaging. The optical system herein may utilize a method of focusing by moving only one

or more lens element of the optical assembly instead, such as moving the stage or the optical assembly in order to move the sample into a focal plane of the optical system. The one or more lens element of the optical assembly may not include an objective lens. In some embodiments, the optical system herein may utilize a method of focusing disclosed herein that eliminates the z-stage that is response for moving the sample along z axis relative to the objective lens in another optical systems.

[0800] The solid support (e.g., flow cell) can comprise a probe configured to bind a nucleic acid molecule, a protein, a polypeptide, or the like. For example, a probe complementary to a nucleic acid molecule can be immobilized (e.g., bound) on a surface of the solid support.

[0801] The solid support (e.g., flow cell) may comprise one or more samples immobilized thereon. For example, the solid support can comprise one or more probe molecules configured to bind one or more samples thereto. The solid support can comprise two or more surfaces with one or more samples immobilized thereto. For example, the solid support can comprise a first surface with a first sample immobilized thereto and a second surface with a second sample immobilized thereto. In some cases, the solid support comprises at least about 2, 3, 4, 5, 6, or more surfaces. In some cases, each surface of the solid support has a different sample immobilized thereto, and each sample can be illuminated and imaged by the optical system. In some cases, the two or more surfaces of the solid support can be axially displaced from each other along an optical axis (e.g., a z-axis) of the optical system. For example, the surfaces of the solid support can be in a stacked configuration with regard to the optical axis of the optical system.

[0802] The optical system can comprise a light source configured to illuminate the flow cell. The illumination can be used in at least a portion of an imaging operation (e.g., a sequencing operation as described elsewhere herein). The illumination may have an irradiance of at least about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 90, 100, or more milliwatts per square millimeter. In some cases, the light source may be a pulsed light source (e.g., a flash lamp, a pulsed laser, a pulsed light emitting diode, etc.). In some cases, the light source can be a continuous light source (e.g., an incandescent source, a fluorescent lamp, a light emitting diode, a continuous laser, etc.).

[0803] The optical system can comprise an optical assembly. The optical assembly can be disposed within an optical path between from the stage and to the light source. The optical assembly can be configured to provide an illumination of the flow cell of greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or more square millimeters. The optical assembly can be configured to illuminate the area of the flow cell with a variation (e.g., a peak-to-valley variation, standard deviation, variance, interquartile

range, mean absolute deviation, coefficient of variation, etc.) of at most about 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or less percent. The optical assembly can be configured to illuminate the area of the flow cell with a variation (e.g., a peak-to-valley variation, standard deviation, variance, interquartile range, mean absolute deviation, coefficient of variation, etc.) of at most 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or less percent. In some embodiments, such illumination variation can be the variation in power cross the area of the flow cell. In some embodiments, such illumination variation can be the variation in irradiance cross the area of the flow cell.

[0804] In some cases, the optical assembly does not comprise an objective lens (e.g., objective lens assembly). For example, the optical assembly may not comprise an objective lens assembly. In some cases, the optical system does not comprise an objective lens. For example, the entire optical system may not comprise an objective lens anywhere in the optical system. In another example, the optical system may not comprise an objective lens in the optical path of the optical system. The optical assembly may not comprise a tube lens. In some cases, the optical system may not comprise a tube lens. For example, there may not be a tube lens in the optical path of the optical system. Despite not having a tube lens and/or an objective lens, the optical assembly or optical system may be able to achieve the large area illumination described herein. For example, the optical system can achieve broad, flat illumination without the use of a tube lens or objective lens.

[0805] As disclosed herein, the optical assembly may be used interchangeably and equivalently as the imager 7516 of FIG. 75 or the optical system. The optical assembly, more specifically, the illumination system of the optical assembly can be configured to transmit the illumination light from the light source to the stage and a sample immobilized on the stage. For example, the illumination system may be positioned above the excitation dichroic filter, e.g., above 2770 as shown in FIG. 91, so that the excitation dichroic filter of the optical assembly may be configured to transmit the illumination from the illumination system to the sample and the stage, via the first segment. In other words, the excitation dichroic filter and the optical elements of the optical assembly that are between the excitation dichroic filter and the stage are within the optical path between the light source and the stage, e.g., first segment 2710 and 2770 in FIG. 91. In some embodiments, the excitation dichroic filter can also be configured to reflect emission light from the sample to an optical path of a detector. In other words, the excitation dichroic filter and the optical elements of the optical assembly that are also between the excitation dichroic filter and the stage are within the optical path between the stage and the detector, e.g., first segment 2710 and 2770 in FIG. 91. The optical assembly can be configured to receive an emission light from the flow cell (e.g., a light produced by the interaction of the illumination light with a label in a

sample in the flow cell). For example, the optical assembly can be configured to receive the emission light and transmit the emission light to a detector. Nonlimiting examples of the detection includes a CCD camera or a CMOS camera. The optical assembly may have a numerical aperture of at least about 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, or more. The emission light may have a wavelength of at least about 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1,000, or more nanometers. The emission light may have a wavelength of at most about 1,000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, or less nanometers. The emission light may have a wavelength in a range as defined by any two of the preceding values. The optical assembly can have a working distance of at least about 1 mm to about 30 mm . The optical assembly can have a working distance of at least about 2 mm to about 25 mm.

[0806] In some cases, the optical assembly can comprise a motion coil housed within the optical assembly. The motion coil can be configured to move a focusing element within an optical path of the optical system. For example, the motion coil can be used to move a focusing element to change the focus of the optical system without moving other portions of the system (e.g., the optical assembly, the stage, etc.). Alternatives to a motion coil include, but are not limited to, piezoelectric actuators, motors (e.g., stepper motors, servo motors, etc.), electrostatic actuators, hydraulic actuators, pneumatic actuators or the like, or any combination thereof. In some cases, the motion coil (or other actuator) can be positioned external to the optical assembly or optical system and be configured to move the focusing element along the optical axis. For example, a motor positioned outside of the optical assembly can be operatively coupled to the focusing element and can adjust the position of the focusing element within the optical assembly. 2760 of FIG. 91 shows an actuator integrated with an optical assembly. An example of an external actuator can be found in FIGS. 93A – 93B, where actuator 2901 is coupled to the focusing element 2903 by coupling element 2902. In some cases, the actuator is coupled directly to the focusing element or a focusing element housing. For example, the actuator can be coupled without the use of a coupling element. In some embodiments, the focusing element includes one or more lenses of the optical assembly. In the particular embodiments as shown in FIG. 93A and FIG. 95, the focusing element is a single lens of the optical assembly. The focusing element can be mechanically coupled with a focusing element housing so that movement of the focusing element housing may cause movement of the focusing element. Various methods may be used for the mechanical coupling between the focusing element and the focusing element housing. Such mechanical coupling can be direct without contact of a third element. Such mechanical coupling can be indirect with contact of a third element in between. For example, as shown in FIG. 93A, the two ends of the focusing element 2903 are clamped directly with the focusing housing element 2904.

[0807] In some embodiments, the sequencing cycle time herein can be further reduced by adjusting the focusing element of the image module disclosed herein without the need of using an objective lens in comparison with imaging using traditional optical systems. In some embodiments, the sequencing cycle time herein can be further reduced by eliminating the need to move the sample stage or objective lens to focus the sample(s) relative to the optical system using the imaging module disclosed herein.

[0808] In some embodiment, the root-mean-square (RMS) wavefront error of the optical systems herein is measured with a flow cell device with four axially displaced surfaces and two axially displaced fluidic channels. In some embodiments, the RMS wavefront error of the optical system is less than the diffraction limit of the optical system. The root-mean-square (RMS) wavefront error of one or more surfaces of the flow cell device is less than 0.09λ, 0.08λ, 0.07λ, 0.06λ, 0.05λ, 0.03λ, or 0.02 λ, and the RMS wavefront error is for the four surfaces of the flow cell and each surface in four different color channels, wherein λ is the center wavelength of a light source. The RMS wavefront error is for a target FOV of about 1.5, 2, 2.5, 3, 3.5, or 4 mm in x or y direction. In some embodiments, the RMS wavefront error is greater for the fourth surface than the first, second, or third surfaces. In some embodiments, the root-mean-square (RMS) wavefront error of one or more surfaces of the flow cell device is less than 20,30,40,50,60,70,80,90, or 100 milli-wavelengths across the FOV. In some embodiments, the RMS wavefront error is diffraction limited and varies by less than 20,30,40,50,60,70,80,90, or 100 milli-wavelengths across the FOV.

[0809] In an exemplary embodiment, the root-mean-square (RMS) wavefront error of the optical system herein with the NA of greater than 0.25 (e.g., NA of 0.5) is measured with a flow cell device with four axially displaced surfaces and two axially displaced fluidic channels and a total thickness of about 240um. In this particular embodiment, the RMS wavefront error of the optical system is less than the diffraction limit of the optical system. The root-mean-square (RMS) wavefront error of is less than 0.09λ or 0.07λ, wherein λ is the center wavelength of a light source. The RMS wavefront error is for the four surfaces of the flow cell and each surface in four different color channels. The RMS wavefront error is for a target FOV of about 1.5 mm in x or y direction.

[0810] In an exemplary embodiment, the root-mean-square (RMS) wavefront error of the optical system herein with the NA of 0.4 or any other NA that is greater than 0.25 is measured with a flow cell device with four axially displaced surfaces and two axially displaced fluidic channels and a total thickness of about 240 or about 350 um. In this particular embodiment, the RMS wavefront error of the optical system is less than the diffraction limit of the optical system. The root-mean-square (RMS) wavefront error of is less than 0.09λ or 0.07λ, wherein λ is the

center wavelength of a light source. The RMS wavefront error is for the four surfaces of the flow cell and each surface in four different color channels. The RMS wavefront error is for a target FOV of about 1.5 mm in x or y direction.

[0811] The optical system may be configured to image the solid support without moving an optical compensator into, out from, or both into and out from the optical path between the solid support and detector of the optical system. For example, an image of a sample can be taken without moving an optical compensator into or out of the optical path between the sample and the detector. The optical assembly may be configured to generate one or more spatial constrictions lateral to the optical paths of light traveling through the optical assembly (e.g., one or more double waists in the light). The one or more spatial constrictions can advantageously: enhance imaging resolution; enhance and the depth of focus of the optical assembly, provide the low illumination variation of the optical assembly at the sample stage, provide wide field of view with low illumination variation, or a combination thereof. The optical assembly may be configured to generate at least one field curvature corrections lateral to the optical path of light traveling through the optical assembly. At least one field curvature correction can similarly improve optical resolution and depth of focus. The optical assembly may be configured to generate more than two field curvature corrections lateral to the optical path of light traveling through the optical assembly.

[0812] FIG. 91 shows an example of an optical assembly, according to some embodiments. The first segment 2710 can comprise a first housing including a first plurality of lenses therewithin. The second segment 2720 can comprise a second housing. The third segment 2730 can comprise a third housing containing a second plurality of lenses therewithin. The first segment and the third segment can be optically aligned (e.g., aligned in a same optical axis). The first segment can be positioned between the third segment and the stage (e.g., solid support 2740). The third segment can be positioned between the first segment and an image sensor (e.g., detector) of the optical system 2750. One or more lens element of the first plurality of lenses can be movable along an optical path of the optical system by at least about 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, or more millimeters. The first plurality of lenses can be movable along an optical path of the optical system by at most about 5.0, 4.9, 4.8, 4.7, 4.6, 4.5, 4.4, 4.3, 4.2, 4.1, 4.0, 3.9, 3.8, 3.7, 3.6, 3.5, 3.4, 3.3, 3.2, 3.1, 3.0, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, or 0 millimeters. One or more lens elements of the first plurality of lenses can be movable along an optical path of the optical system over a distance in a range as defined by any two of the preceding values. For example, a single lens

element of the first plurality of lenses can be movable along an optical path of the optical system in a range of about 0 to about 25 millimeters in one direction or both directions along the optical axis. The optical assembly may comprise a dichroic 2770 configured to transmit an illumination light from outside the optical assembly to the solid support.

[0813] In some cases, the first plurality of lenses comprises one or more asymmetric convex-convex lenses. In some cases, the second plurality of lenses comprises one or more asymmetric concave-concave lenses. The use of both the convex-convex and concave-concave lenses and the adjustment between the lenses can be used to tune the focal plane of the optical system (e.g., move the focal plane to image various surfaces of the solid support).

[0814] In some cases, an actuator (e.g., a motion coil) can be coupled to a portion of the optical assembly (e.g., the first, second, or third housings of the optical assembly), and the focusing element can be coupled to the portion of the optical assembly the actuator is coupled to. In this way, the actuator can adjust a focus of the optical assembly. The optical assembly can be configured to generate at least one field curvature correction lateral to an optical path of the first, second, or third segments.

[0815] FIG. 94 shows an example of the optical elements of an optical assembly and the associated focus paths, according to some embodiments. The optical assembly can be configured to focus light onto and between a solid substrate 3001 and a detector 3002. For example, illumination light can come through dichroic filter 3003 and be focused on the solid substrate 3001. Once the illumination light interacts with a sample on the solid support, the resultant sample light can be refocused up the optical assembly, reflected off of the dichroic filter, and transmitted through internal focus group 3004. The internal focus group may be configured to adjust a focus of the optical assembly, thereby permitting imaging of a plurality of surfaces of the solid support. The internal focus group may be coupled to an actuator as described elsewhere herein. The signal light can be transmitted through multi-bandpass filter 3005. The multi-bandpass filter may have an optical density plot as shown in the inset graph of FIG. 94. The multi-bandpass filter may be configured to transmit sample light from a label in a sample while rejecting other light to reduce noise. Passing through a field flattener 3006, the signal light can be detected by detector 3002 and further analyzed as described elsewhere herein.

[0816] Similarly, FIG. 95 shows an alternate lens configuration for illuminating and collecting light from a solid support (e.g., flow cell, slide, etc.) 3101. Lens group 3102 can be configured to both focus incoming illumination light (e.g., illumination light transmitted through dichroic 3103) onto the solid substrate while also focusing signal light from the solid substrate back up through notch filter 3104 and through focusing element 3105 (part of lens group 3106). The

focusing element can be as described elsewhere herein. Lens group 3017 can be configured to focus the signal light onto detector 3108 for processing as described elsewhere herein.

[0817] FIG. 88 shows a flow chart of a method 2400 of analyzing a biological molecule, according to some embodiments. In an operation 2410, the method 2400 may comprise providing a solid support comprising the biological molecule. The solid support may be a flow cell. The biological molecule may comprise a label. The biological molecule may comprise, for example, nucleic acid molecules, proteins, polypeptides, carbohydrates, lipids, or the like. The label may be changed depending on the identity of the biological molecule. For example, the label for a nucleic acid and a protein may be different to enable binding to the different molecules. In another example, for a nucleic acid, the label can be hybridized to the nucleic acid.

[0818] The label may be an optical label (e.g., a fluorescent label, a luminescent label, a Raman label, scattering label, plasmonic label, etc.), a magnetic label, or the like. In some cases, the probe is integral to the biological molecule. For example, a protein comprising green fluorescent protein can be the biological molecule and the label. In some cases, prior to operation 2410, the method 2400 may comprise binding the biological molecule to a probe in the solid support and/or coupling the label to the biological molecule. For example, the biological molecule can be flowed into a flow cell already comprising a probe, bind to the probe, and then a fluorescent label can be coupled to the biological molecule once it is bound to the probe. In another example, a biological molecule already comprising the probe can be flowed into the flow cell and bound to the probe. As another example, the sample to be sequenced and imaged may be immobilized on the flow cell device and the label or probe may be flowed into the flow cell during various sequencing reactions. Details of examples of sequencing the sample (s) are disclosed below.

[0819] In another operation 2420, the method 2400 may comprise using an optical system comprising a light source to provide illumination to the biological molecule comprising the label, thereby generating a signal light or a change thereof. The illumination may be provided over an area of the flow cell that is greater than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, or more square millimeters. The illumination may have a variation (e.g., a peak-to-valley variation, standard deviation, variance, interquartile range, mean absolute deviation, coefficient of variation, etc.) of at most about 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or less percent.

[0820] The optical system may be as described elsewhere herein. For example, the optical system may not comprise an objective lens or tube lens. The solid support may not be moved along an optical axis of the optical system. For example, a flow cell may be stationary along the z axis of the flow cell (e.g., the optical axis of the optical system) while being movable in the x

and y axes of the flow cell. In some cases, a plurality of images of the flow cell can be acquired without moving the flow cell along the optical axis. For example, a first image at a first focal depth can be acquired and a second image at a second focal depth can be acquired without moving the solid support.

[0821] A motion coil or other actuator as described elsewhere herein may be used within the optical system to move a focusing element within an optical path of the optical system, thereby changing a focus of the optical system on the solid support. For example, an actuator can move the focusing element along the optical path to change a parameter of the optical system, thereby changing the focus of the optical system from one side of a flow cell to another. The light source may be a light source as described elsewhere herein (e.g., a pulsed light source). The optical system may have an illumination efficiency (e.g., an efficiency of the illumination light transmitted through the optical system) of at least about 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.9, or more percent.

[0822] In another operation 2430, the method 2400 may comprise detecting, using a detector of the optical system, the signal light or the change thereof. The signal light may have a wavelength of at least about 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1,000, or more nanometers. The signal light may have a wavelength of at most about 1,000, 950, 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, or less nanometers. The signal light may have a wavelength in a range as defined by any two of the preceding values. The optical system may have a numerical aperture of at least about 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, or more. In some cases, the label can be removed from the biological molecule. For example, a hybridized label can be dehybridized from a nucleic acid molecule.

[0823] In another operation 2440, the method 2400 may comprise processing at least in part the signal light or the change thereof to analyze the biological molecule. The processing may be as described elsewhere herein (e.g., using an identity of the label to determine a portion of the biological molecule, etc.). An image of the solid support may comprise a field of view of greater than about 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 80, 100, 120, 150, 200, or more square millimeters. The processing may comprise processing at least in part the signal light or the change thereof to generate one or more images of the solid support and analyze the one or more images of the solid support to generate base calls of the sample.

[0824] In some cases, the method 2400 may comprise repeating operations 2420 - 2440 for an additional biological molecule coupled to an additional surface of the solid support. For example, a plurality of biological molecules can be coupled to the solid support with a plurality of labels each affixed to the plurality of biological molecules, and the optical system can image each of the labels. In some cases, the method 2400 may comprise repeating operations 2410-

2440 for an additional label that binds to another portion of the biological molecule. For example, a first label can identify a first nucleotide of a nucleic acid, the first nucleotide can be removed from the nucleic acid, and a second label can be hybridized to the nucleic acid molecule at a second nucleotide. In this example, the second label can be identified in a similar way to the first, providing information related to the second nucleotide of the nucleic acid molecule.

Illumination systems

[0825] The illumination system herein may advantageously provide an extra-wide illumination field that is no less than 10 mm^2, 20 mm^2, 30 mm^2, 40 mm^2, 50 mm^2, 60 mm^2, 70 mm^2, 80 mm^2, 100 mm^2, 150 mm^2, or 200 mm^2 at a sample plane. In some embodiments, the illumination system may advantageously provide an illumination field that is 2x, 4x, 5x, 6x, 8x, 10x, 15x, 20x, 40x, 50x, or larger than the illumination fields generated by other illumination systems in NGS optical systems. The illumination system may advantageously provide an illumination power density that is no less than 30, 40, 50, 60, 70, or 100 milli-Watts/mm^2 at the sample plane. In some embodiments, the illumination system is configured to generate an illumination field at the sample stage that is greater than 20, 30, 40, 50, 80, 100, or 200 mm^2 with less than ± 2%, 5%, 8%, 10%, or 12% variance or standard deviation in illumination power density across the illumination field. In some embodiments, the variance or standard deviation is measured as a percentage to an average power density, a maximum power density, or a median power density.

[0826] The illumination system advantageously enables imaging of wide FOVs that are 2x, 4x, 5x, 6x, 8x, 10x, 15x, 20x, 40x, 50x, 100x, or larger than the largest FOVs provided by existing optical systems for NGS applications, thereby increasing system throughput and flexibility in NGS applications. With the capability of illuminating a wide field and imaging across some or all of the wide illumination field, the illumination system and imaging module herein can advantageously eliminate the problem of photon bleaching in un-imaged areas associated with some optical systems.

[0827] In some embodiments, the illumination system may provide a power efficiency of no less than 65%, 70%, 75%, or 80%. In other words, the power loss within the illumination system can be less than 20%, 25%, 30%, or 35%. In some embodiments, the illumination subsystem, the light beam delivery subsystem, or both may each provide a power efficiency of no less than 65%, 70%, 75%, 80%, 85%, or 90%. In some embodiments, one or more optical elements in the illumination system may each provide a power efficiency of no less than 65%, 70%, 75%, 80%, 85%, 90% or 94%, e.g., power efficiency of the optical fiber(s), the lens arrays, etc. In some embodiments, the power efficiency can be determined as the ratio or percentage of the power exiting the optical element to the power entering the optical element.

[0828] The sample plane herein can be where the sample is positioned and can be orthogonal to a z-axis or optical axis of the imaging module. In some embodiments, the sample plane overlaps with a focal plane of the objective lens of the imaging module.

[0829] In some embodiments, the illumination system includes an illumination subsystem and a light beam delivery subsystem optically coupled to the illumination system.

Illumination subsystems

[0830] The illumination subsystem may include a light source, alone or in combination with a despeckler of the light source.

[0831] The light source can comprise one or more lasers. The lasers can be of various types. Some non-limiting examples of the lasers include: gas lasers, solid-state lasers, fiber lasers, dye lasers, and semiconductor lasers (laser diodes). The one or more lasers may comprise one or more laser diodes. The one or more lasers may emit light of multiple wavelengths. In some embodiments, each laser or laser diode may emit light of a predetermined color, e.g., red, green, or blue. In some embodiments, each laser or laser diode may emit light within a wavelength range that corresponds to a predetermined color, e.g., red, green, or blue. The wavelength range of the predetermined color may be less than 0.1Hz, 1Hz, 10Hz, 20Hz, 50Hz, or more. In some embodiments, the one or more lasers or laser diodes may emit light of multiple colors or wavelength ranges. In some embodiments, each laser or laser diode may emit light of multiple colors, e.g., a white light.

[0832] In some embodiments, the light source comprises one or more multi-color laser arrays. Each multi-color laser array can include lasers arranged in an array in any direction(s) in the x-y plane, e.g., a 2D array. The lasers in the array can be of different colors so that each laser can have a different color with its immediately adjacent neighbor(s) in the array. In some embodiments, the multi-color laser array comprises an array of lasers that emits laser light at 2, 3, 4, 5, or 6 wavelengths or in 2, 3, 4, 5, or 6 wavelength ranges. Each wavelength or wavelength range can correspond to a different color. In some embodiments, the multi-color laser array comprises lasers that emit light of 2, 3, or 4 color wavelengths or wavelength ranges at least in a direction that is orthogonal to a z axis. FIG. 66 shows an example of an embodiment of the multi-color laser array with laser diodes generating at least three different colors, e.g., blue, red, and green.

[0833] In some embodiments, the light source comprises a single laser or a laser array that emit light of a single wavelength, e.g., a blue light or a green light. In some embodiments, the light source is configured to emit light with a single wavelength with a predetermined bandwidth, e.g., a blue light of 460 nm ± 5nm.

[0834] In some embodiments, the illumination subsystem may further comprise one or more optical fibers that can be coupled to the light source for transmitting light therefrom. In some embodiments, a single fiber is coupled to a corresponding laser or laser diode, either multi-colored or single colored. The optical fibers may be of various fiber lengths. For example, one or more of the optical fibers may be 0.5 m to 5 m long. In some embodiments, one or more optical fibers may include a fiber core. The fiber core may have a maximum dimension (e.g., diameter) that is 50 um to 2000 um in its cross section. The cross-section can be orthogonal to the longitudinal axis extending along the length of the fiber. In some embodiments, the cross section of the fiber core can be circular or substantially circular. FIGS. 65, 66, 68, and 71 show examples of embodiments of the laser diode(s) and the optical fiber(s) coupled to the laser diode(s).

[0835] In some embodiments, the illumination subsystem further comprises a single optical fiber as shown in FIG. 65. The single optical fiber can be a multi-mode fiber. The multi-mode fiber is configured to transmit lights of different colors, different wavelengths, or different wavelength ranges in the same fiber. The single fiber may include a fiber core with a maximum dimension (e.g., diameter) of 400 um to 2000 um in its cross section orthogonal to the longitudinal axis of the fiber. The single fiber may include a fiber core with a maximum dimension (e.g., diameter) of 600 um to 1600 um. The single fiber may include a fiber core with a maximum dimension (e.g., diameter) of 800 um to 1300 um.

[0836] In some embodiments, the illumination subsystem may comprise a plurality of optical fibers. Each optical fiber may be optically coupled to one or more corresponding lasers of the light source. The one or more corresponding lasers may emit light of a same wavelength or wavelength range. The one or more corresponding lasers may emit light of a same color. In some embodiments, the illumination subsystem further comprises one or more dichroic filters, optical lens elements, or both.

[0837] FIG. 67 shows an example of an embodiment in which the light source comprises a laser diode array of red, green, and blue colors. An individual fiber is coupled to a laser, and light with different colors and from different fibers can be combined together using various optical elements such as dichroic filters and lens elements.

[0838] In some embodiments, the light source comprises one or more light beam combiners. Each light beam combiner is configured to combine two different light beams (e.g., different polarization or other beam characteristics) into a combined light beam. In some embodiments, the light beam combiner can be polarization light beam combiners. In some embodiments, the light source may include two or more lasers that emit light at a same wavelength or in a same wavelength range. Each light beam combiner may combine light emitted from such two or more

lasers at the same light wavelength or in the same light wavelength range into a combined beam. In some embodiments, each light beam combiner may combine light emitted from two or more lasers emitting light of a same color into a combined light beam. In some embodiments, the light beam combiner is configured to increase power coupling into a fiber or at sample plane by combining two light beams into a combined light beam. In some embodiments, the power of the combined light beam is greater than each individual light beam before the combination. FIG. 69 shows an example of an embodiment combining two light beams of the same color from two lasers into a combined light beam with a greater power than each individual beam.

[0839] In some embodiments, the optical fiber, either coupled to a single color or multiple color laser, may comprise a core with a non-circular cross-section that is orthogonal to the z -axis. The non-circular cross-section may be of various shapes, e.g., an oval, a triangle, a diamond, a pentagon, a hexagon shape, etc. For example, the fiber core may include a rectangular or square cross-section. FIG. 71 shows an example of an embodiment of the optical fiber with a rectangular core which may advantageously facilitate delivery of more uniform light power at the sample plane in a rectangular shape that better matches the imaging FOV, which is also rectangular.

[0840] In some embodiments, the light source may be coupled to a liquid light guide with a liquid core. In some embodiments, the one or more liquid light guides are optically coupled to the light source in the absence of an optical fiber. In some embodiments, each laser may be coupled to a liquid light guide. In other embodiments, multiple individual lasers can be coupled into a single liquid light guide. FIG. 72 shows an example of an embodiment of the liquid light guide. In some embodiments, the liquid light guide can facilitate delivery of high optical power with homogenization across the wide illumination field at the sample plane. In some embodiments, the one or more liquid light guides comprise a liquid core. The liquid core may have a maximum dimension of 0.5 mm to 10 mm in the cross-section orthogonal to the z-axis. In some embodiments, the one or more liquid light guides comprise a liquid core of a maximum dimension of 0.2 mm to 20 mm in the cross-section orthogonal to the z-axis. In some embodiments, the liquid core comprises a cross-section that is circular. In some embodiments, the liquid core comprises a cross-section that is non-circular. In some embodiments, the liquid core may comprise a cross-section of various non-circular shapes.

[0841] In some embodiments, the illumination subsystem further comprises one or more coupling elements, e.g., optical lenses. The one or more coupling lenses can be positioned between the light source and an optical fiber, e.g., as shown in FIG. 66. The coupling lens(es) may be configured to couple the laser light from the light source to the optical fiber, the liquid light guide, or other optical element(s) for transmitting light, e.g., a collimator. The coupling

lens(es) may include one or more of: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens.

[0842] FIGS. 90-91 shows a non-limiting example of the single channel time-sequential color imaging module or optical assemblies disclosed herein. The single channel time-sequential color imaging module herein may be advantageously used for imaging optical signal at different wavelengths, so that it is configured to performing imaging of multiple color channels in traditional systems without requiring additional image sensors and other optical elements such as the associated dichroic beam splitters and excitation notch filters. Compared to systems wherein signals are acquired by different channels, images of optical signals at different wavelengths, can be acquired in a sequential fashion in a single channel. The single channel time-sequential color imaging module advantageously reduces the system volume, complexity, and cost over existing optical systems.

[0843] FIG. 90 is a perspective view of the imaging module, showing the housing of different segments (e.g., 3 segments) of the imaging module. FIG. 91 is a cross-sectional view showing different lens elements of the different segments and their relative positions to each other. The autofocus light beam and excitation/illumination beam can be injected into the single channel time-sequential color imaging module through the excitation dichroic in FIGS. 90-91.

[0844] The imaging module can have a "double-waist" design that includes at least two constrictions of the optical paths traveling through the imaging module, e.g., between the sample and the image sensor. For example, the two constrictions are shown in FIG. 95, one of the constrictions occurs in the first segment, and the other one may occur in the third segment. Compared to some imaging systems, these constrictions may induce over-corrected or a backward curving component to the field curvature correction, thereby enabling a flat-field imaging of the sample over a much wider dimension than these imaging systems may allow. This wider field may advantageously translate into greatly improved image acquisition times to cover a specific sample area, thereby offering the ability to build a high-throughput sequencing system. In this particular embodiment, the sample area or field-of-view that can be imaged within a single image is increased by a factor of 5x, 10x, 13x, 15x, 20x, 40x, 50x, or more over some imaging systems.

[0845] In some embodiments, a triple notch filter or a double notch filter (not shown) can be embedded in the collimated space between two lens elements, e.g., 5 and 7 in FIG. 92. The maximum angle of optical paths (with the optical axis) at this location is controlled to be less than 10, 8, 6, or 5 degrees to enable OD6 rejection of the excitation wavelengths. In some embodiments, a double-notch or triple-notch filter may be added in this location to suppress

possible leakage from the excitation wavelengths, e.g., from the illumination system to the sample. For example, the triple or double notch filter may sit on top of 9 in FIG. 92, and the housing may provide an accessible hard aperture stop. The accessible hard aperture stop may be accessible from outside the imaging module.

[0846] In some embodiments, the imaging module here enables autofocusing at least along the optical axis by moving one or more elements along the optical axis. In some embodiments, a lens element may move longitudinally to enable multiple surface imaging with a z-motion in a range from 0 to 5 mm, 0 to 3 mm, 0 to 2 mm, 0 to 1 mm, 0 to 0.8 mm, 0 to 0.6 mm, 0 to 0.5 mm, or 0 to 0.4 mm. The movement of an internal lens element can advantageously eliminate the z-stage assembly for moving the entire objective lens relative to the sample and the associated problems with integration.

[0847] In some embodiments, the imaging module may include a lens element for aberration correction. The lens element may be aspheric. For example, 8 in FIG. 92, is a lens element for aberration correction. In some embodiments, this lens element may enable spherical aberration correction to help simultaneously improve aberration correction and improve transmission. In some embodiments, this lens element, or any other optical element, can be manufactured using glass types with low autofluorescence. In some embodiments, this lens element may include an all-spherical design.

[0848] In some embodiments, the imaging module or the optical assembly herein includes an illumination system. The illumination system may include the illumination subsystem and the light beam delivery system. FIG. 65 shows a nonlimiting example of the illumination system with excitation/illumination light.

Despecklers

[0849] Due to the coherent feature of laser light, the interference of light waves with the same frequency may happen and cause undesired laser speckle noise. The speckle noise may cause inhomogeneity in the illumination field, thus error in DNA sequencing results. As such, there is a need for effective and easy-to-implement speckle reduction. Commercially available despecklers are expensive, e.g., $1000 per unit. The despecklers herein may provide a low-cost, easy to implement, and effective way of reducing the speckle noise of the light source.

[0850] In some embodiments, disclosed herein are optical systems, comprising: a stage configured to hold one or more samples immobilized on a solid support; a light source configured to illuminate a FOV of the one or more samples; and a despeckler optically coupled to said light source and disposed within an optical path from said light source to said stage.

[0851] In some embodiments, the optical system further comprises an additional light source optically coupled into the despeckler. In some embodiments, light from the additional light

source is configured to illuminate the FOV with a different wavelength of light from the light source. In some embodiments, at least about 4 light sources are coupled into said despeckler. In some embodiments, despeckler is a vibrational despeckler. In some embodiments, the despeckler is a passive despeckler. In some embodiments, the passive despeckler comprises a diffuse scattering plate. In some embodiments, the despeckler is a tension despeckler. The despeckler may be configured to reduce speckle noise to at most about 5%.

[0852] In some embodiments, various laser-based illumination sources may suffer from speckle noise, and that speckle intensity may be controlled to minimize possible errors in sequencing analysis based on image intensities. The source coherence (e.g., speckle) may be reduced by using predetermined fiber length and/or fiber core size, e.g., a 1-3 m length of fiber with a 200-800 um core. In some embodiments, the illumination system may allow mode mixing to reduce speckle to prespecified levels. In some embodiment, a time-variant diffuser reduction method may be integrated into the beam path to improve the source coherency.

[0853] In some embodiments, a single optical fiber may be coupled to a corresponding laser or laser diode. In some embodiments, the characteristics of the optical fiber may be predetermined in order to reduce speckle of the laser. Some non-limiting characteristics of the optical fiber may include: fiber length, fiber bending radius, fiber core shape, fiber core size, fiber attachment to a vibration source, etc.

[0854] In some embodiments, the despeckler is comprised of an optical fiber, which is optically coupled to the light source. In some embodiments, the despeckler is coupled to or associated with the optical fiber to mitigate speckle of the light source. In some embodiments, the despeckler is coupled to or associated with the optical fiber so that the speckle reduction can be generated at some or all regions of the optical fiber.

[0855] In some embodiments, the despeckler comprises a mechanical vibration source, e.g., a vibration motor. The vibration source may produce vibration at a predetermined frequency or frequency range. In some embodiments, the mechanical vibration source is configured to vibrate at one or more frequencies in an audible sound frequency range, an ultrasound frequency range, or both. In some embodiments, the mechanical vibration source is configured to vibrate at one or more frequencies from 10 to 500 Hz. For example, the vibration source may vibrate at a single frequency, with a standard deviation that is less than 1%, 5%, or 10% of the single frequency. As another example, the vibration source may vibrate at a frequency randomly selected in a frequency bandwidth, e.g., 80-90 Hz. In some embodiments, the mechanical vibration source is configured to generate vibrating motions in one, two, or three dimensions. In some embodiments, the mechanical vibration source is configured to generate vibrating motions that includes translation, rotation, or both in two or three dimensions. In some embodiments, the

mechanical vibration source is configured to but is not limited to generate linear or non-linear vibrations, random or deterministic vibrations, and/or undamped or damped vibrations.

[0856] In some embodiments, at least a portion of the optical fiber(s) is wound or coiled for one or more rounds, as shown in FIGS. 9A-9D. The wound up or coiled up portion may include a radius that is no less than the minimum bend radius of the optical fiber to avoid damage to the optical fiber. For example, the wound or coiled up portion may include a radius of 60mm, 65mm, 70 mm or more with a minimum bend radius of 60 mm. The wound up or coiled up portion may be, but is not required to be, perfectly circular, as shown in FIGS. 9A -9D. In some embodiments, the wound or coiled up portion may include at least 2, 3, 4, 5, or more rounds. In some embodiments, the number of rounds of the wound or coiled up portion may be limited by the length of the optical fiber and the minimum bend radius of the optical fiber to enable a maximum possible number of rounds. In some embodiments, the number of rounds of the wound or coiled up portion may be maximized based on the length of the optical fiber and the minimum bend radius of the optical fiber. In some embodiments, at least a portion of the optical fiber at or near its ends is not wound up or coiled up. The non-coiled portion of the optical fiber at each end thereof may be less than 2%, 4%, 5%, 8%, or 10% of the total fiber length. The non-coiled portion of the optical fiber at each end thereof may be less than 0.05m, 0.1m, 0.15m, 0.2m, or 0.3 m.

[0857] In some embodiments, at least part of the optical fiber is loosely or fixedly attached to the mechanical vibration source. For example, the optical fiber can be taped to an off-the-shelf fan with or without wounding or coiling up for one or more rounds. The off-the-shelf fan may be the cooling fan for the CPU or other part(s) of the sequencing system described herein. As shown in FIGS. 73C-73D, the optical fiber is wound or coiled up around the off-the-shelf fan or further taped to the fan at some location(s). As shown in FIG. 73B, the fiber is coiled up on a wheel, and the vibration source is fixedly attached to a center of the wheel, at the bottom.

[0858] The mechanical vibration source can be various machineries that are capable of producing vibration motion(s). The mechanical vibration source can be various off-the-shelf machines that are much more cost-efficient than commercially available despecklers. As nonlimiting examples, the mechanical vibration source can include one or more of: an eccentric rotating mass (ERM) vibration motor, a linear resonant actuator, a coin vibration motor, a cell phone vibration motor, a sonic or ultrasonic vibration motor (e.g., such as one used for an electric toothbrush), an orbital gear or gear set, an orbital weight, etc.

[0859] In some embodiments, the despeckler is physically isolated from other elements of the imaging module, except the optical fiber, to minimize the influence of the despeckler's motion on sequencing reactions and/or imaging quality. For example, the despeckler is not coupled to

the optical fiber at or near either end of the optical fiber, e.g., less than 0.1m. As an example, the despeckler is not positioned within a pre-determined threshold distance from the other elements of the imaging module, e.g., at least 0.1m, 0.2 m, 0.5m, or more. In some embodiments, the despeckler is physically isolated from the sample stage, the objective lens, and/or the one or more image sensors, so that mechanical motion of the despeckler is independent from the sample state, the objective lens, and the one or more image sensors. As an example, the despeckler is at least 0.1m, 0.2 m, 0.5m, or more away from the sample stage, the objective lens, and/or the one or more image sensors. In some embodiments, the despeckler is configured to reduce speckle noise to be no more than 4%, 4.5%, 5%, or 5.5%. In some embodiments, the despeckler is configured to reduce speckle noise by at least 10%, 15%, 20%, 30%, 35%, 40% or more so that the speckle noise after despeckler reduction can be less than 40%, 50%, 55%, 60%, 65%, 70%, or 70% of the speckle noise before reduction.

[0860] Table 1 in FIG. 74 shows the effect of different embodiments of the despecklers herein on the speckle noise in optical fibers. Two optical fibers that are each coupled to a green and red light source are examined. The speckle noise was reduced from 6.53% or 6.32% to less than 4.5% with winding of the optical fiber for 3 rounds and vibration of at least the wound-up portion by an off-the-shelf fan. The speckle reduction effect is better when keeping the fan in a standing position rather than having it laid down on a tabletop or an otherwise horizontal surface. FIGS. 73C-73D show the fan while it is standing or laid down.

[0861] In some embodiments, the speckle noise can be calculated using various methods that determine the level of uniformity of the optical beam, e.g., standard deviation of beam intensity at the sample plane. For example, a 2D beam profile within an imaging FOV can be separated into multiple regions, standard deviation of intensity can be determined for each individual region, and the average standard deviation across all the regions can be the speckle noise level.

[0862] In some embodiments, the despeckler is positioned in an optical path between a collimator and the objective lens of the imaging module, e.g. as shown in FIG. 70. The despeckler is configured to generate microscopic motion. The despeckler at such a larger beam location may advantageously avoid high light power density on the despeckler to avoid damage to the despeckler. In some embodiments, the despeckler here may include a single despeckler. In some embodiments, the despeckler here may include a combination of one or more first despecklers associated with the optical fiber(s) and one or more second large-beam despecklers positioned after the collimator but before the objective lens or sample plane in the optical path. In some embodiments, the large-beam despeckler is positioned where a maximum dimension (e.g., diameter or diagonal) of an optical beam at its cross section (orthogonal to the z axis) is greater than 1 mm, 2 mm, 5 mm, 8 mm, 10 mm, or 20 mm.

[0863] In an aspect, the present disclosure provides an optical system. The optical system can comprise a stage configured to hold a solid support (e.g., flow cell). The stage may be as described elsewhere herein. The optical system may comprise a light source configured to illuminate the solid support as described elsewhere herein. The optical system may comprise a despeckler optically coupled to the light source and disposed within an optical path from the light source to the stage.

[0864] The despeckler may be as described elsewhere herein. The despeckler may be configured to reduce speckle noise, act as a coupler for light sources, or a combination thereof. For example, the despeckler can be configured to receive light from multiple light sources and combine the light from the multiple light sources into a single light beam. In this example, a variety of excitation wavelengths can be combined to a single optical path and despeckled simultaneously. At least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more light sources can be optically coupled to a single despeckler. In some cases, each light source can be optically coupled into a different despeckler. For example, four light sources can each be coupled into four despecklers, thereby despeckling the light from each light source.

[0865] The despeckler may comprise one or more of a diffuse despeckler (e.g., a despeckler comprising a diffuser such as a stationary diffuser, a rotational diffuser, etc.), a spatial light modulator, a phase despeckler (e.g., a despeckler configured to change the phase of the light), a polarization despeckler, a vibrational despeckler (e.g., a despeckler configured to vibrate one or more optical elements such as mirrors, fiber optics, lenses, etc.), a tension despeckler (e.g., a despeckler configured to modulate a tension of an optical fiber to induce despeckling) or the like, or any combination thereof. The despeckler may be configured to reduce speckle noise of the optical system by at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99, or more percent. The despeckler may be configured to reduce speckle noise in the optical system to at most about 25, 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or less percent. Use of a despeckler can improve image quality of the optical system by reducing the speckle noise generated in the optical system, enhancing contrast or the like, or a combination thereof. Use of a vibrational despeckler may provide unexpected benefits to the optical system, as vibration may normally be avoided due to the effects of the vibration on the resolution of the optical system. By instead using vibration (e.g., vibration already present in the system from fans, motors, etc. already present in the system), the methods and system of the present disclosure can provide enhanced illumination profiles and imaging.

[0866] FIG. 89 shows a flow chart of a method 2500 for analyzing a biological sample, according to some embodiments. In an operation 2510, the method 2500 may comprise providing a solid support (e.g., flow cell) comprising the biological sample. The biological

sample may comprise a label as described elsewhere herein. The biological sample may be as described elsewhere herein (e.g., the biological sample may comprise a nucleic acid molecule, a protein, a polypeptide, etc.). The label may be an optical label as described elsewhere herein. The biological sample may comprise a two-dimensional biological sample. The biological sample may comprise a three-dimensional biological sample.

[0867] In another operation 2520, the method 2500 may comprise using an optical system comprising a light source to provide illumination to the biological sample comprising the label, thereby generating a signal light or a change thereof. The optical system may be as described elsewhere herein. For example, the optical system may comprise a despeckler. The illumination may be provided through the despeckler oriented in an optical path of the optical system. The despeckler may be as described elsewhere herein. For example, the despeckler may be a vibrational despeckler. In some cases, an additional light source can be used to illuminate the solid support. The additional light source may provide a different wavelength of light to the flow cell from the light source. For example, the light source can provide a first wavelength configured to excite a first label, and the additional light source can provide a second wavelength configured to excite a second label. The additional light source may be optically coupled to the despeckler. For example, the light source and the additional light source can both be optically coupled to the same despeckler, and the output of the despeckler can comprise light from both light sources.

[0868] In another operation 2530, the method 2500 may comprise detecting, using a detector of the optical system, the signal light or the change thereof. The detecting may be as described elsewhere herein. For example, the detecting may comprise directing the signal light or change thereof to the detector using an optical assembly. In some cases, the detecting may comprise time-gated detecting.

[0869] In another operation 2540, the method 2500 may comprise processing at least in part the signal light or the change thereof to analyze the biological sample. The processing may comprise use of one or more computer systems as described elsewhere herein. The processing may comprise generating one or more base calls of the sample.

[0870] In some cases, the method 2500 may comprise repeating operations 2520 - 2540 for an additional biological sample coupled to an additional surface of the solid support. For example, a plurality of biological molecules can be coupled to the solid support with a plurality of labels each affixed to the plurality of biological molecules, and the optical system can image each of the labels. In some cases, the method 2500 may comprise repeating operations 2510-2540 for an additional label that binds to another portion of the biological molecule. For example, a first label can identify a first nucleotide of a nucleic acid, the first nucleotide can be removed from

the nucleic acid, and a second label can be hybridized to the nucleic acid molecule at a second nucleotide. In this example, the second label can be identified in a similar way to the first, providing information related to the second nucleotide of the nucleic acid molecule.

Light beam delivery subsystems

[0871] The light beam delivery subsystems herein may include one or more collimators and one or more optical lens elements. In some embodiments, the power existing the light beam delivery subsystem is greater than 5, 8, 10, 12, or 14 Watts for one or more wavelengths or wavelength ranges. In some embodiments, a power at a sample plane is greater than 5, 8, 10, 12, or 14 Watts for one or more wavelengths or wavelength ranges. In some embodiments, a power existing the light beam delivery subsystem is greater than 5, 8, 10, 12, or 14 Watts for one or more colors. In some embodiments, a power at a sample plane is greater than 5, 8, 10, 12, or 14 Watts for one or more colors.

[0872] In some embodiments, the one or more collimators may be spaced apart along the z-axis or in the x-y plane. In some embodiments, the light beam delivery system may include a single collimator. FIGS. 65 and 70 shows examples of embodiments of the light beam delivery subsystem. As shown in FIG. 65, the one or more optical lens elements comprise one or more multi-lens arrays (e.g., MLA1 and MLA2). In some embodiments, each multi-lens array comprises one or more of: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens. Each of the multi-lens arrays may comprise multiple lens elements at least in a direction that is orthogonal to a z-axis. For example, the multiple lens elements of the array can be distributed along the x or y-axis or any direction in a x-y plane orthogonal to the z-axis (FIG. 65). In some embodiments, the one or more optical lens elements comprise: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens, or a combination thereof.

[0873] In some embodiments, the one or more optical lens elements comprise: a first multi-lens array (MLA1) and a second multi-lens array (MLA2) that are positioned along a z-axis between the collimator and an entrance pupil of the illumination system, as shown in FIG. 65. In this particular embodiment, the illumination system includes a wide-field illumination module with fiber-coupled diode laser inputs interleaved at an angle on adjacent columns on the first multi-element lens array pair, MLA1 and MLA2. Multiple images of the light source are created in the external or entrance pupil to form a uniform illumination field at the sample field, e.g., the flow cell. The illumination system can comprise a fiber coupled laser diode. The illuminator design is based on generating multiple source images in the entrance pupil plane of the imaging module. The output of the fiber-couple laser is optically connected to a collimator and then

segmented in pupil space by a number of multi-element lens arrays to form these secondary illumination sources. The imaging group, G3, in FIG. 65, is a surrogate for the imaging module between the illumination system and the sample(s). The imaging group G3 can be shared by the imaging and illumination optical paths. In some embodiments, the illumination system is configured to generate secondary illumination sources that overlap at the sample stage or sample positioned thereon, thus averaging the individual intensities to provide intensity with improved uniformity compared to some methods.

[0874] In some embodiments, various laser-based illumination sources may suffer from speckle and that speckle intensity may be controlled. The source coherent (e.g., speckle) may be mitigated by using a 1-3 m length of fiber with a 200-800 um core. In some embodiments, the illumination system may allow mode mixing to reduce speckle to prespecified levels. In some embodiment, a time-variant diffuser reduction method may be integrated into the beam path to improve the source coherency. FIG. 67A-67C illustrates the intensity profiles at the sample using the illumination system herein.

Sequencing systems

[0875] FIG. 75 illustrates a block diagram of a system 7500 for imaging sequencing reactions of sample(s) on a flow cell, according to an embodiment. The system 7500 has a sequencing system 7510 that may include a flow cell 7512, a sequencer 7514, an imager (e.g., the optical system herein) 7516, data storage 7522, and user interface 7524. The sequencing system 7510 may be connected to a cloud 7530. The sequencing system 7510 may include one or more of dedicated processors 7518, Field-Programmable Gate Array(s) (FPGA(s)) 7520, and a computer system 7526.

[0876] In some embodiments, the flow cell 7512 is configured to capture DNA fragments and form DNA sequences for base-calling on the flow cell. The flow cell 7512 can include a support as disclosed herein. The support can be a solid support. The support can include a surface coating thereon as disclosed herein. The surface coating can be a polymer coating as disclosed herein.

[0877] A flow cell 7512 can include multiple tiles or otherwise imaging areas thereon, and each tile may be separated into a grid of subtiles. Each subtile can include a plurality of clusters or polonies thereon. As a nonlimiting example, a flow cell can have 424 tiles, and each tile can be divided into a 6 x 9 grid, therefore 54 subtiles.

[0878] The flow cell images herein are images of a sample immobilized on a support, e.g., a flow cell. The flow cell image as disclosed herein can be an image including signals of a plurality of clusters or polonies. The flow cell image can include one or more tiles of signals or one or more subtiles of signals. In some embodiments, a flow cell image can be an image that

includes all the tiles and approximately all signals thereon. The flow cell image can be acquired from a channel during an imaging or sequencing cycle using the imager 7516. In some embodiments, each tile may include millions of polonies or clusters. As a nonlimiting example, a tile can include about 1 to 10 million of clusters or polonies. Each polony can be a collection of many copies of DNA fragments. The clusters or polonies may appear as bright spot expanding from less than a pixel to a couple of pixels.

[0879] The flow cell images may be of various sizes or field-of-views (FOVs). In some embodiments, each of the flow cell images comprises a wide field-of-view (FOV) that is greater than 20 mm^2, 30 mm^2, 40 mm^2, or 50 mm^2. In some embodiments, each of the flow cell images comprises a field-of-view (FOV) that completely overlaps with or contained within the illumination field generated by the illumination system in the sample plane. In some embodiments, each of the flow cell images comprises a field-of-view (FOV) that overlaps with at least 80%, 85%, 90%, or 95% of an illumination field generated by the illumination system at the sample plane. In some embodiments, each of the flow cell images comprises a field-of-view (FOV) with a size that is at least 80% , 85%, 90%, or 95% of the size of the illumination field generated by the illumination system at the sample plane.

[0880] In some embodiments, to capture such wide FOVs, the image sensor may be of a wider size than other image sensors for some NGS sequencing systems. In some embodiments, the image sensor may include a sensor size that is greater than 20 mm^2, 30 mm^2, 40 mm^2, or 50 mm^2. In some NGS systems, the flow cell images are generally in a range from 1 mm^2 to 8 mm^2. As a result, an illumination field that is greater than 10 mm^2 is not preferred in these NGS systems to avoid or reduce undesired photon bleaching in neighboring areas to the image FOVs. The illumination system herein generates an illumination field that is 2x, 5x, 10x, or larger than the illumination fields in these NGS systems. In some embodiments, the imager 116 is capable of generating FOVs of flow cell images that is comparable to the size of the wide-illumination field herein. The FOVs and the illumination field may be customized so that they overlap or substantially overlap with each other. Additionally, the illumination field may be customized so that its shape can match the shape of the FOV to facilitate such overlap. With such overlap, photon bleaching in the unimaged area of the sample(s) caused by a illumination field wider than the FOV of the flow cell images is avoided or minimized.

[0881] The sequencer 7514 may be configured to flow a nucleotide mixture onto the flow cell 7512, cleave blockers from the nucleotides in between flowing steps, and perform other steps for the formation of the DNA sequences on the flow cell 7512. The nucleotides may have fluorescent elements attached that emit light or energy in a wavelength that indicates the type of

nucleotide. Each type of fluorescent element may correspond to a particular nucleotide base (e.g., A, G, C, T). The fluorescent elements may emit light in visible wavelengths.

[0882] For example, each nucleotide base may be assigned a color. In some cases, adenine may be red, cytosine may be blue, guanine may be green, and thymine may be yellow. The color or wavelength of the fluorescent element for each nucleotide may be selected so that the nucleotides are distinguishable from one another based on the wavelengths of light emitted by the fluorescent elements.

[0883] The imager 7516 may be configured to capture images of the flow cell 7512 after each flowing step. The imager 7516 may include one or more imaging modules disclosed herein. For example, the imager may include an optical system comprising 4 different imaging modules, each for capturing flow cell images from a different color channel.

[0884] In an embodiment, the imager 7516 includes a camera configured to capture digital images, such as a CMOS or a CCD camera. The camera may be configured to capture images at the wavelengths of the fluorescent elements bound to the nucleotides.

[0885] The resolution of the imager 7516 controls the level of detail in the flow cell images, including pixel size. In existing systems, this resolution is very important, as it controls the accuracy with which a spot-finding algorithm identifies the cluster centers. One way to increase the accuracy of spot finding is to improve the resolution of the imager 7516, or improve the processing performed on images taken by the imager 7516. The methods described herein may detect cluster centers in pixels other than those detected by a spot-finding algorithm. These methods allow for improved accuracy in detection of cluster centers without increasing the resolution of the imager 7516. The resolution of the imager may even be less than existing systems with comparable performance, which may reduce the cost of the sequencing system 7510.

[0886] In an embodiment, the images of the flow cell may be captured in groups, where each image in the group is taken at a wavelength or in a spectrum that matches or includes only one of the fluorescent elements. In another embodiment, the images may be captured as single images that capture at least a portion (e.g., a portion, all, etc.) of the wavelengths of the fluorescent elements.

[0887] The sequencing system 7500 may be configured to identify cluster locations on the flow cell 7512 based on the flow cell images. The processing for identifying the cluster may be performed by the dedicated processors 7518, the FPGA(s) 7520, the computing system 7526, or a combination thereof. Identifying or determining the cluster locations may involve performing traditional cluster finding in combination with the cluster finding methods described more particularly herein.

[0888] General purpose processors provide interfaces to run a variety of program in an operating system, such as Windows™ or Linux™. Such an operating system can provides great flexibility to a user.

[0889] In some embodiments, the dedicated processors 7518 may be configured to perform operations of the cluster finding methods described herein. They may not be general-purpose processors, but instead custom processors with specific hardware or instructions for performing those operations. Dedicated processors directly run specific software without an operating system. The lack of an operating system reduces overhead, at the cost of the flexibility in what the processor may perform. A dedicated processor may make use of a custom programming language, which may be designed to operate more efficiently than the software run on general-purpose processors. This may increase the speed at which the operations are performed and allow for real time processing.

[0890] In some embodiments, the FPGA(s) 7520 may be configured to perform operations of the cluster finding methods described herein. An FPGA is programmed as hardware that will only perform a specific task. A special programming language may be used to transform software operations into hardware componentry. Once an FPGA is programmed, the hardware directly processes digital data that is provided to it without running software. The FPGA instead uses logic gates and registers to process the digital data. Because there is no overhead required for an operating system, an FPGA generally processes data faster than a general purpose processor. Similar to dedicated processors, this is at the cost of flexibility.

[0891] The lack of software overhead may also allow an FPGA to operate faster than a dedicated processor, although this will depend on the exact processing to be performed and the specific FPGA and dedicated processor.

[0892] A group of FPGA(s) 7520 may be configured to perform the operations in parallel. For example, a number of FPGA(s) 7520 may be configured to perform a processing operation for an image, a set of images, or a cluster location in one or more images. Each FPGA(s) 7520 may perform its own part of the processing operation at the same time, reducing the time needed to process data. This may allow the processing operations to be completed in real time. Further discussion of the use of FPGAs is provided below.

[0893] Compared to methods that may need to store the data before it can be processed, which may require more memory or accessing a computer system located in the cloud 7530, performing the processing steps in real time may allow the system to use less memory, as the data may be processed as it is received.

[0894] In some embodiments, the data storage 7522 is used to store information used in the identification of the cluster locations. This information may include the images themselves or

information derived from the images captured by the imager 7516. The DNA sequences determined from the base-calling may be stored in the data storage 7522. Parameters identifying cluster locations may also be stored in the data storage 7522.

[0895] The user interface 7524 may be used by a user to operate the sequencing system or access data stored in the data storage 7522 or the computer system 7526.

[0896] The computer system 7526 may control the general operation of the sequencing system and may be coupled to the user interface 7524. It may also perform operations in the identification of cluster locations and base-calling. In some embodiments, the computer system 7526 is a computer system. The computer system 7526 may store information regarding the operation of the sequencing system 7510, such as configuration information, instructions for operating the sequencing system 7510, or user information. The computer system 7526 may be configured to pass information between the sequencing system 110 and the cloud 7530.

[0897] As discussed above, the sequencing system 7510 may have dedicated processors 7518, FPGA(s) 7520, or the computer system 7526. The sequencing system may use one, two, or all of these elements to accomplish necessary processing described above. In some embodiments, when these elements are present together, the processing tasks are split between them. For example, the FPGA(s) 7520 may be used to perform the cluster center finding methods described herein, while the computer system 7526 may perform other processing functions for the sequencing system 7510. Various combinations of these elements can allow various system embodiments that balance efficiency and speed of processing with cost of processing elements.

[0898] The cloud 7530 may be a network, remote storage, or some other remote computing system separate from the sequencing system 7510. The connection to cloud 7530 may allow access to data stored externally to the sequencing system 7510 or allow for updating of software in the sequencing system 7510.

Supports and low non-specific coatings

[0899] In some embodiments, NGS sequencing compositions and methods, e.g., pairwise sequencing, employ a support comprising a plurality of oligonucleotide surface primers immobilized thereon. In some embodiments, the support is passivated with a low non-specific binding coating. The surface coatings described herein exhibit very low non-specific binding to reagents typically used for nucleic acid capture, amplification, and sequencing workflows, such as dyes, nucleotides, enzymes, and nucleic acid primers. The surface coatings exhibit low background fluorescence signals or high contrast-to-noise (CNR) ratios compared to some surface coatings.

[0900] The low non-specific binding coating comprises one layer or multiple layers (FIG. 75). In some embodiments, the plurality of surface primers is immobilized to the low non-specific

binding coating. In some embodiments, at least one surface primer is embedded within the low non-specific binding coating. The low non-specific binding coating enables improved nucleic acid hybridization and amplification performance. In general, the supports comprise a substrate (or support structure), one or more layers of a covalently or non-covalently attached low-binding, chemical modification layer such as silane layers or polymer films, and one or more covalently or non-covalently attached surface primers that can be used for tethering single-stranded nucleic acid library molecules to the support. In some embodiments, the formulation of the coating, e.g., the chemical composition of one or more layers, the coupling chemistry used to cross-link the one or more layers to the support and/or to each other, and the total number of layers, may be varied such that non-specific binding of proteins, nucleic acid molecules, and other hybridization and amplification reaction components to the coating is minimized or reduced relative to a comparable monolayer. The formulation of the coating described herein may be varied such that non-specific hybridization on the coating is minimized or reduced relative to a comparable monolayer. The formulation of the coating may be varied such that non-specific amplification on the coating is minimized or reduced relative to a comparable monolayer. The formulation of the coating may be varied such that specific amplification rates and/or yields on the coating are maximized. Amplification levels suitable for detection are achieved in no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, or more than 30 amplification cycles in some cases disclosed herein.

[0901] The support structure that comprises the one or more chemically-modified layers, e.g., layers of a low non-specific binding polymer, may be independent or integrated into another structure or assembly. For example, in some embodiments, the support structure may comprise one or more surfaces within an integrated or assembled microfluidic flow cell. The support structure may comprise one or more surfaces within a microplate format, e.g., the bottom surface of the wells in a microplate. In some embodiments, the support structure comprises the interior surface (such as the lumen surface) of a capillary. In some embodiments, the support structure comprises the interior surface (such as the lumen surface) of a capillary etched into a planar chip.

[0902] The attachment chemistry used to graft a first chemically-modified layer to the surface of the support will generally be dependent on both the material from which the surface is fabricated and the chemical nature of the layer. In some embodiments, the first layer may be covalently attached to the surface. In some embodiments, the first layer may be non-covalently attached, e.g., adsorbed to the support through non-covalent interactions such as electrostatic interactions, hydrogen bonding, or van der Waals interactions between the support and the molecular components of the first layer. In either case, the support may be treated prior to attachment or

deposition of the first layer. A variety of surface preparation techniques may be used to clean or treat the surface. For example, glass or silicon surfaces may be acid-washed using a Piranha solution (a mixture of sulfuric acid H_2SO_4 and hydrogen peroxide H_2O_2), base treated with KOH and NaOH, and/or cleaned using an oxygen plasma treatment method.

[0903] Silane chemistries constitute non-limiting approaches for covalently modifying the silanol groups on glass or silicon surfaces to attach more reactive functional groups (e.g., amines or carboxyl groups), which may then be used in coupling linker molecules (e.g., linear hydrocarbon molecules of various lengths, such as C6, C12, or C18 hydrocarbons, or linear polyethylene glycol (PEG) molecules) or layer molecules (e.g., branched PEG molecules or other polymers) to the surface. Examples of suitable silanes that may be used in creating any of the disclosed low binding coatings include, but are not limited to, (3-Aminopropyl) trimethoxysilane (APTMS), (3-Aminopropyl) triethoxysilane (APTES), any of a variety of PEG-silanes (e.g., comprising molecular weights of 1K, 2K, 5K, 10K, 20K, etc.), amino-PEG silane (e.g., comprising a free amino functional group), maleimide-PEG silane, biotin-PEG silane, and the like.

[0904] A variety of molecules including, but not limited to, amino acids, peptides, nucleotides, oligonucleotides, other monomers or polymers, or combinations thereof may be used in creating the one or more chemically-modified layers on the support, where the choice of components used may be varied to alter one or more properties of the layers such as the surface density of functional groups and/or tethered oligonucleotide primers, the hydrophilicity/hydrophobicity of the layers, or the three three-dimensional nature (e.g., "thickness") of the layers. Examples of polymers that may be used to create one or more layers of low non-specific binding material in any of the disclosed coatings include, but are not limited to, polyethylene glycol (PEG) of various molecular weights and branching structures, streptavidin, polyacrylamide, polyester, dextran, poly-lysine, and poly-lysine copolymers, or any combination thereof. Examples of conjugation chemistries that may be used to graft one or more layers of material (e.g. polymer layers) to the surface and/or to cross-link the layers to each other include, but are not limited to, biotin-streptavidin interactions (or variations thereof), His tag – Ni/NTA conjugation chemistries, methoxy ether conjugation chemistries, carboxylate conjugation chemistries, amine conjugation chemistries, NHS esters, maleimides, thiol, epoxy, azide, hydrazide, alkyne, isocyanate, and silane.

[0905] The low non-specific binding surface coating may be applied uniformly across the support. Alternatively, the surface coating may be patterned, such that the chemical modification layers are confined to one or more discrete regions of the support. For example, the coating may be patterned using photolithographic techniques to create an ordered array or random pattern of

chemically-modified regions on the support. Alternately or in combination, the coating may be patterned using, e.g., contact printing and/or ink-jet printing techniques. In some embodiments, an ordered array or random pattern of chemically-modified regions may comprise at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, or 10,000 or more discrete regions.

[0906] In some embodiments, the low nonspecific binding coatings comprise hydrophilic polymers that are non-specifically adsorbed or covalently grafted to the support. Passivation may be performed utilizing poly(ethylene glycol) (PEG, also known as polyethylene oxide (PEO) or polyoxyethylene) or other hydrophilic polymers with different molecular weights and end groups that are linked to a support using, for example, silane chemistry. The end groups distal from the surface can include, but are not limited to, biotin, methoxy ether, carboxylate, amine, NHS ester, maleimide, and bis-silane. In some embodiments, two or more layers of a hydrophilic polymer, e.g., a linear polymer, branched polymer, or multi-branched polymer, may be deposited on the surface. In some embodiments, two or more layers may be covalently coupled to each other or internally cross-linked to improve the stability of the resulting coating. In some embodiments, surface primers with different nucleotide sequences and/or base modifications (or other biomolecules, e.g., enzymes or antibodies) may be tethered to the resulting layer at various surface densities. In some embodiments, for example, both surface functional group density and surface primer concentration may be varied to attain a specified surface primer density range. Additionally, surface primer density can be controlled by diluting the surface primers with other molecules that carry the same functional group. For example, amine-labeled surface primers can be diluted with amine-labeled polyethylene glycol in a reaction with an NHS-ester coated surface to reduce the final primer density. Surface primers with different lengths of linker between the hybridization region and the surface attachment functional group can also be applied to control surface density. Example of suitable linkers include poly-T and poly-A strands at the 5' end of the primer (e.g., 0 to 20 bases), PEG linkers (e.g., 3 to 20 monomer units), and carbon-chain (e.g., C6, C12, C18, etc.). To measure the primer density, fluorescently-labeled primers may be tethered to the surface and a fluorescence reading then compared with that for a dye solution of known concentration.

[0907] In some embodiments, the low nonspecific binding coatings comprise a functionalized polymer coating layer covalently bound at least to a portion of the support via a chemical group on the support, a primer grafted to the functionalized polymer coating, and a water-soluble protective coating on the primer and the functionalized polymer coating. In some embodiments, the functionalized polymer coating comprises a poly(N-(5-azidoacetamidylpentyl)acrylamide-co-acrylamide (PAZAM).

[0908] In order to scale primer surface density and add additional dimensionality to hydrophilic or amphoteric coatings, supports comprising multi-layer coatings of PEG and other hydrophilic polymers have been developed. By using hydrophilic and amphoteric surface layering approaches that include, but are not limited to, the polymer/co-polymer materials described below, it is possible to increase primer loading density on the support significantly. Some PEG coating approaches use monolayer primer deposition, which have been generally reported for single molecule applications but do not yield high copy numbers for nucleic acid amplification applications. As described herein, "layering" can be accomplished using traditional crosslinking approaches with any compatible polymer or monomer subunits such that a surface comprising two or more highly crosslinked layers can be built sequentially. Examples of suitable polymers include, but are not limited to, streptavidin, poly acrylamide, polyester, dextran, poly-lysine, and copolymers of poly-lysine and PEG. In some embodiments, the different layers may be attached to each other through any of a variety of conjugation reactions including, but not limited to, biotin-streptavidin binding, azide-alkyne click reaction, amine-NHS ester reaction, thiol-maleimide reaction, and ionic interactions between positively charged polymer and negatively charged polymer. In some embodiments, high primer density materials may be constructed in solution and subsequently layered onto the surface in multiple steps.

[0909] Examples of materials from which the support structure may be fabricated include, but are not limited to, glass, fused-silica, silicon, a polymer (e.g., polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET)), or any combination thereof. Various compositions of both glass and plastic support structures are contemplated.

[0910] The support structure may be rendered in a variety of geometries and dimensions, and may comprise a variety of materials. For example, the support structure may be locally planar (e.g., comprising a microscope slide or the surface of a microscope slide). Globally, the support structure may be cylindrical (e.g., comprising a capillary or the interior surface of a capillary), spherical (e.g., comprising the outer surface of a non-porous bead), or irregular (e.g., comprising the outer surface of an irregularly-shaped, non-porous bead or particle). In some embodiments, the surface of the support structure used for nucleic acid hybridization and amplification may be a solid, non-porous surface. In some embodiments, the surface of the support structure used for nucleic acid hybridization and amplification may be porous, such that the coatings described herein penetrate the porous surface, and nucleic acid hybridization and amplification reactions performed thereon may occur within the pores.

[0911] The support structure that comprises the one or more chemically-modified layers, e.g., layers of a low non-specific binding polymer, may be independent or integrated into another structure or assembly. For example, the support structure may comprise one or more surfaces within an integrated or assembled microfluidic flow cell. The support structure may comprise one or more surfaces within a microplate format, e.g., the bottom surface of the wells in a microplate. In some embodiments, the support structure comprises the interior surface (such as the lumen surface) of a capillary. In some embodiments the support structure comprises the interior surface (such as the lumen surface) of a capillary etched into a planar chip.

[0912] As noted, the low non-specific binding supports of the present disclosure exhibit reduced non-specific binding of proteins, nucleic acids, and other components of the hybridization and/or amplification formulation used for solid-phase nucleic acid amplification. The degree of non-specific binding exhibited by a given support surface may be assessed either qualitatively or quantitatively. For example, exposure of the surface to fluorescent dyes (e.g., cyanins such as Cy3, or Cy5, etc., fluoresceins, coumarins, rhodamines, etc. or other dyes disclosed herein), fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (e.g. polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging, may be used as a qualitative tool for comparison of non-specific binding on supports comprising different surface formulations. In some embodiments, exposure of the surface to fluorescent dyes, fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (e.g. polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging, may be used as a quantitative tool for comparison of non-specific binding on supports comprising different surface formulations. Care can be taken to ensure that the fluorescence imaging is performed under conditions where fluorescence signal is linearly related (or related in a predictable manner) to the number of fluorophores on the support surface (e.g., under conditions where signal saturation and/or self-quenching of the fluorophore is not an issue) and suitable calibration standards are used. In some embodiments, other techniques such as radioisotope labeling and counting methods may be used for quantitative assessment of the degree to which non-specific binding is exhibited by the different support surface formulations of the present disclosure.

[0913] Some surfaces disclosed herein exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein. Some surfaces disclosed herein exhibit a ratio of specific to nonspecific fluorescence of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20,

25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

[0914] The degree of non-specific binding exhibited by the disclosed low-binding supports may be assessed using a standardized protocol for contacting the surface with a labeled protein (e.g., bovine serum albumin (BSA), streptavidin, a DNA polymerase, a reverse transcriptase, a helicase, a single-stranded binding protein (SSB), etc., or any combination thereof), a labeled nucleotide, a labeled oligonucleotide, etc., under a standardized set of incubation and rinse conditions, followed by detection of the amount of label remaining on the surface and comparison of the signal resulting therefrom to an appropriate calibration standard. In some embodiments, the label may comprise a fluorescent label. In some embodiments, the label may comprise a radioisotope. In some embodiments, the label may comprise any other detectable label. In some embodiments, the degree of non-specific binding exhibited by a given support surface formulation may thus be assessed in terms of the number of non-specifically bound protein molecules (or nucleic acid molecules or other molecules) per unit area. In some embodiments, the low-binding supports of the present disclosure may exhibit non-specific protein binding (or non-specific binding of other specified molecules, (e.g., cyanins such as Cy3, or Cy5, etc., fluoresceins, coumarins, rhodamines, etc. or other dyes disclosed herein)) of less than 0.001 molecule per μm^2, less than 0.01 molecule per μm^2, less than 0.1 molecule per μm^2, less than 0.25 molecule per μm^2, less than 0.5 molecule per μm^2, less than 1 molecule per μm^2, less than 10 molecules per μm^2, less than 100 molecules per μm^2, or less than 1,000 molecules per μm^2. A given support surface of the present disclosure may exhibit non-specific binding falling anywhere within this range, for example, of less than 86 molecules per μm^2. For example, some modified surfaces disclosed herein exhibit nonspecific protein binding of less than 0.5 molecule/μm^2 following contact with a 1 μM solution of Cy3 labeled streptavidin (GE Amersham) in phosphate buffered saline (PBS) buffer for 15 minutes, followed by 3 rinses with deionized water. Some modified surfaces disclosed herein exhibit nonspecific binding of Cy3 dye molecules of less than 0.25 molecules per μm^2. In independent nonspecific binding assays, 1 μM labeled Cy3 SA (ThermoFisher), 1 μM Cy5 SA dye (ThermoFisher), 10 μM Aminoallyl-dUTP-ATTO-647N (Jena Biosciences), 10 μM Aminoallyl-dUTP-ATTO-Rhol 1 (Jena Biosciences), 10 μM Aminoallyl-dUTP-ATTO-Rhol 1 (Jena Biosciences), 10 μM 7-Propargylamino-7-deaza-dGTP-Cy5 (Jena Biosciences, and 10 μM 7-Propargylamino-7-deaza-dGTP-Cy3 (Jena Biosciences) were incubated on the low binding coated supports at 37° C. for 15 minutes in a 384 well plate format. Each well was rinsed 2-3× with 50 ul deionized RNase/DNase Free water and 2-3× with 25 mM ACES buffer pH 7.4. The 384 well plates were imaged on a GE Typhoon instrument using the Cy3, AF555, or Cy5 filter sets (according to dye

test performed) as specified by the manufacturer at a PMT gain setting of 800 and resolution of 50-100 μm. For higher resolution imaging, images were collected on an Olympus IX83 microscope (e.g., inverted fluorescence microscope) (Olympus Corp., Center Valley, Pa.) with a total internal reflectance fluorescence (TIRF) objective (100×, 1.5 NA, Olympus), a CCD camera (e.g., an Olympus EM-CCD monochrome camera, Olympus XM-10 monochrome camera, or an Olympus DP80 color and monochrome camera), an illumination source (e.g., an Olympus 100W Hg lamp, an Olympus 75W Xe lamp, or an Olympus U-HGLGPS fluorescence light source), and excitation wavelengths of 532 nm or 635 nm. Dichroic mirrors were purchased from Semrock (IDEX Health & Science, LLC, Rochester, N.Y.), e.g., 405, 488, 532, or 633 nm dichroic reflectors/beamsplitters, and band pass filters were chosen as 532 LP or 645 LP concordant with the appropriate excitation wavelength. Some modified surfaces disclosed herein exhibit nonspecific binding of dye molecules of less than 0.25 molecules per μm^2. In some embodiments, the coated support was immersed in a buffer (e.g., 25 mM ACES, pH 7.4) while the image was acquired.

[0915] In some embodiments, the surfaces disclosed herein exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein. In some embodiments, the surfaces disclosed herein exhibit a ratio of specific to nonspecific fluorescence signals for a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

[0916] The low-background surfaces consistent with the disclosure herein may exhibit specific dye attachment (e.g., Cy3 attachment) to non-specific dye adsorption (e.g., Cy3 dye adsorption) ratios of at least 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1, or more than 50 specific dye molecules attached per molecule nonspecifically adsorbed. Similarly, when subjected to an excitation energy, low-background surfaces consistent with the disclosure herein to which fluorophores, e.g., Cy3, have been attached may exhibit ratios of specific fluorescence signal (e.g., arising from Cy3-labeled oligonucleotides attached to the surface) to non-specific adsorbed dye fluorescence signals of at least 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1, or more than 50:1.

[0917] In some embodiments, the degree of hydrophilicity (or "wettability" with aqueous solutions) of the disclosed support surfaces may be assessed, for example, through the measurement of water contact angles in which a small droplet of water is placed on the surface and its angle of contact with the surface is measured using, for example, an optical tensiometer. In some embodiments, a static contact angle may be determined. In some embodiments, an

advancing or receding contact angle may be determined. In some embodiments, the water contact angle for the hydrophilic, low-binding support surfaced disclosed herein may range from about 0 degrees to about 30 degrees. In some embodiments, the water contact angle for the hydrophilic, low-binding support surfaced disclosed herein may no more than 50 degrees, 40 degrees, 30 degrees, 25 degrees, 20 degrees, 18 degrees, 16 degrees, 14 degrees, 12 degrees, 10 degrees, 8 degrees, 6 degrees, 4 degrees, 2 degrees, or 1 degree. In many cases the contact angle is no more than 40 degrees. A given hydrophilic, low-binding support surface of the present disclosure may exhibit a water contact angle having a value of anywhere within this range.

[0918] In some embodiments, the hydrophilic surfaces disclosed herein facilitate reduced wash times for bioassays, often due to reduced nonspecific binding of biomolecules to the low-binding surfaces. In some embodiments, adequate wash steps may be performed in less than 60, 50, 40, 30, 20, 15, 10, or less than 10 seconds. For example, adequate wash steps may be performed in less than 30 seconds.

[0919] Some low-binding surfaces of the present disclosure exhibit significant improvement in stability or durability to prolonged exposure to solvents and elevated temperatures, or to repeated cycles of solvent exposure or changes in temperature. For example, the stability of the disclosed surfaces may be tested by fluorescently labeling a functional group on the surface, or a tethered biomolecule (e.g., an oligonucleotide primer) on the surface, and monitoring fluorescence signal before, during, and after prolonged exposure to solvents and elevated temperatures, or to repeated cycles of solvent exposure or changes in temperature. In some embodiments, the degree of change in the fluorescence used to assess the quality of the surface may be less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% over a time period of 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 15 hours, 20 hours, 25 hours, 30 hours, 35 hours, 40 hours, 45 hours, 50 hours, or 100 hours of exposure to solvents and/or elevated temperatures (or any combination of these percentages as measured over these time periods). In some embodiments, the degree of change in the fluorescence used to assess the quality of the surface may be less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% over 5 cycles, 10 cycles, 20 cycles, 30 cycles, 40 cycles, 50 cycles, 60 cycles, 70 cycles, 80 cycles, 90 cycles, 100 cycles, 200 cycles, 300 cycles, 400 cycles, 500 cycles, 600 cycles, 700 cycles, 800 cycles, 900 cycles, or 1,000 cycles of repeated exposure to solvent changes and/or changes in temperature (or any combination of these percentages as measured over this range of cycles).

[0920] In some embodiments, the surfaces disclosed herein may exhibit a high ratio of specific signal to nonspecific signal or other background. For example, when used for nucleic acid

amplification, some surfaces may exhibit an amplification signal that is at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, or greater than 100-fold greater than a signal of an adjacent unpopulated region of the surface. Similarly, some surfaces exhibit an amplification signal that is at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, or greater than 100-fold greater than a signal of an adjacent amplified nucleic acid population region of the surface.

[0921] In some embodiments, fluorescence images of the disclosed low background surfaces when used in nucleic acid hybridization or amplification applications to create polonies of hybridized or clonally-amplified nucleic acid molecules (e.g., that have been directly or indirectly labeled with a fluorophore) exhibit contrast-to-noise ratios (CNRs) of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 20, 210, 220, 230, 240, 250, or greater than 250.

[0922] One or more types of primer may be attached or tethered to the support surface. In some embodiments, the one or more types of adapters or primers may comprise spacer sequences, adapter sequences for hybridization to adapter-ligated target library nucleic acid sequences, forward amplification primers, reverse amplification primers, sequencing primers, and/or molecular barcoding sequences, or any combination thereof. In some embodiments, 1 primer or adapter sequence may be tethered to at least one layer of the surface. In some embodiments, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 different primer or adapter sequences may be tethered to at least one layer of the surface.

[0923] In some embodiments, the tethered adapter and/or primer sequences may range in length from about 10 nucleotides to about 100 nucleotides. In some embodiments, the tethered adapter and/or primer sequences may be at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides in length. In some embodiments, the tethered adapter and/or primer sequences may be at most 100, at most 90, at most 80, at most 70, at most 60, at most 50, at most 40, at most 30, at most 20, or at most 10 nucleotides in length. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some embodiments the length of the tethered adapter and/or primer sequences may range from about 20 nucleotides to about 80 nucleotides. The length of the tethered adapter and/or primer sequences may have any value within this range, e.g., about 24 nucleotides.

[0924] In some embodiments, the resultant surface density of primers (e.g., capture primers) on the low binding support surfaces of the present disclosure may range from about 100 primer molecules per μm^2 to about 100,000 primer molecules per μm^2. In some embodiments, the resultant surface density of primers on the low binding support surfaces of the present disclosure may range from about 1,000 primer molecules per μm^2 to about 1,000,000 primer molecules per

μm^2. In some embodiments, the surface density of primers may be at least 1,000, at least 10,000, at least 100,000, or at least 1,000,000 molecules per μm^2. In some embodiments, the surface density of primers may be at most 1,000,000, at most 100,000, at most 10,000, or at most 1,000 molecules per μm2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some embodiments the surface density of primers may range from about 10,000 molecules per μm^2 to about 100,000 molecules per μm^2. The surface density of primer molecules may have any value within this range, e.g., about 455,000 molecules per μm^2. In some embodiments, the surface density of target library nucleic acid sequences initially hybridized to adapter or primer sequences on the support surface may be less than or equal to that indicated for the surface density of tethered primers. In some embodiments, the surface density of clonally-amplified target library nucleic acid sequences hybridized to adapter or primer sequences on the support surface may span the same range as that indicated for the surface density of tethered primers.

[0925] Local densities as listed above do not preclude variation in density across a surface, such that a surface may comprise a region having an oligo density of, for example, 500,000/μm^2, while also comprising at least a second region having a substantially different local density.

Contrast to noise ratio (CNR)

[0926] In some embodiments, the performance of nucleic acid hybridization, amplification reactions, or a combination thereof using the disclosed reaction formulations and low-binding supports may be assessed using fluorescence imaging techniques, where the contrast-to-noise ratio (CNR) of the images provides a key metric in assessing amplification specificity and non-specific binding on the support. CNR is defined as: CNR=(Signal-Background)/Noise. The background term is taken to be the signal measured for the interstitial regions surrounding a particular feature (diffraction limited spot, DLS) in a specified region of interest (ROI). While signal-to-noise ratio (SNR) is often considered to be a benchmark of overall signal quality, it can be shown that improved CNR can provide a significant advantage over SNR as a benchmark for signal quality in applications that require rapid image capture (e.g., sequencing applications for which cycle times must be minimized), as shown in the example below. At high CNR the imaging time required to reach accurate discrimination (and thus accurate base-calling in the case of sequencing applications) can be drastically reduced even with moderate improvements in CNR. Improved CNR in imaging data on the imaging integration time provides a method for more accurately detecting features such as clonally-amplified nucleic acid colonies on the support surface.

[0927] In some ensemble-based sequencing approaches, the background term may be measured as the signal associated with `interstitial` regions. In addition to "interstitial" background

(Binter), "intrastitial" background (Bintra) exists within the region occupied by an amplified DNA colony. The combination of these two background signals dictates the achievable CNR, and subsequently directly impacts the optical instrument requirements, architecture costs, reagent costs, run-times, cost/genome, and ultimately the accuracy and data quality for cyclic array-based sequencing applications. The Binter background signal arises from a variety of sources; a few examples include auto-fluorescence from consumable flow cells, non-specific adsorption of detection molecules that yield spurious fluorescence signals that may obscure the signal from the ROI, and the presence of non-specific DNA amplification products (e.g., those arising from primer dimers). In some next generation sequencing (NGS) applications, this background signal in the current field-of-view (FOV) is averaged over time and subtracted. The signal arising from individual DNA colonies (e.g., (Signal)-B(interstial) in the FOV) yields a discernable feature that can be classified. In some embodiments, the intrastitial background (B(intrastitial)) can contribute a confounding fluorescence signal that is not specific to the target of interest, but is present in the same ROI thus making it far more difficult to average and subtract.

[0928] Nucleic acid amplification on the low-binding coated supports described herein may decrease the B(interstitial) background signal by reducing non-specific binding, may lead to improvements in specific nucleic acid amplification, and may lead to a decrease in non-specific amplification that can impact the background signal arising from both the interstitial and intrastitial regions. Compared to some methods, in some embodiments, the disclosed low-binding coated supports, optionally used in combination with the disclosed hybridization and/or amplification reaction formulations, may lead to improvements in CNR by a factor of 2, 5, 10, 100, 250, 500 or 1000-fold. Although described here in the context of using fluorescence imaging as the read-out or detection mode, the same principles apply to the use of the disclosed low-binding coated supports and nucleic acid hybridization and amplification formulations for other detection modes as well, including both optical and non-optical detection modes.

Methods for sequencing

[0929] The present disclosure provides methods for autofocusing optical systems that can be used for sequencing immobilized or non-immobilized template nucleotide acid molecules. In some embodiments, the immobilized template molecules comprise a plurality of nucleic acid template molecules having one copy of a target sequence of interest. In some embodiments, nucleic acid template molecules having one copy of a target sequence of interest can be generated by conducting bridge amplification using linear library molecules. In some embodiments, the immobilized template molecules comprise a plurality of nucleic acid template molecules each having two or more tandem copies of a target sequence of interest (e.g.,

concatemers). In some embodiments, nucleic acid template molecules comprising concatemer molecules can be generated by conducting rolling circle amplification of circularized linear library molecules. In some embodiments, the non-immobilized template molecules comprise circular molecules. In some embodiments, methods for sequencing employ soluble (e.g., non-immobilized) sequencing polymerases or sequencing polymerases that are immobilized to a support.

[0930] In some embodiments, the sequencing reactions employ detectably labeled nucleotide analogs. In some embodiments, the sequencing reactions employ a two-stage sequencing reaction comprising binding detectably labeled multivalent molecules and incorporating nucleotide analogs. In some embodiments, the sequencing reactions employ non-labeled nucleotide analogs. In some embodiments, the sequencing reactions employ phosphate chain-labeled nucleotides.

Multivalent Molecules

[0931] The present disclosure provides methods for autofocusing optical systems that can be used for sequencing template nucleic acid molecules. In some embodiments, the sample immobilized or otherwise positioned on the support may include at least one multivalent molecule. In some embodiments, the sample that is used for autofocusing the optical system may include at least one multivalent molecule. In some embodiments, the sequencing methods utilizing the optical system for imaging may employ at least one multivalent molecule. In some embodiments, the sequencing methods utilizing the optical system for imaging may include autofocusing of the optical system before imaging one or more surfaces in a sequencing cycle of the sequencing run.

[0932] In some embodiments, the multivalent molecule comprises a plurality of nucleotide arms attached to a core and having any configuration including a starburst, helter skelter, or bottle brush configuration (e.g., FIG. 76). The multivalent molecule comprises: (1) a core; and (2) a plurality of nucleotide arms which comprise (i) a core attachment moiety, (ii) a spacer comprising a PEG moiety, (iii) a linker, and (iv) a nucleotide unit, wherein the core is attached to the plurality of nucleotide arms, wherein the spacer is attached to the linker, and wherein the linker is attached to the nucleotide unit. In some embodiments, the nucleotide unit comprises a base, sugar and at least one phosphate group, and the linker is attached to the nucleotide unit through the base. In some embodiments, the linker comprises an aliphatic chain or an oligo ethylene glycol chain where both linker chains having 2-6 subunits. In some embodiments, the linker also includes an aromatic moiety. An example of a nucleotide arm is shown in FIG. 80. Examples of multivalent molecules are shown in FIGS. 76-79. An example of a spacer is shown

in FIG. 81 (top) and examples of linkers are shown in FIG. 81 (bottom) and FIG. 82. Examples of nucleotides attached to a linker are shown in FIGS. 83-86. An example of a biotinylated nucleotide arm is shown in FIG. 87.

[0933] In some embodiments, a multivalent molecule comprises a core attached to multiple nucleotide arms, and wherein the multiple nucleotide arms have the same type of nucleotide unit which is selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

[0934] In some embodiments, a multivalent molecule comprises a core attached to multiple nucleotide arms, where each arm includes a nucleotide unit. The nucleotide unit comprises an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and one or more phosphate groups (e.g., 1-10 phosphate groups). The plurality of multivalent molecules can comprise one type of multivalent molecule having one type of nucleotide unit selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP. The plurality of multivalent molecules can comprise at a mixture of any combination of two or more types of multivalent molecules, where individual multivalent molecules in the mixture comprise nucleotide units selected from the group consisting of dATP, dGTP, dCTP, dTTP,dUTP, or a combination thereof.

[0935] In some embodiments, the nucleotide unit comprises a chain of one, two or three phosphorus atoms where the chain is attached to the 5' carbon of the sugar moiety via an ester or phosphoramide linkage. In some embodiments, at least one nucleotide unit is a nucleotide analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene, or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH_3. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

[0936] In some embodiments, the multivalent molecule comprises a core attached to multiple nucleotide arms, and wherein individual nucleotide arms comprise a nucleotide unit which is a nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the nucleotide unit comprises a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' position. In some embodiments, the chain terminating moiety can inhibit polymerase-catalyzed incorporation of a subsequent nucleotide unit or free nucleotide in a nascent strand during a primer extension reaction. In some embodiments, the chain terminating moiety is attached to the 3' sugar position where the sugar comprises a ribose or deoxyribose sugar moiety. In some embodiments, the chain terminating moiety is removable/cleavable from the 3' sugar position to generate a nucleotide having a 3'OH sugar group which is extendable with a subsequent nucleotide in a polymerase-catalyzed

nucleotide incorporation reaction. In some embodiments, the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group. In some embodiments, the chain terminating moiety is cleavable/removable from the nucleotide unit, for example by reacting the chain terminating moiety with a chemical agent, pH change, light or heat. In some embodiments, the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh3)4) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the chain terminating moieties aryl and benzyl are cleavable with H2 Pd/C. In some embodiments, the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). In some embodiments, the chain terminating moiety carbonate is cleavable with potassium carbonate (K2CO3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some embodiments, the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0937] In some embodiments, the nucleotide unit comprises a chain terminating moiety (e.g., blocking moiety) at the sugar 2' position, at the sugar 3' position, or at the sugar 2' and 3' positions. In some embodiments, the chain terminating moiety comprises an azide, azido or azidomethyl group. In some embodiments, the chain terminating moiety comprises a 3'-O-azido or 3'-O-azidomethyl group. In some embodiments, the chain terminating moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[0938] In some embodiments, the nucleotide unit comprising a chain terminating moiety which is selected from the group consisting of 3'-deoxy nucleotides, 2',3'-dideoxynucleotides, 3'-methyl, 3'-azido, 3'-azidomethyl, 3'-O-azidoalkyl, 3'-O-ethynyl, 3'-O-aminoalkyl, 3'-O-fluoroalkyl, 3'-fluoromethyl, 3'-difluoromethyl, 3'-trifluoromethyl, 3'-sulfonyl, 3'-malonyl, 3'-amino, 3'-O-amino, 3'-sulfhydral, 3'-aminomethyl, 3'-ethyl, 3'butyl, 3'-tert butyl, 3'-Fluorenylmethyloxycarbonyl, 3' tert-Butyloxycarbonyl, 3'-O-alkyl hydroxylamino group, 3'-phosphorothioate, and 3-O-benzyl, and derivatives thereof.

[0939] In some embodiments, the multivalent molecule comprises a core attached to multiple nucleotide arms, wherein the nucleotide arms comprise a spacer, linker and nucleotide unit, and wherein the core, linker and/or nucleotide unit is labeled with detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the multivalent molecule can correspond to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) of the nucleotide unit to permit detection and identification of the nucleotide base.

[0940] In some embodiments, at least one nucleotide arm of a multivalent molecule has a nucleotide unit that is attached to a detectable reporter moiety. In some embodiments, the detectable reporter moiety is attached to the nucleotide base. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the multivalent molecule can correspond to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) of the nucleotide unit to permit detection and identification of the nucleotide base.

[0941] In some embodiments, the core of a multivalent molecule comprises an avidin-like or streptavidin-like moiety and the core attachment moiety comprises biotin. In some embodiments, the core comprises a streptavidin-type or avidin-type moiety which includes an avidin protein, as well as any derivatives, analogs and other non-native forms of avidin that can bind to at least one biotin moiety. Other forms of avidin moieties include native and recombinant avidin and streptavidin as well as derivatized molecules, e.g. non-glycosylated avidin and truncated streptavidins . For example, avidin moiety includes de-glycosylated forms of avidin, bacterial streptavidin produced by Streptomyces (e.g., Streptomyces avidinii), as well as derivatized forms, for example, N-acyl avidins, e.g., N-acetyl, N-phthalyl and N-succinyl avidin, and the commercially-available products EXTRAVIDIN, CAPTAVIDIN, NEUTRAVIDIN and NEUTRALITE AVIDIN.

[0942] In some embodiments, any of the methods for sequencing nucleic acid molecules described herein can include forming a binding complex, where the binding complex comprises (i) a polymerase, a nucleic acid template molecule duplexed with a primer, and a nucleotide, or the binding complex comprises (ii) a polymerase, a nucleic acid template molecule duplexed with a primer, and a nucleotide unit of a multivalent molecule. In some embodiments, the binding complex has a persistence time of greater than about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1 second. The binding complex has a persistence time of greater than about 0.1-0.25 seconds, or about 0.25-0.5 seconds, or about 0.5-0.75 seconds, or about 0.75-1 second, or about 1-2 seconds, or about 2-3 seconds, or about 3-4 second, or about 4-5 seconds, and/or wherein the method is or may be carried out at a temperature of at or above 15 °C, at or above 20 °C, at or

above 25 °C, at or above 35 °C, at or above 37 °C, at or above 42 °C at or above 55 °C at or above 60 °C, or at or above 72 °C, or at or above 80 °C, or within a range defined by any of the foregoing. The binding complex (e.g., ternary complex) remains stable until subjected to a condition that causes dissociation of interactions between any of the polymerase, template molecule, primer and/or the nucleotide unit or the nucleotide. For example, a dissociating condition comprises contacting the binding complex with any one or any combination of a detergent, EDTA and/or water. In some embodiments, the present disclosure provides said method wherein the binding complex is deposited on, attached to, or hybridized to, a surface showing a contrast-to-noise ratio in the detecting step of greater than 20. In some embodiments, the present disclosure provides said method wherein the contacting is performed under a condition that stabilizes the binding complex when the nucleotide or nucleotide unit is complementary to a next base of the template nucleic acid, and destabilizes the binding complex when the nucleotide or nucleotide unit is not complementary to the next base of the template nucleic acid.

[0943] In some embodiments, the present disclosure provides a multivalent molecule comprising a core attached to at least one nucleotide-arm. In some embodiments, the at least one nucleotide-arm can comprise a core attachment moiety. In some embodiments, the at least one nucleotide-arm can comprise a spacer. In some embodiments, the at least one nucleotide-arm can comprise a linker. In some embodiments, the at least one nucleotide-arm can comprise a nucleotide unit. In some embodiments, the at least one nucleotide-arm can comprise a core attachment moiety, a spacer, a linker, and a nucleotide unit. In some embodiments, the core can comprise a bead, particle or nanoparticle. In some embodiments, the core can comprise an alkyl, alkenyl, or alkynyl core such as may be present in a branched polymer or dendrimer. In some embodiments the core can comprise a moiety that mediates conjugation of the core to the nucleotide-arm. In some embodiments, the core can be attached to a plurality of nucleotide-arms. In some cases, the core can be attached to between about 1 to about 50 nucleotide arms. In some cases, the core is attached to between about 2 to about 20 nucleotide-arms. In some cases, the core is attached to between about 2 to about 4 nucleotide-arms. In some cases, the core is attached to between about 4 to about 10 nucleotide-arms. In some cases, the core is attached to between about 10 to about 15 nucleotide-arms. In some cases, the core is attached to between about 15 to about 20 nucleotide-arms. Examples of multivalent molecules are shown in FIGS. 76-79.

[0944] The present disclosure provides a multivalent molecule comprising a core attached to at least one biotinylated nucleotide-arm. In some embodiments, the at least one biotinylated nucleotide-arm can comprise a core attachment moiety. In some embodiments, the at least one

biotinylated nucleotide-arm can comprise a spacer. In some embodiments, the at least one biotinylated nucleotide-arm can comprise a linker. In some embodiments, the at least one biotinylated nucleotide-arm can comprise a nucleotide unit. In some embodiments, the at least one biotinylated nucleotide-arm can comprise a core attachment moiety, a spacer, a linker, and a nucleotide unit. In some embodiments the core can comprise a streptavidin-type or avidin-type moiety, and the biotin unit of the biotinylated nucleotide-arm can mediate conjugation of the core to the biotinylated nucleotide-arm. A streptavidin-type or avidin-type core can be a tetrameric biotin-binding protein that can bind one, two, three or up to four biotinylated nucleotide-arms.

[0945] In some embodiments, the core can comprise a streptavidin-type or avidin-type moiety, including streptavidin or avidin protein, as well as any derivatives, analogs and other non-native forms of streptavidin or avidin that can bind to at least one biotin moiety. The streptavidin or avidin moiety can comprise native or recombinant forms, as well as mutant versions and derivatized molecules. Mutant versions of streptavidin and avidin can comprise any one or any combination of two or more of amino acid insertions, deletions, substitutions, or truncations. Mutant versions can also include fusion polypeptides. Many different forms of streptavidin and avidin are commercially available.

[0946] The multivalent molecules can be configured using a streptavidin or avidin core having a high affinity for the biotin moiety on a biotinylated nucleotide-arm to reduce dissociation of the nucleotide-arms from the core. A mixture of multivalent molecules can be prepared, where the mixture contains two or more sub-populations of multivalent molecules and each sub-population contains multivalent molecules having one type of nucleotide units (e.g., dATP, dGTP, dCTP, dTTP or dUTP). Multivalent molecules that are configured to have high affinity between the core and nucleotide-arms can reduce undesirable dissociation of nucleotide-arms from the core, and exchange of nucleotide arms between different cores. Exchange of nucleotide arms during a sequencing reaction can lead to incorrect based calling and reduced sequencing accuracy. In some embodiments, multivalent molecules having increased stability (e.g., reduced dissociation of biotinylated nucleotide-arms) can comprise a dye labeled streptavidin, where the streptavidin subunits carry a Lys121Arg mutation which can exhibit reduced dissociation of a biotinylated nucleotide-arm from the streptavidin core.

[0947] The streptavidin moiety can comprise full-length or truncated forms having a high affinity for binding biotin. For example, the streptavidin moiety can exhibit a dissociation constant (K_d) of about 10^{-14} mol/L, or about 10^{-15} mol/L. In some embodiments, the streptavidin moiety can comprise any amino acid substitution mutation at a site that can be labeled with a dye. For example, the dye-labeling site can comprise lysine at position 121 which may overlap

with a biotin binding site. In some embodiments, a dye attached to streptavidin at Lys121 may block or inhibit biotin binding to the dye-labeled streptavidin. A multivalent molecule comprising a dye labeled streptavidin carrying lysine at position 121 may exhibit dissociation of a biotinylated nucleotide-arm from the streptavidin core. A multivalent molecule having increased stability can comprise a dye labeled streptavidin carrying a Lys121Arg mutation which can exhibit reduced dissociation of a biotinylated nucleotide-arm from the streptavidin core.

[0948] In some embodiments, the streptavidin moiety can comprise any amino acid substitution that increases the affinity for binding biotin (e.g., increases the K_d to about 10^{-16} mol/L), improves retention of biotin at temperatures up to about 60 °C, or about 65 °C, or about 70 °C or about 80 °C, or a combination of increases the affinity for binding biotin and improves retention of biotin.

[0949] The avidin moiety can comprise full-length or truncated forms having a high affinity for binding biotin. For example, the avidin moiety can exhibit a dissociation constant (K_d) of about 10^{-14} mol/L, or about 10^{-15} mol/L. In some embodiments, the avidin can comprise substitutions of any one or any combination of the eight arginine residues. The avidin can comprise partially de-glycosylated forms and non-glycosylated forms. The avidin moiety can include derivatized forms, for example, N-acyl avidins, e.g., N-acetyl, N-phthalyl and N-succinyl avidin, and the commercially-available products including EXTRAVIDIN, CAPTAVIDIN (selective nitration of tyrosine residues at the four biotin-binding sites to generate avidin that reversibly binds biotin), NEUTRAVIDIN (having chemically de-glycosylated and include modified arginine residues), and NEUTRALITE AVIDIN (five of the eight arginine residues are replaced with neutral amino acids, two of the lysine residues are replaced with glutamic acid, and Asp17 is replaced with isoleucine). Amino acids having neutral non-polar side chains include alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline and valine. Amino acids having neutral polar side chains include asparagine, cysteine, glutamine, serine, threonine, tryptophan and tyrosine.

[0950] In some embodiments, the core can be labeled with a detectable reporter moiety. The core can be streptavidin or avidin which are homo-tetramers. Each subunit in the homo-tetramer can include at least one lysine residue which can be conjugated to a fluorophore. A labeling reaction can employ N-hydroxysuccinimide (NHS) ester-conjugated fluorophores. The maximum number of fluorophores that can be attached to a streptavidin or avidin subunit can be dictated by the number of lysine residues in the subunit.

[0951] When preparing labeled streptavidin or avidin cores, the labeling reaction can be optimized to achieve a predetermined degree of labeling (sometimes abbreviated as DoL). The

degree of labeling can be expressed as a molar ratio in the form of label/protein. Dye-core conjugates with a lower degree of labeling will exhibit weaker fluorescent intensities. Dye-core conjugates with very high degree of labeling (e.g., DoL > 6) may exhibit reduced fluorescence due to self-quenching from the conjugated fluorophore. In some embodiments, the predetermined degree of labeling for streptavidin or avidin cores may depend upon the dye. Fluorescent dyes include but are not limited to: CF647, CF680, CF570 and CF532 dyes from Biotium; AF647, AF680, AF568 and AF532 from Thermo Fisher Scientific; IFluor 647, IFluor 680, IFlour 568 and IFlour 532 from AATBio; DY648P1, DY679P1, DY585 and DY530 from Dyomics; and AFDy 647, IRFlour 680LT, AFDye 568 and AFDye 532 from Fluoroprobes. The predetermined degree of labeling can be about 1 – 10, or about 3 – 8, or about 3.5 – 7, or about 1.6 - 4.

[0952] Red fluorophores are brighter (higher intensity) than green dyes, which can cause color bleeding when imaging both red-labeled and green-labeled multivalent molecules on the same support (e.g., flow cell). The degree of labeling of a sub-population of multivalent molecules can be increased or decreased to achieve improved signal balance from a mixture of labeled multivalent molecules. For example, the degree of labeling of a sub-population of multivalent molecules labeled with a red fluorophore can be decreased compared to the degree of labeling of a sub-population of multivalent molecules labeled with a green fluorophore. In some embodiments, the degree of labeling of a sub-population of multivalent molecules labeled with a red fluorophore can be about 1-3, or about 2 – 3, or about 3 - 6. In some embodiments, the degree of labeling of a sub-population of multivalent molecules labeled with a green fluorophore can be about 4 - 7.

[0953] Solution fluorescence measurements can be used to determine the relative brightness of the labeled streptavidin or avidin cores. Alternatively, the degree of labeling can be determined by employing a functional assay (e.g., a flow cell trap assay) in which clonally-amplified template molecules immobilized on a flow cell are contacted with primers, polymerases and fluorescently-labeled multivalent molecules, under a condition suitable for binding the multivalent molecules to complexed polymerases without incorporating the nucleotide units into the primer, and signal intensity can be detected.

[0954] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise the same type of nucleotide units. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or

biotinylated nucleotide arms, where all of the attached arms have a nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

[0955] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise different types of nucleotide units. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where at least a first attached arm can have a first nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP, and a second attached arm can have a second nucleotide unit selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP, where the first and second nucleotide units are different.

[0956] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise the same type of spacer For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where all of the attached arms have the same spacer.

[0957] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise different types of spacers. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where at least a first attached arm can have a first type of spacer, and a second attached arm can have a second type of spacer, where the first and second spacer units are different. In some embodiments, the first and second type of linker can be selected from any of the spacers described herein.

[0958] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise the same type of linker. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where all of the attached arms have the same linker. In some embodiments, the linker can be selected from any of the linkers described herein (e.g., FIG. 81).

[0959] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core

attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise different types of linkers. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where at least a first attached arm can have a first type of linker, and a second attached arm can have a second type of linker, where the first and second linker units are different. In some embodiments, the first and second type of linker can be selected from any of the linkers described herein (e.g., FIG. 81).

[0960] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of e-arms. In some embodiments, the plurality of nucleotide-arms can comprise the same type of spacer and linker. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where all of the attached arms have the same spacer and linker. In some embodiments, the spacer and linker can be selected from any of the spacers and linkers described herein.

[0961] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise the same type of reactive group. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where all of the attached arms have the same reactive group. In some embodiments, the reactive group can comprise an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group.

[0962] In some embodiments, the reactive group in the linker can be reactive with a chemical reagent. For example, the reactive groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The reactive groups aryl and benzyl can be reactive with H2 Pd/C. The reactive groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The reactive group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The reactive groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0963] In some embodiments, the nucleotide-arms can have the same type of reactive group in the linker where the reactive group can comprise an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl group in the linker can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP),bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[0964] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise different types of reactive groups in the linkers. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where at least a first attached arm can have a first type of reactive group in a first linker unit, and a second attached arm can have a second type of reactive group in a second linker unit, where the first and second reactive groups are different.

[0965] In some embodiments, the first reactive group in the first linker unit, and the second reactive group in the second linker unit, can be selected in any combination from a group consisting of an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, and silyl group.

[0966] In some embodiments, the first and second reactive groups can be reactive with a chemical agent. For example, the reactive groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The reactive groups aryl and benzyl can be reactive with H2 Pd/C. The reactive groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The reactive group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The reactive groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0967] In some embodiments, the nucleotide-arms can have the different types of reactive groups in the linkers where the reactive group can comprise an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl group in the linker can be

reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[0968] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise a nucleotide unit with the same type of sugar 3'OH group. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where all of the attached arms have a nucleotide unit having the same type of sugar 3'OH group.

[0969] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise a nucleotide unit with the same type of sugar 3' blocking group (e.g., chain terminating moiety For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where all of the attached arms can have a nucleotide unit having the same type of sugar 3' blocking group. In some embodiments, the sugar 3' blocking group can comprise an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group. In some embodiments, the sugar 3' blocking group can comprise a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, or a 3'-O-benzyl group. In some embodiments, the sugar 3' blocking group can comprise an azide, azido or azidomethyl group.

[0970] In some embodiments, the sugar 3' blocking group can be reactive with a chemical reagent. For example, the sugar 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The sugar 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The sugar 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The sugar 3' blocking group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The sugar 3' blocking groups urea and silyl can be reactive with

tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0971] In some embodiments, the sugar 3' blocking group (e.g., azide, azido and azido methyl) can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[0972] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise a nucleotide unit with different sugar 3' blocking groups. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where at least a first attached arm can have a first nucleotide unit having a first 3' blocking group, and a second attached arm can have a second nucleotide unit having a second 3' blocking group, where the first and second 3' blocking groups are different.

[0973] In some embodiments, the first 3' blocking group in the first nucleotide unit, and the second 3' blocking group in the second nucleotide unit, can be selected in any combination from a group consisting of an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group. In some embodiments, the first 3' blocking group in the first nucleotide unit, and the second 3' blocking group in the second nucleotide unit, can be selected in any combination from a group consisting of an 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, or a 3'-O-benzyl group. In some embodiments, the first 3' blocking group in the first nucleotide unit, and the second 3' blocking group in the second nucleotide unit, can be selected in any combination from a group consisting of an azide, azido or azidomethyl group.

[0974] In some embodiments, the first and second 3' blocking groups can be reactive with a chemical reagent. For example, the 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The 3' blocking group carbonate can be reactive with potassium carbonate

(K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The 3' blocking groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0975] In some embodiments, the first and second 3' blocking groups (e.g., azide, azido and azido methyl) can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[0976] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can comprise a core attached to a plurality of nucleotide-arms. In some embodiments, the plurality of nucleotide-arms can comprise a nucleotide unit with a first sugar 3' OH blocking groups. In some embodiments, the plurality of nucleotide-arms can comprise a nucleotide unit with a second 3' OH blocking group. In some cases, the first and second 3' OH blocking groups can be different. For example, a multivalent molecule can comprise a core (e.g., streptavidin or avidin core) attached to a plurality of nucleotide arms or biotinylated nucleotide arms, where (a) at least a first arm can comprise a first nucleotide unit having a sugar moiety which includes a 3'OH group, (b) at least second arm can comprise a second nucleotide unit having a first 3' blocking group, and (c) at least third arm can comprise a third nucleotide unit having a second blocking group, wherein the first and second 3' blocking groups are different from each other.

[0977] In some embodiments, the first 3' blocking group in the first nucleotide unit, and the second 3' blocking group in the second nucleotide unit, can be selected in any combination from a group consisting of an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, or silyl group. In some embodiments, the first 3' blocking group in the first nucleotide unit, and the second 3' blocking group in the second nucleotide unit, can be selected in any combination from a group consisting of an 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, or a 3'-O-benzyl group. In some embodiments, the first 3' blocking group in the first nucleotide unit, and the second 3' blocking group in the second nucleotide unit, can be selected in any combination from a group consisting of an azide, azido or azidomethyl group.

[0978] In some embodiments, the first and second 3' blocking groups can be reactive with a chemical reagent. For example, the 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-

Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The 3' blocking group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The 3' blocking groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[0979] In some embodiments, the first and second 3' blocking groups (e.g., azide, azido and azido methyl) can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[0980] The present disclosure provides compositions, systems, methods, and kits comprising a multivalent molecule. In some embodiments, the multivalent molecule can have a core. In some embodiments, the core can be labeled with at least one detectable reporter moiety to form a labeled core. In some embodiments, a labeled core attached to two or more nucleotide-arms can comprise a labeled multivalent molecule. In some embodiments, a streptavidin or avidin core can be labeled with 1-6 or more reporter moieties. In some embodiments, the reporter moiety can comprise a fluorophore.

[0981] A mixture of multivalent molecules having different units in their nucleotide-arms, where distinction between the different multivalent molecules can be achieved. In some embodiments, the core of a first multivalent molecule can be labeled with a reporter moiety to distinguish it from a second labeled (or non-labeled) multivalent molecule. For example, a unit in a nucleotide-arm of the labeled first multivalent molecule can differ from a unit in a nucleotide-arm of a labeled second multivalent molecule. Any unit in the first multivalent molecule (e.g., spacer, linker, reactive group, nucleotide base, sugar 3'OH, 3' blocking group, or a combination thereof) can differ from a corresponding unit in the second multivalent molecule, where the first and second reporter moieties correspond to the differentiating unit. In some embodiments, the first and second reporter moieties can be spectrally distinguishable from each other.

[0982] In some embodiments, the core of a first multivalent molecule can be labeled with a first reporter moiety that corresponds to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) in the attached nucleotide-arms, and the core of a second multivalent molecule can be labeled with a second reporter moiety that corresponds to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP)

in the attached nucleotide-arms, where the base in the first multivalent molecule and the base in the second multivalent molecule are different. In some embodiments, the first and second reporter moieties are spectrally distinguishable from each other. In some embodiment, detection of the first reporter moiety indicates a binding event, an incorporation event, or a combination of binding and incorporation events of the first multivalent molecule having the first base, and detection of the second reporter moiety indicates a binding event, an incorporation event, or a combination of binding and incorporation events of the second multivalent molecule having the second base. The binding event can be a multivalent molecule binding to a complexed polymerase. The incorporation event can be a nucleotide unit incorporating into the terminal 3' end of an extendible primer in a complexed polymerase, where the nucleotide unit is part of a multivalent molecule.

Mixture of multivalent molecules

[0983] The present disclosure provides separate batches (sub-populations) of labeled multivalent molecules. In some embodiments, the separate batches of labeled multivalent molecules can be prepared using a different reporter moiety for each batch. In some embodiments, the different reporter moiety reporter moiety can correspond to a particular base in the nucleotide arms. A particular batch can be distinguishable from other batches based on the reporter moiety attached to the core. Two, three, four, five or more separate batches (sub-populations) can be mixed together to form a plurality of labeled multivalent molecules comprising two or more sub-populations of spectrally distinguishable multivalent molecules. In some embodiments, at least one batch of multivalent molecules in the mixture can be non-labeled (e.g., dark multivalent molecules).

[0984] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules which can comprise a mixture of at least two sub-populations of multivalent molecules labeled with different reporter moieties. In some embodiments, at least a first sub-population of multivalent molecules can be labeled with a first reporter moiety that corresponds to a first nucleotide unit on the nucleotide-arms. In some embodiments, at least a second sub-population of multivalent molecules can be labeled with a second reporter moiety that corresponds to a second nucleotide unit on the nucleotide-arms. In some cases, the first and second reporter moieties can differ from each other. In some embodiments, the plurality of multivalent molecules can further comprise at least a third sub-population of multivalent molecules which is labeled with a third reporter moiety, wherein the first, second and third reporter moieties can differ from each other. In some embodiments, the plurality of multivalent molecules can further comprise at least a fourth sub-population of multivalent molecules which is labeled with a fourth reporter moiety, wherein the first, second, third and fourth reporter

moieties can differ from each other. In some embodiments, additional sub-populations (e.g., fifth, sixth, seventh, eighth, nineth, tenth or more) of labeled multivalent molecules can be added into the mixture. In some embodiments, the reporter moiety can be a fluorophore. In some embodiments, a first sub-population of multivalent molecules can be labeled with a first fluorophore and a second fluorophore of multivalent molecules can be labeled with a second fluorophore. In some cases, the first fluorophore and the second fluorophore can be different.

[0985] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules which can comprise a mixture of at least two sub-populations of multivalent molecules labeled with different reporter moieties. In some embodiments, at least a first sub-population of multivalent molecules can be labeled with a first reporter moiety that corresponds to a first nucleotide unit on the nucleotide-arms. In some embodiments, at least a second sub-population of multivalent molecules can be non-labeled (e.g., a dark multivalent molecule).

[0986] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules which comprises a mixture of at least three sub-populations of multivalent molecules labeled with different reporter moieties. In some embodiments, at least a first sub-population of multivalent molecules can be labeled with a first reporter moiety that corresponds to a first nucleotide unit on the nucleotide-arms. In some embodiments, at least a second sub-population of multivalent molecules can be labeled with a second reporter moiety that corresponds to a second nucleotide unit on the nucleotide-arms. In some embodiments, at least a third sub-population of multivalent molecules can be non-labeled (e.g., a dark multivalent molecule). In some embodiments, the first and second reporter moieties can differ from each other.

[0987] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules which comprises a mixture of at least four sub-populations of multivalent molecules labeled with different reporter moieties. In some embodiments, the mixture of multivalent molecules can have at least a first sub-population of multivalent molecules can be labeled with a first reporter moiety that corresponds to a first nucleotide unit on the nucleotide-arms. In some embodiments, the mixture of multivalent molecules can have at least a second sub-population of multivalent molecules can be labeled with a second reporter moiety that corresponds to a second nucleotide unit on the nucleotide-arms. In some embodiments, the mixture of multivalent molecules can have at least a third sub-population of multivalent molecules is labeled with a third reporter moiety. In some embodiments, the mixture of multivalent molecules can have at least a fourth sub-population of multivalent molecules can

be non-labeled (e.g., a dark multivalent molecule). In some cases, the first, second and third reporter moieties can differ from each other.

[0988] An embodiment comprises: a mixture of four different types of multivalent molecules comprising (1) a first sub-population of multivalent molecules each comprising a dATP nucleotide unit and a core labeled with a first type of fluorophore, (2) a second sub-population of multivalent molecules each comprising a dGTP nucleotide unit and a core labeled with a second type of fluorophore, (3) a third sub-population of multivalent molecules each comprising a dCTP nucleotide unit and a core labeled with a third type of fluorophore, and (4) a fourth sub-population of multivalent molecules each comprising a dTTP nucleotide unit and a core labeled with a fourth type of fluorophore, where the first, second, third and fourth fluorophores can be spectrally distinguishable. In some embodiments, any one of the sub-populations of multivalent molecules can be non-labeled for use as "dark" multivalent molecules.

[0989] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a streptavidin or avidin core bound to 2-5 biotinylated nucleotide-arms.

[0990] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm having one type of nucleotide unit comprising dATP, dGTP, dCTP, dTTP or dUTP. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms, where the nucleotide-arms have one type of nucleotide unit comprising dATP, dGTP, dCTP, dTTP or dUTP. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms, where the biotinylated nucleotide-arms have one type of nucleotide unit comprising dATP, dGTP, dCTP, dTTP or dUTP.

[0991] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of two or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can have at least a first multivalent molecule in the plurality. In some cases, the at least the first multivalent molecule can comprise a core bound to at least one nucleotide-arm having a first type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP or dUTP. In some embodiments, the plurality of multivalent molecules can have at least a

second multivalent molecule. In some embodiments, the plurality of multivalent molecules can comprise at least a first multivalent molecule in the plurality and at least a second multivalent molecule. In some cases, the at least second multivalent molecule can comprise a core bound to at least one nucleotide-arm having a second type of nucleotide that differs from the first nucleotide in the first multivalent molecule. In some embodiments, the first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP or dUTP. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a second type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP or dUTP, where the first and second type of nucleotides are different. In some embodiments, the mixture can comprise two, three, four, five, or more different types of multivalent molecules having nucleotides selected in any combination from a group consisting of dATP, dGTP, dCTP, dTTP or dUTP.

[0992] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm. In some embodiments, the at least one nucleotide arm that are bound to a core can have the same spacer. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms.

[0993] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm. In some embodiments, the at least one nucleotide-arm that are bound to a core can have the same linker. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms.

[0994] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm. In some embodiments, all of the nucleotide arms that are bound to a core can have the same spacer and linker. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms.

[0995] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of two or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can comprise at least a first multivalent molecule in the plurality can comprise a core bound to at least one nucleotide-arm having a first type of spacer. In some embodiments, the plurality of multivalent molecules can comprise at least a second multivalent molecule can comprise a core bound to at least one nucleotide-arm having a second type of spacer. In some embodiments, the plurality of multivalent molecules can comprise a mixture of the at least the first multivalent molecule and the at least the second multivalent molecule. In some cases, the second type of spacer in the second multivalent molecule can differ from the first spacer in the first multivalent molecule. In some embodiments, the first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of spacer. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a second type of spacer, where the first and second type of spacers are different.

[0996] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of two or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can comprise at least a first multivalent molecule in the plurality comprises a core bound to at least one nucleotide-arm having a first type of linker. In some embodiments, the plurality of multivalent molecules can comprise at least a second multivalent molecule comprises a core bound to at least one nucleotide-arm having a second type of linker. In some embodiments, the plurality of multivalent molecules can comprise a mixture of the at least the first multivalent molecule and the at least the second multivalent molecule. In some cases, the second type of linker in the second multivalent molecule can differ from the first linker in the first multivalent molecule. In some embodiments, the first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of linker. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a second type of linker, where the first and second type of spacers are different.

[0997] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm. In some embodiments, all of the nucleotide arms that are bound to a core can have the same reactive group in the linker. In some embodiments, individual multivalent molecules in the plurality can comprise a core

bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms. In some embodiments, the reactive group can comprise alkyl, alkenyl, alkynyl, allyl, aryl, benzyl, azide, amine, amide, keto, isocyanate, phosphate, thio, disulfide, carbonate, urea, or silyl group. In some embodiments, the individual multivalent molecules can comprise a reactive group that can be reactive with a chemical agent. For example, the reactive groups alkyl, alkenyl, alkynyl and allyl are reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The reactive groups aryl and benzyl can be reactive with H2 Pd/C. The reactive groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The reactive group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The reactive groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride. In some embodiments, the reactive group can comprise an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl group in the linker can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[0998] The present disclosure provides compositions, systems and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of two or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can have at least a first multivalent molecule (a first subpopulation) in the plurality. In some embodiments, the at least the first subpopulation can comprise a core bound to at least one nucleotide-arm having a first type of reactive group in the linker. In some embodiments, the plurality of multivalent molecules can have at least a second multivalent molecule (a second subpopulation) comprises a core bound to at least one nucleotide-arm having a second type of reactive group in the linker. In some cases, the first reactive group in the first type of linker in the first sub-population differ from the second reactive group in the second type of linker in the second sub-population. In some embodiments, the first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of reactive group in the linker. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a second type

of reactive group in the linker, where the first reactive group differs from the second reactive group.

[0999] In some embodiments, the first and second reactive can be selected, in any combination, from a group consisting of alkyl, alkenyl, alkynyl, allyl, aryl, benzyl, azide, amine, amide, keto, isocyanate, phosphate, thio, disulfide, carbonate, urea, and silyl group. In some embodiments, the individual multivalent molecules can comprise a first or second reactive group that can be reactive with a chemical agent. For example, the reactive groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The reactive groups aryl and benzyl can be reactive with H2 Pd/C. The reactive groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The reactive group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The reactive groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride. In some embodiments, the first or second reactive can be selected, in any combination, from a group consisting of an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl reactive group in the linker can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[1000] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm, wherein all of the nucleotide arms that are bound to a core can have a nucleotide unit having the same sugar 3'OH group. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms.

[1001] The present disclosure provides compositions, systems, methods, and kits comprising a plurality (e.g., a population) of multivalent molecules, wherein individual multivalent molecules in the plurality can comprise a core bound to at least one nucleotide-arm, wherein all of the nucleotide arms that are bound to a core can have a nucleotide unit having the sugar 3'OH group substituted with the same 3' blocking group. In some embodiments, individual multivalent

molecules in the plurality can comprise a core bound to 2-5 nucleotide-arms. In some embodiments, individual multivalent molecules in the plurality can comprise a core bound to 2-5 biotinylated nucleotide-arms. In some embodiments, the sugar 3'blocking group can comprise alkyl, alkenyl, alkynyl, allyl, aryl, benzyl, azide, amine, amide, keto, isocyanate, phosphate, thio, disulfide, carbonate, urea, or silyl group. In some embodiments, the individual multivalent molecules can comprise a 3' blocking group that can be reactive with a chemical agent. For example, the 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The 3' blocking group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The 3' blocking groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride. In some embodiments, the 3' blocking group can comprise a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, or a 3'-O-benzyl group. In some embodiments, the 3' blocking group can comprise an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl 3' blocking group can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[1002] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of two or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can comprise at least a first multivalent molecule in the plurality can comprise a core bound to at least one nucleotide-arm having a first nucleotide unit with a first type of sugar 3'OH blocking group (chain terminating moiety). In some embodiments, the plurality of multivalent molecules can comprise at least a second multivalent molecule comprises a core bound to at least one nucleotide-arm having a second nucleotide unit having a second type of sugar 3' blocking group (chain terminating moiety). In some embodiments, the plurality can comprise the first multivalent molecule and the second multivalent molecule. In some cases, the first 3' blocking group can differ from the second 3' blocking group. In some embodiments, the

first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of 3' blocking group. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a second type of 3' blocking group, where the first 3' blocking group differs from the second 3' blocking group.

[1003] In some embodiments, the first and second 3' blocking group can be selected, in any combination, from a group consisting of alkyl, alkenyl, alkynyl, allyl, aryl, benzyl, azide, amine, amide, keto, isocyanate, phosphate, thio, disulfide, carbonate, urea, and silyl group. In some embodiments, the individual multivalent molecules can comprise a first or second 3' blocking group that can be reactive with a chemical agent. For example, the 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The 3' blocking group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The 3' blocking groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride. In some embodiments, the first and second 3' blocking group can be selected, in any combination, from a group consisting of a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, and a 3'-O-benzyl group. In some embodiments, the first or second 3' blocking group can be selected, in any combination, from a group consisting of an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl 3' blocking group is reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[1004] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of two or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can comprise at least a first multivalent molecule in the plurality can comprise a core bound to at least one nucleotide-arm having a first nucleotide unit with a sugar 3' OH group. In some embodiments, the plurality of multivalent molecules can comprise at least a second

multivalent molecule comprising a core bound to at least one nucleotide-arm having a second nucleotide unit having a first type of sugar 3' blocking group. In some embodiments, the plurality of multivalent molecules can comprise the first multivalent molecule and the second multivalent molecule. In some embodiments, the first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a sugar 3' OH group. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of 3' blocking group.

[1005] In some embodiments, the first 3' blocking group can be selected, in any combination, from a group consisting of alkyl, alkenyl, alkynyl, allyl, aryl, benzyl, azide, amine, amide, keto, isocyanate, phosphate, thio, disulfide, carbonate, urea, and silyl group. In some embodiments, the individual multivalent molecules can comprise a first 3' blocking group that can be reactive with a chemical agent. For example, the 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The 3' blocking group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The 3' blocking groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride. In some embodiments, the first 3' blocking group can be selected, in any combination, from a group consisting of a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, and a 3'-O-benzyl group. In some embodiments, the first 3' blocking group can be selected, in any combination, from a group consisting of an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl 3' blocking group can be reactive with a chemical agent. In some embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

[1006] The present disclosure provides compositions, systems, methods, and kits comprising a plurality of multivalent molecules comprising a mixture (sub-populations) of three or more different types of multivalent molecules. In some embodiments, the plurality of multivalent molecules can comprise at least a first multivalent molecule. In some embodiments, the at least

the first multivalent molecule can comprise a core bound to at least one nucleotide-arm having a first nucleotide unit with a sugar 3' OH group. In some embodiments, the plurality of multivalent molecules can comprise at least a second multivalent molecule. In some embodiments, the at least the second multivalent molecule can comprise a core bound to at least one nucleotide-arm having a second nucleotide unit having a first type of sugar 3' blocking group. In some embodiments, the plurality of multivalent molecules can comprise at least a third multivalent molecule. In some embodiments, the at least third multivalent molecule can comprise a core bound to at least one nucleotide-arm having a third nucleotide unit having a second type of sugar 3' blocking group. In some cases, the first and second 3' blocking groups are different. In some embodiments, the first multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a sugar 3' OH group. In some embodiments, the second multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a first type of 3' blocking group. In some embodiments, the third multivalent molecule can comprise a core bound to 2-5 biotinylated nucleotide arms, where the biotinylated-arms can have a second type of 3' blocking group.

[1007] In some embodiments, the first and second 3' blocking groups can be selected, in any combination, from a group consisting of alkyl, alkenyl, alkynyl, allyl, aryl, benzyl, azide, amine, amide, keto, isocyanate, phosphate, thio, disulfide, carbonate, urea, and silyl group. In some embodiments, the individual multivalent molecules can comprise a first or second 3' blocking group that can be reactive with a chemical agent. For example, the 3' blocking groups alkyl, alkenyl, alkynyl and allyl can be reactive with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). The 3' blocking groups aryl and benzyl can be reactive with H2 Pd/C. The 3' blocking groups amine, amide, keto, isocyanate, phosphate, thio, disulfide can be reactive with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). The 3' blocking group carbonate can be reactive with potassium carbonate (K_2CO_3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). The 3' blocking groups urea and silyl can be reactive with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride. In some embodiments, the first and second 3' blocking groups can be selected, in any combination, from a group consisting of a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, and a 3'-O-benzyl group. In some embodiments, the first and second 3' blocking groups can be selected, in any combination, from a group consisting of an azide, azido or azidomethyl group. In some embodiments, the azide, azido or azidomethyl 3' blocking group can be reactive with a chemical agent. In some

embodiments, the chemical agent can comprise a phosphine compound. In some embodiments, the phosphine compound can comprise a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound can comprise Tris(2-carboxyethyl)phosphine (TCEP), bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP).

Methods for sequencing using phosphate chain-labeled nucleotides

[1008] In some embodiments, the methods herein can be used for autofocusing of optical systems that can be used for sequencing using immobilized sequencing polymerases which bind non-immobilized template molecules. The present disclosure provides methods for sequencing using immobilized sequencing polymerases which bind non-immobilized template molecules, wherein the sequencing reactions are conducted with phosphate-chain labeled nucleotides. In some embodiments, the sequencing methods comprise an operation (a): providing a support having a plurality of sequencing polymerases immobilized thereon. In some embodiments, the sequencing polymerase comprises a processive DNA polymerase. In some embodiments, the sequencing polymerase comprises a wild type or mutant DNA polymerase, including, for example, a Phi29 DNA polymerase. In some embodiments, the support comprise a plurality of separate compartments and a sequencing polymerase is immobilized to the bottom of a compartment. In some embodiments, the separate compartments comprise a silica bottom through which light can penetrate. In some embodiments, the separate compartments comprise a silica bottom configured with a nanophotonic confinement structure comprising a hole in a metal cladding film (e.g., aluminum cladding film). In some embodiments, the hole in the metal cladding has a small aperture, for example, approximately 70 nm. In some embodiments, the height of the nanophotonic confinement structure is approximately 100 nm. In some embodiments, the nanophotonic confinement structure comprises a zero mode waveguide (ZMW). In some embodiments, the nanophotonic confinement structure contains a liquid.

[1009] In some embodiments, the sequencing method further comprises an operation (b): contacting the plurality of immobilized sequencing polymerases with a plurality of single stranded circular nucleic acid template molecules and a plurality of oligonucleotide sequencing primers, under a condition suitable for individual immobilized sequencing polymerases to bind a single stranded circular template molecule, and suitable for individual sequencing primers to hybridize to individual single stranded circular template molecules, thereby generating a plurality of polymerase/template/primer complexes. In some embodiments, the individual sequencing primers hybridize to a universal sequencing primer binding site on the single stranded circular template molecule.

[1010] In some embodiments, the sequencing method further comprises an operation (c): contacting the plurality of polymerase/template/primer complexes with a plurality of phosphate chain labeled nucleotides each comprising an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and a phosphate chain comprising 3-20 phosphate groups, where the terminal phosphate group is linked to a detectable reporter moiety (e.g., a fluorophore). The first, second and third phosphate groups can be referred to as alpha, beta and gamma phosphate groups. In some embodiments, a particular detectable reporter moiety which is attached to the terminal phosphate group corresponds to the nucleotide base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) to permit detection and identification of the nucleo-base. In some embodiments, the plurality of polymerase/template/primer complexes are contacted with the plurality of phosphate chain labeled nucleotides under a condition suitable for polymerase-catalyzed nucleotide incorporation. In some embodiments, the sequencing polymerases are capable of binding a complementary phosphate chain labeled nucleotide and incorporating the complementary nucleotide opposite a nucleotide in a template molecule. In some embodiments, the polymerase-catalyzed nucleotide incorporation reaction cleaves between the alpha and beta phosphate groups thereby releasing a multi-phosphate chain linked to a fluorophore.

[1011] In some embodiments, the sequencing method further comprises an operation (d): detecting the fluorescent signal emitted by the phosphate chain labeled nucleotide that is bound by the sequencing polymerase and incorporated into the terminal end of the sequencing primer. In some embodiments, the operation (d) further comprises identifying the phosphate chain labeled nucleotide that is bound by the sequencing polymerase and incorporated into the terminal end of the sequencing primer.

[1012] In some embodiments, the sequencing method further comprises an operation (e): repeating steps (c) – (d) at least once. In some embodiments, sequencing methods that employ phosphate chain labeled nucleotides can be conducted according to the methods described in U.S. Patent Nos. 7,170,050; 7,302,146; and/or 7,405,281, each of which is incorporated by reference in its entirety.

Analyte detection complexes comprising target barcode sequences for cell painting

[1013] Antibody bridge circle complexes and/or bipartite complexes with target barcodes for identifying cellular structures: The present disclosure provides compositions comprising a plurality analyte detection complexes wherein individual analyte detection complexes comprise a target barcode sequence (1200) that is distinguishable from other target barcode sequences of other analyte detection complexes in the plurality. In some embodiments, the plurality of analyte detection complexes comprises a plurality of antibody bridge circle complexes (1700) and/or a plurality of bipartite complexes (1800).

[1014] In some embodiments, the target barcode sequences (1200) in the analyte detection complexes can be designed to enable simultaneously detecting and identifying two or more cellular target analytes (e.g., cellular structures) by conducting a single sequencing cycle and employing multi-color imaging. In some embodiments, the target barcode sequences (1200) in the analyte detection complexes can be used for cell painting.

[1015] In some embodiments, the cellular target analyte(s) are located inside the sample(s) or on the membrane of the sample(s), wherein the target analyte(s) comprise at least one polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the target analyte(s) comprise at least one polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the target analyte(s) comprise at least one polypeptide, enzyme or lipid located in or on a cellular structure including without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

Antibody Bridge Circle Complexes

[1016] In some embodiments, the plurality of analyte detection complexes comprises a plurality of antibody bridge circle complexes (1700) wherein individual antibody bridge complexes comprise a primary antibody which binds a target analyte wherein the primary antibody is attached to a bridge circle complex (1600). In some embodiments, the bridge circle complex (1600) comprises a circularized barcoded oligonucleotide (1400) and a bridge oligonucleotide (1500) (e.g., FIGS. 109-113, 114-116).

[1017] In some embodiments, individual antibody bridge circle complexes (1700) comprise a covalently closed circular barcoded oligonucleotide (1400) which is hybridized to a bridge oligonucleotide (1500) wherein one end of the bridge oligonucleotide is attached to a primary antibody (e.g., FIGS. 109-113, 114-116).

[1018] In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise a single-stranded oligonucleotide. In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise DNA, RNA or chimeric DNA/RNA. In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise canonical nucleotides or nucleotide analogs, or a combination thereof. In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise at least one locked nucleic acid (LNA).

[1019] In some embodiments, the covalently closed circular barcoded oligonucleotide (1400) comprises any combination of: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof), (ii) a target barcode sequence (1200), (iii) a sample index sequence (1210), (iv) a batch barcode sequence (1220), and (v) a universal circularized region

(1300) that binds at least a portion of the bridge oligonucleotide (1500). In some embodiments, the universal circularized region (1300) can be sub-divided in sub-regions comprising a first sub-region (1310) and a second sub-region (1320) wherein the first sub-region (1310) binds a portion of the bridge oligonucleotide (1500), and the second sub-region (1320) binds a different portion of the same bridge oligonucleotide (1500). In some embodiments, the universal circularized region (1300) further comprises a compaction oligonucleotide binding site sequence (1315) (or a complementary sequence thereof) (e.g., FIGS. 109-113, 114-116). In some embodiments, the sample index sequence (1210) can be used to distinguish cellular samples from different sources, e.g., in a multiplex assay. In some embodiments, a batch barcode sequence (1220) can be used for batch sequencing.

[1020] In some embodiments, individual bridge oligonucleotides (1500) comprise a universal sequence region (1500) which binds a portion of the circularized barcoded oligonucleotide (1400) (e.g., FIGS. 109-113, 114-116).

[1021] A shown in FIG. 109A, in some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; and (iii) a universal circularized region (1300) that binds a universal sequence region of the bridge oligonucleotide (1500). In some embodiments, the bridge oligonucleotide (1500) comprises an oligonucleotide having a universal sequence region (1500) that binds the universal circularized region (1300) of a circularized barcoded oligonucleotide (1400).

[1022] A shown in FIG. 110A, in some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; and (iii) a universal circularized region (1300) that binds a universal sequence region of the bridge oligonucleotide (1500). In some embodiments, the bridge oligonucleotide (1500) comprises an oligonucleotide having a universal sequence region (1500) that binds the universal circularized region (1300) of a circularized barcoded oligonucleotide (1400), and a linker region (1505).

[1023] As shown in FIG. 111A, in some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; and (iii) a universal circularized region (1300) which can be sub-divided into two sub-regions, such as for example a first sub-region (1310) and a second sub-region (1320). In some embodiments, the first sub-region (1310) binds a portion of the bridge oligonucleotide (1520), and the second sub-region (1320) binds another portion of the bridge oligonucleotide (1510). In some embodiments, the universal sequence region (1500) of the bridge

oligonucleotide can be sub-divided into a first sub-region (1510) and a second sub-region (1520).

[1024] As shown in FIG. 112A, in some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; and (iii) a universal circularized region (1300) which can be sub-divided into two sub-regions, such as for example a first sub-region (1310) and a second sub-region (1320). In some embodiments, the first sub-region (1310) binds a portion of the bridge oligonucleotide (1520), and the second sub-region (1320) binds another portion of the bridge oligonucleotide (1510). In some embodiments, the bridge oligonucleotide (1500) comprises a linker region (1505) and a universal sequence region which can be sub-divided into a first sub-region (1510) and a second sub-region (1520).

[1025] As shown in FIG. 113A, in some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; (iii) a sample index sequence (1210); (iv) a batch barcode sequence (1220); (v) a universal circularized first sub-region (1310) which binds a portion of a bridge oligonucleotide (1500); (vi) a compaction oligonucleotide binding site (1315) (or complementary sequence thereof); and (vii) a universal circularized region second sub-region (1320) which binds another portion (1510) of the same bridge oligonucleotide. In some embodiments, the bridge oligonucleotide (1500) comprises a linker region (1505), first sub-region (1510) of a universal sequence region, a compaction oligonucleotide binding site (1515) (or complementary sequence thereof), and a second sub-region (1520) of the universal sequence region.

[1026] In some embodiments, the second antibody bridge circle complex (1700-2) comprises a second bridge circle complex (1600-2) attached to the second primary antibody. In some embodiments, in FIGS. 115A and 115B, the first and second target analytes are different target analytes. In some embodiments, in FIGS. 115A and 115B, the first and second primary antibodies comprise different primary antibodies. In some embodiments, in FIGS. 115A and 115B, the first and second bridge circle complexes ((1600-1) and (1600-2)) comprise different bridge circle complexes.

[1027] In some embodiments, the first antibody bridge circle complex (1700-1) comprises a first primary antibody having an antigen binding site that binds a first epitope of the target analyte. In some embodiments, the first antibody bridge circle complex (1700-1) comprises a first bridge circle complex (1600-1) attached to the first primary antibody. In some embodiments, the second antibody bridge circle complex (1700-2) comprises a second primary antibody having an

antigen binding site that binds a different epitope of the same target analyte. In some embodiments, the second antibody bridge circle complex (1700-2) comprises a second bridge circle complex (1600-2) attached to the second primary antibody. In some embodiments, the first and second primary antibodies comprise different primary antibodies. In some embodiments, the first and second bridge circle complexes ((1600-1) and (1600-2)) comprise different bridge circle complexes.

[1028] In some embodiments, individual bridge oligonucleotides (1500) comprise single-stranded oligonucleotides. In some embodiments, individual bridge oligonucleotides (1500) comprise DNA, RNA or chimeric DNA/RNA. In some embodiments, individual bridge oligonucleotides (1500) comprise canonical nucleotides or nucleotide analogs, or a combination thereof. In some embodiments, individual bridge oligonucleotides (1500) comprise at least one locked nucleic acid (LNA). In some embodiments, the universal sequence region (1500) can be 5-100 nucleotides in length, or any range therebetween. In some embodiments, the universal sequence region (1500) can be 15-75 nucleotides in length, or any range therebetween.

[1029] In some embodiments, one end of the bridge oligonucleotide (1500) is attached to a primary antibody (e.g., FIGS. 109-113, 114-116).

[1030] In some embodiments, the universal sequence region (1500) of the bridge oligonucleotide comprises a first sub-region (1510) and a second sub-region (1520). In some embodiments, the first sub-region (1510) binds the second sub-region of the universal circularized region (1320). In some embodiments, the second sub-region (1520) binds the first sub-region of the universal circularized region (1310) (e.g., FIGS. 111-113).

[1031] In some embodiments, the bridge oligonucleotides (1500) comprise an optional compaction oligonucleotide binding region (1515), or a complementary sequence thereof (e.g., FIG. 113A and 113B). In some embodiments, the compaction oligonucleotide binding region (1515) can be located between the first sub-region (1510) and the second sub-region (1520).

[1032] In some embodiments, the bridge oligonucleotides (1500) comprise a 3′ OH extendible end, or a 3′ non-extendible end that can be converted into a 3′ OH extendible end. In some embodiments, the bridge oligonucleotides (1500) comprise a 5′ end that inhibits ligation.

[1033] In some embodiments, the bridge oligonucleotides (1500) comprise one or more phosphorothioate linkage(s) at their 5′ and/or 3′ ends, for example to confer exonuclease resistance. In some embodiments, the bridge oligonucleotides (1500) comprise one or more phosphorothioate linkage(s) at an internal position, for example to confer endonuclease resistance. In some embodiments, the bridge oligonucleotides (1500) comprise one or more 2'-O-methylcytosine bases at their 5′ and/or 3′ ends. In some embodiments, the bridge oligonucleotides (1500) comprise one or more 2'-O-methylcytosine bases at an internal position.

In some embodiments, the 5′ end of individual bridge oligonucleotides (1500) is phosphorylated. In some embodiments, the 5′ end of individual bridge oligonucleotides (1500) is non-phosphorylated. In some embodiments, the 3′ end of individual bridge oligonucleotides (1500) comprises a terminal 3′ OH group or a terminal 3′ blocking group.

[1034] In some embodiments, individual bridge oligonucleotides (1500) comprise an optional linker region (1505). In some embodiments, the optional linker region (1505) is located at one end of the bridge oligonucleotide (e.g., FIGS. 110, 112 and 113). In some embodiments, the linker region (1505) is attached to an antibody (e.g., FIGS. 110, 112 and 113). In some embodiments, the linker region (1505) comprises a polynucleotide having a sequence that does not hybridize to any portion of the circularized barcoded oligonucleotide (1400). In some embodiments, the linker region (1505) comprises a spacer, for example an 18-carbon spacer (e.g., a hexa-ethylene glycol spacer), one or more C3 spacer phosphoramidites, or a spacer 9 comprising a trimethylene glycol spacer. In some embodiments, the linker region (1505) comprises a polyethylene glycol moiety, including, but not limited to, a PEG2, a PEG3 or a PEG4 spacer.

[1035] In some embodiments, the plurality of analyte detection complexes (e.g., a set of analyte detection complexes) comprises at least a first and second sub-population of antibody bridge circle complexes (e.g., FIGS. 115A and 115B). In some embodiments, individual antibody bridge circle complexes in the first sub-population (1700-1) comprise a first target barcode sequence (1200-1) that corresponds to a first target analyte. In some embodiments, the first target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the first target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1036] In some embodiments, individual antibody bridge circle complexes in the second sub-population (1700-2) comprise a second target barcode sequence (1200-2) that corresponds to a second target analyte, wherein the first and second target barcode sequences are different, and wherein the first and second target analytes are different target analytes. In some embodiments, the second target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the second target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1037] In some embodiments, the first and second sub-population of antibody bridge circle complexes ((1700-1) and (1700-2)) can be employed to detect and identify their cognate target analytes. In some embodiments, the plurality of antibody bridge circle complexes ((1700-1) and (1700-2)) can be co-located inside the sample(s) and can bind their cognate target analytes to form a plurality of analyte-complexes inside the sample(s), the analyte-complexes can be subjected to rolling circle amplification reaction to generate a plurality of concatemer molecules comprising first and second sub-populations of concatemer molecules. In some embodiments,

individual concatemer molecules in the first and second sub-population comprise a plurality of tandem copies of the first or second target barcode sequences ((1200-1) or (1200-2)). In some embodiments, the target barcode regions of the concatemer molecules in the first and second sub-populations can be sequenced to detect and identify the target analytes.

[1038] In a set of at least two different target barcode sequences (1200), which are part of a set of at least two different antibody bridge circle complexes (1700) that bind their cognate first or second target analytes, the sequences of the target barcodes can be designed so that sequencing the same corresponding nucleo-base position of all of the target barcodes in the set, a first target barcode in the set generates a first color signal in one particular sequencing cycle that is distinguishable from a second color signal of all of the other target barcodes in the same sequencing cycle, wherein the first color signal of the first target barcode identifies the first target analyte (e.g., see the table at FIG. 121). In some embodiments, the first and second target barcodes can be sequenced essentially simultaneously or can be sequenced sequentially (e.g., sequenced in separate batches) to detect and identify the target analytes. In some embodiments, only a portion of the target barcodes in the set need to be sequenced in order to identify the first and second target analytes (e.g., FIG. 121).

[1039] In some embodiments, the set comprises 2-10 different antibody bridge circle complexes each comprising a different target barcode sequence, or the set comprises 10-50 different antibody bridge circle complexes each comprising a different target barcode sequence, or the set comprises 50-100 different antibody bridge circle complexes each comprising a different target barcode sequence, or the set comprises 100-500 different antibody bridge circle complexes each comprising a different target barcode sequence, or the set comprises 500-1,000 different antibody bridge circle complexes each comprising a different target barcode sequence, or the set comprises 1,000-5,000 different antibody bridge circle complexes each comprising a different target barcode sequence, or the set comprises more than 5,000 different antibody bridge circle complexes each comprising a different target barcode sequence.

Bipartite complexes

[1040] In some embodiments, the plurality of analyte detection complexes comprises a plurality of bipartite complexes (1800) wherein individual bipartite complexes comprise a primary antibody which can bind a target analyte and the primary antibody is bound to a secondary antibody. In some embodiments, the secondary antibody is attached to a bridge circle complex (1600). In some embodiments, the bridge circle complex (1600) comprises a circularized barcoded oligonucleotide (1400) and a bridge oligonucleotide (1500) (e.g., FIGS. 117-122).

[1041] In some embodiments, the primary antibody can bind a target analyte. In some embodiments, the bridge circle complex (1600) comprises a circularized barcoded

oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500). In some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; and (iii) a universal circularized region (1300) that binds a universal sequence region of the bridge oligonucleotide (1500). In some embodiments, the bridge oligonucleotide (1500) comprises an oligonucleotide having a universal sequence region (1500) that binds the universal circularized region (1300) of a circularized barcoded oligonucleotide (1400).

[1042] In some embodiments, individual bipartite complexes (1800) comprise a covalently closed circular barcoded oligonucleotide (1400) which is hybridized to a bridge oligonucleotide (1500) wherein one end of the bridge oligonucleotide is attached to a secondary antibody wherein the secondary antibody is bound to a primary antibody (e.g., FIGS. 117-122).

[1043] In some embodiments, individual bridge oligonucleotides (1500) comprise a universal sequence region (1500) which binds a portion of the circularized barcoded oligonucleotide (1400) (e.g., FIGS. 117-112).

[1044] In some embodiments, e.g., as shown in FIG. 117, individual covalently closed circular barcoded oligonucleotides (1400) comprise a single-stranded oligonucleotide. In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise DNA, RNA or chimeric DNA/RNA. In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise canonical nucleotides or nucleotide analogs, or a combination thereof. In some embodiments, individual covalently closed circular barcoded oligonucleotides (1400) comprise at least one locked nucleic acid (LNA).

[1045] In some embodiments, the covalently closed circular barcoded oligonucleotide (1400) comprises any combination of: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof), (ii) a target barcode sequence (1200), (iii) a sample index sequence (1210), (iv) a batch barcode sequence (1220), and (v) a universal circularized region (1300) that binds at least a portion of the bridge oligonucleotide (1500). In some embodiments, the universal circularized region (1300) can be sub-divided in sub-regions comprising a first sub-region (1310) and a second sub-region (1320) wherein the first sub-region (1310) binds a portion of the bridge oligonucleotide (1500), and the second sub-region (1320) binds a different portion of the same bridge oligonucleotide (1500). In some embodiments, the universal circularized region (1300) further comprises a compaction oligonucleotide binding site sequence (1315) (or a complementary sequence thereof) (e.g., FIGS. 117-122). In some embodiments, the sample index sequence (1210) can be used to distinguish cellular samples from different

sources, e.g., in a multiplex assay. In some embodiments, a batch barcode sequence (1220) can be used for batch sequencing.

[1046] In some embodiments, e.g. as shown in FIG. 118, the primary antibody can bind a target analyte. In some embodiments, the bridge circle complex (1600) comprises a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500). In some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; and (iii) a universal circularized region (1300) which can be sub-divided into two sub-regions, such as for example a first sub-region (1310) and a second sub-region (1320). In some embodiments, the first sub-region (1310) binds a portion of the bridge oligonucleotide (1520), and the second sub-region (1320) binds another portion of the bridge oligonucleotide (1510). In some embodiments, the universal sequence region (1500) of the bridge oligonucleotide can be sub-divided into a first sub-region (1510) and a second sub-region (1520).

[1047] In some embodiments, e.g., as shown in FIG. 119, the bridge circle complex (1600) comprises a circularized barcoded oligonucleotide (1400) hybridized to a linear bridge oligonucleotide (1500). In some embodiments, the circularized barcoded oligonucleotide (1400) comprises: (i) a sequencing primer binding site sequence (1100) (or a complementary sequence thereof); (ii) a target barcode sequence (1200) that corresponds to a target analyte; (iii) a sample index sequence (1210); (iv) a batch barcode sequence (1220); (v) a universal circularized first sub-region (1310) which binds a portion of a bridge oligonucleotide (1500); (vi) a compaction oligonucleotide binding site (1315) (or complementary sequence thereof); and (vii) a universal circularized region second sub-region (1320) which binds another portion (1510) of the same bridge oligonucleotide. In some embodiments, the bridge oligonucleotide (1500) comprises a linker region (1505), first sub-region (1510) of a universal sequence region, a compaction oligonucleotide binding site (1515) (or complementary sequence thereof), and a second sub-region (1520) of the universal sequence region.

[1048] In some embodiments, e.g., as shown in FIG. 120A, the first primary antibody can bind a first target analyte. In some embodiments, the first bridge circle complex (1600-1) comprises a first circularized barcoded oligonucleotide (1400-1) hybridized to a first bridge oligonucleotide (1500-1). In some embodiments, the first circularized barcoded oligonucleotide (1400-1) comprises: (i) a first sequencing primer binding site sequence (1100-1) (or a complementary sequence thereof); (ii) a first target barcode sequence (1200-1) that corresponds to the first target analyte; and (iii) a first universal circularized region (1300-1) that binds a universal sequence region of the first bridge oligonucleotide (1500-1). In some embodiments, the first bridge

oligonucleotide (1500-1) comprises an oligonucleotide having a first universal sequence region (1500-1) that binds the first universal circularized region (1300-1) of the first circularized barcoded oligonucleotide (1400-1).

[1049] In some embodiments, e.g., as shown in FIG. 120B, the second bridge circle complex (1600-2) comprises a second circularized barcoded oligonucleotide (1400-2) hybridized to a second bridge oligonucleotide (1500-2). In some embodiments, the second circularized barcoded oligonucleotide (1400-2) comprises: (i) a second sequencing primer binding site sequence (1100-2) (or a complementary sequence thereof); (ii) a second target barcode sequence (1200-2) that corresponds to the second target analyte; and (iii) a second universal circularized region (1300-2) that binds a universal sequence region of the second bridge oligonucleotide (1500-2). In some embodiments, the second bridge oligonucleotide (1500-2) comprises an oligonucleotide having a second universal sequence region (1500-2) that binds the second universal circularized region (1300-2) of the second circularized barcoded oligonucleotide (1400-2). In some embodiments, in FIGS. 120A and 120B, the first and second target analytes are different target analytes. In some embodiments, in FIGS. 120A and 120B, the first and second primary antibodies comprise different primary antibodies. In some embodiments, in FIGS. 120A and 120B, the first and second bridge circle complexes ((1600-1) and (1600-2)) comprise different bridge circle complexes. In some embodiments, in FIGS. 120A and 120B, the first and second secondary antibodies comprise the same type of secondary antibody or different types of secondary antibodies.

[1050] In some embodiments, individual bridge oligonucleotides (1500) comprise single-stranded oligonucleotides. In some embodiments, individual bridge oligonucleotides (1500) comprise DNA, RNA or chimeric DNA/RNA. In some embodiments, individual bridge oligonucleotides (1500) comprise canonical nucleotides or nucleotide analogs, or a combination thereof. In some embodiments, individual bridge oligonucleotides (1500) comprise at least one locked nucleic acid (LNA). In some embodiments, the universal sequence region (1500) can be 5-100 nucleotides in length, or any range therebetween. In some embodiments, the universal sequence region (1500) can be 15-75 nucleotides in length, or any range therebetween.

[1051] In some embodiments, one end of the bridge oligonucleotide (1500) is attached to a secondary antibody (e.g., FIGS. 117-122).

[1052] In some embodiments, the universal sequence region (1500) of the bridge oligonucleotide comprises a first sub-region (1510) and a second sub-region (1520). In some embodiments, the first sub-region (1510) binds the second sub-region of the universal circularized region (1320). In some embodiments, the second sub-region (1520) binds the first sub-region of the universal circularized region (1310) (e.g., FIGS. 118-119).

[1053] In some embodiments, the bridge oligonucleotides (1500) comprise an optional compaction oligonucleotide binding region (1515), or a complementary sequence thereof (e.g., FIG. 119). In some embodiments, the compaction oligonucleotide binding region (1515) can be located between the first sub-region (1510) and the second sub-region (1520).

[1054] In some embodiments, the bridge oligonucleotides (1500) comprise a 3′ OH extendible end, or a 3′ non-extendible end that can be converted into a 3′ OH extendible end. In some embodiments, the bridge oligonucleotides (1500) comprise a 5′ end that inhibits ligation.

[1055] In some embodiments, the bridge oligonucleotides (1500) comprise one or more phosphorothioate linkage(s) at their 5′ and/or 3′ ends, for example to confer exonuclease resistance. In some embodiments, the bridge oligonucleotides (1500) comprise one or more phosphorothioate linkage(s) at an internal position, for example to confer endonuclease resistance. In some embodiments, the bridge oligonucleotides (1500) comprise one or more 2'-O-methylcytosine bases at their 5′ and/or 3′ ends. In some embodiments, the bridge oligonucleotides (1500) comprise one or more 2'-O-methylcytosine bases at an internal position. In some embodiments, the 5′ end of individual bridge oligonucleotides (1500) is phosphorylated. In some embodiments, the 5′ end of individual bridge oligonucleotides (1500) is non-phosphorylated. In some embodiments, the 3′ end of individual bridge oligonucleotides (1500) comprises a terminal 3′ OH group or a terminal 3′ blocking group.

[1056] In some embodiments, individual bridge oligonucleotides (1500) comprise an optional linker region (1505). In some embodiments, the optional linker region (1505) is located at one end of the bridge oligonucleotide (e.g., FIG. 119). In some embodiments, the linker region (1505) is attached to an antibody (e.g., FIG. 119). In some embodiments, the linker region (1505) comprises a polynucleotide having a sequence that does not hybridize to any portion of the circularized barcoded oligonucleotide (1400). In some embodiments, the linker region (1505) comprises a spacer, for example an 18-carbon spacer (e.g., a hexa-ethylene glycol spacer), one or more C3 spacer phosphoramidites, or a spacer 9 comprising a trimethylene glycol spacer. In some embodiments, the linker region (1505) comprises a polyethylene glycol moiety, including, but not limited to, a PEG2, a PEG3 or a PEG4 spacer.

[1057] In some embodiments, the plurality of analyte detection complexes (e.g., a set of analyte detection complexes) comprises at least a first and second sub-population of bipartite complexes (e.g., FIGS. 120A and 120B). In some embodiments, individual bipartite complexes in the first sub-population (1800-1) comprise a first target barcode sequence (1200-1) that corresponds to a first target analyte. In some embodiments, the first target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the first target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1058] In some embodiments, individual bipartite complexes in the second sub-population (1800-2) comprise a second target barcode sequence (1200-2) that corresponds to a second target analyte, wherein the first and second target barcode sequences are different, and wherein the first and second target analytes are different target analytes. In some embodiments, the second target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the second target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1059] In some embodiments, the first and second sub-population of bipartite complexes ((1800-1) and (1800-2)) can be employed to detect and identify their cognate target analytes. In some embodiments, the plurality of bipartite complexes ((1800-1) and (1800-2)) can be co-located inside the sample(s) and can bind their cognate target analytes to form a plurality of analyte-complexes inside the sample(s), the analyte-complexes can be subjected to rolling circle amplification reaction to generate a plurality of concatemer molecules comprising first and second sub-populations of concatemer molecules. In some embodiments, individual concatemer molecules in the first and second sub-population comprise a plurality of tandem copies of the first or second target barcode sequences ((1200-1) or (1200-2)). In some embodiments, the target barcode regions of the concatemer molecules in the first and second sub-populations can be sequenced to detect and identify the target analytes.

[1060] In a set of at least two different target barcode sequences (1200), which are part of a set of at least two different bipartite complexes (1800) that bind their cognate first or second target analytes, the sequences of the target barcodes can be designed so that sequencing the same corresponding nucleo-base position of all of the target barcodes in the set, a first target barcode in the set generates a first color signal in one particular sequencing cycle that is distinguishable from a second color signal of all of the other target barcodes in the same sequencing cycle, wherein the first color signal of the first target barcode identifies the first target analyte (e.g., see the table at FIG. 121). In some embodiments, the first and second target barcodes can be sequenced essentially simultaneously or can be sequenced sequentially (e.g., sequenced in separate batches) to detect and identify the target analytes. In some embodiments, only a portion of the target barcodes in the set need to be sequenced in order to identify the first and second target analytes (e.g., FIG. 121).

[1061] In some embodiments, the set comprises 2-10 different bipartite complexes each comprising a different target barcode sequence, or the set comprises 10-50 different bipartite complexes each comprising a different target barcode sequence, or the set comprises 50-100 different bipartite complexes each comprising a different target barcode sequence, or the set comprises 100-500 different bipartite complexes each comprising a different target barcode sequence, or the set comprises 500-1,000 different bipartite complexes each comprising a

different target barcode sequence, or the set comprises 1,000-5,000 different bipartite complexes each comprising a different target barcode sequence, or the set comprises more than 5,000 different bipartite complexes each comprising a different target barcode sequence.

A set of at least two different target barcodes for cell painting

[1062] The present disclosure provides compositions comprising a plurality analyte detection complexes wherein individual analyte detection complexes comprise a target barcode sequence (1200) that can be used for cell painting. In some embodiments, the plurality of analyte detection complexes comprises a plurality of antibody bridge circle complexes (1700) and/or a plurality of bipartite complexes (1800). Embodiments of antibody bridge circle complexes (1700) are shown in FIGS. 109-113, 114-116. Embodiments of bipartite complexes are shown in FIGS. 117-120.

[1063] In some embodiments, the target barcode sequence (1200) can be carried on a linear oligonucleotide or can be carried on a circularized oligonucleotide (1400).

[1064] In some embodiments, the plurality of analyte detection complexes comprise at least a first and second sub-population of analyte detection complexes (e.g., a set comprising at least a first and second sub-population of oligonucleotides), (a) wherein individual oligonucleotides in the first sub-population comprise a first target barcode sequence which corresponds to a first target analyte and wherein the first target barcode is at least 2 nucleotides in length, wherein individual oligonucleotides in the second sub-population comprise a second target barcode sequence which corresponds to a second target analyte and wherein the second target barcode is at least 2 nucleotides in length, and wherein the first and second target barcode sequences comprise different sequences; (b) wherein one nucleo-base in a first position in the first target barcode sequence comprises a nucleo-base that generates a first color signal in a first sequencing cycle, and wherein one nucleo-base in a corresponding first position in the second target barcode sequence comprises a nucleo-base that generates a second color signal in the same first sequencing cycle; (c) wherein one nucleo-base in a second position in the first target barcode sequence comprise a nucleo-base that generates the second color signal in a second sequencing cycle, and wherein one nucleo-base in a corresponding second position in the second target barcode sequence comprises a nucleo-base that generates the first color signal in the same second sequencing cycle; and (d) wherein the first color signal in the first corresponding position of the first and second target barcode sequences in the first sequencing cycle identifies the first target analyte, and wherein the first color signal in the second corresponding position of the first and second target barcode sequences in the second sequencing cycle identifies the second target analyte (e.g., see the table at FIG. 121).

A set of at least three different target barcodes for cell painting

[1065] The present disclosure provides compositions comprising a plurality analyte detection complexes wherein individual analyte detection complexes comprise a target barcode sequence (1200) that can be used for cell painting. In some embodiments, the plurality of analyte detection complexes comprises a plurality of antibody bridge circle complexes (1700) and/or a plurality of bipartite complexes (1800). Embodiments of antibody bridge circle complexes (1700) are shown in FIGS. 109-113, 114-116. Embodiments of bipartite complexes are shown in FIGS. 117-122.

[1066] In some embodiments, the target barcode sequence (1200) can be carried on a linear oligonucleotide or can be carried on a circularized oligonucleotide (1400).

[1067] In some embodiments, the plurality of analyte detection complexes comprise at least a first, second and third sub-population of analyte detection complexes (e.g., a set comprising at least a first, second and third sub-population of oligonucleotides), (a) wherein individual oligonucleotides in the first sub-population comprise a first target barcode sequence (1200-1) which corresponds to a first target analyte and wherein the first target barcode is at least 3 nucleotides in length, wherein individual oligonucleotides in the second sub-population comprise a second target barcode sequence (1200-2) which corresponds to a second target analyte and wherein the second target barcode is at least 3 nucleotides in length, wherein individual oligonucleotides in the third sub-population comprise a third target barcode sequence (1200-3) which corresponds to a third target analyte and wherein the third target barcode is at least 3 nucleotides in length, and wherein the first, second and third target barcode sequences comprise different sequences; (b) wherein one nucleo-base in a first position in the first target barcode sequence comprises a nucleo-base that generates a first color signal in a first sequencing cycle, and wherein one nucleo-base in a corresponding first position in the second and third target barcode sequences comprise a nucleo-base that generates a second color signal in the same first sequencing cycle; (c) wherein one nucleo-base in a second position in the first and third target barcode sequences comprise a nucleo-base that generates the second color signal in a second sequencing cycle, and wherein one nucleo-base in a corresponding second position in the second target barcode sequence comprise a nucleo-base that generates the first color signal in the same second sequencing cycle; (d) wherein one nucleo-base in a third position in the first and second target barcode sequences comprise a nucleo-base that generates the second color signal in a third sequencing cycle, and wherein one nucleo-base in a corresponding third position in the third target barcode sequence comprise a nucleo-base that generates the first color signal in the same third sequencing cycle; and (e) wherein the first color signal in the first corresponding position of the first, second and third target barcode sequences in the first sequencing cycle identifies the first target analyte, wherein the first color signal in the second corresponding position of the first, second and third target barcode sequences in the second

sequencing cycle identifies the second target analyte, and wherein the first color signal in the third corresponding position of the first, second and third target barcode sequences in the third sequencing cycle identifies the third target analyte (e.g., see the table at FIG. 121).

Methods for detecting and identifying target analytes using barcoded analyte detection complexes – cell painting

[1068] The present disclosure provides methods for simultaneously detecting and identifying two or more cellular target analytes (e.g., cellular structures) by conducting a single sequencing cycle and employing multi-color imaging. In some embodiments, the target barcode sequences (1200) in the analyte detection complexes can be used for cell painting.

[1069] The present disclosure provides methods for detecting and identifying target analytes comprising sequencing a plurality of barcoded concatemer molecules inside a cellular sample, wherein individual barcoded concatemer molecules in the plurality comprise tandem repeat polynucleotide units wherein each polynucleotide unit comprises a sequencing primer binding site (1100) and a target barcode sequence (1200) (or complementary sequences thereof), and wherein the target barcode sequences correspond to their cognate target analytes. In some embodiments, the plurality of barcoded concatemer molecules can be generated by conducting rolling circle amplification on covalently closed circular barcoded oligonucleotides. For example, the plurality of barcoded concatemer molecules can be generated by conducting rolling circle amplification on a plurality of analyte detection complexes comprising a plurality of antibody bridge circle complexes (1700) and/or a plurality of bipartite complexes (1800). In some embodiments, individual barcoded concatemers correspond to a target analyte. In some embodiments, the target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the target analyte comprises a polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the target analyte comprises a polypeptide, enzyme or lipid located in or on a cellular structure including without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

[1070] In some embodiments, the sample(s) comprises a plurality of barcoded concatemer molecules located inside the sample(s), including at least a first and second sub-population of barcoded concatemer molecules. In some embodiments, the sample(s) comprises additional sub-populations of barcoded concatemer molecules, for example 3-5,000 sub-populations of barcoded concatemer molecules.

[1071] In some embodiments, individual concatemers in the first sub-population comprise multiple copies of a sequencing primer binding site and a first target barcode sequence (1200-1) (or a complementary sequence thereof). In some embodiments, the plurality of concatemers in the first sub-population correspond to a plurality of first target analytes. In some embodiments, the first target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the first target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1072] In some embodiments, individual concatemers in the second sub-population comprise multiple copies of a sequencing primer binding site and a second target barcode sequence (1200-2) (or a complementary sequence thereof), wherein the first and second target barcode sequences are distinguishable from each other. In some embodiments, the barcoded concatemer molecules in the first and second sub-populations comprise sequencing primer binding site sequences that are the same or different sequences. In some embodiments, the plurality of concatemers in the second sub-population correspond to a plurality of second target analytes. In some embodiments, the second target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the second target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1073] In some embodiments, the sample(s) comprises a third sub-population of barcoded concatemer molecules, wherein individual concatemers in the third sub-population comprise multiple copies of a sequencing primer binding site and a third target barcode sequence (1200-3) (or a complementary sequence thereof), wherein the first, second and third target barcode sequences are distinguishable from each other. In some embodiments, the barcoded concatemer molecules in the first, second and third sub-populations comprise sequencing primer binding site sequences that are the same or different sequences. In some embodiments, the plurality of concatemers in the third sub-population correspond to a plurality of third target analytes. In some embodiments, the third target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the third target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide.

[1074] In some embodiments, the sample(s) comprises additional sub-populations of barcoded concatemer molecules wherein the barcoded concatemer molecules in each sub-population comprise a target barcode sequence that is distinguishable from the target barcode sequence in other sub-populations of barcoded concatemer molecules. In some embodiments, the barcoded concatemer molecules in the additional sub-populations comprise sequencing primer binding site sequences that are the same or different sequences. In some embodiments, the barcoded concatemer molecules in each sub-population corresponds to their cognate target analyte. In some embodiments, the sample(s) comprises 2-10 sub-populations of barcoded concatemer

molecules, or 10-50 sub-populations of barcoded concatemer molecules, or 50-100 sub-populations of barcoded concatemer molecules, or 100-500 sub-populations of barcoded concatemer molecules, or 500-1,000 sub-populations of barcoded concatemer molecules, or 1,000-5,000 sub-populations of barcoded concatemer molecules, or more than 5,000 sub-populations of barcoded concatemer molecules.

[1075] In some embodiments, in a single sequencing cycle, simultaneously sequencing two or more sub-populations of barcoded concatemer molecules located inside a cellular sample, and imaging the multi-color signals generated by the sequencing, can be used for simultaneously identifying two or more cellular target analytes. In some embodiments, the simultaneously sequencing and multi-color imaging can be used for cell painting.

[1076] The present disclosure provides methods for detecting and identifying target analytes comprises step (a): sequencing a plurality of barcoded concatemer molecules inside a cellular sample by conducting at least two sequencing cycles wherein in each sequencing cycle, sequencing the same corresponding nucleo-base position of all of the target barcodes in the plurality of sub-populations of barcoded concatemer molecules, wherein a first target barcode of a barcoded concatemer molecule in the first sub-population generates a first color signal in one particular sequencing cycle and the target barcodes of barcoded concatemer molecules in the other sub-populations generate a second color signal in the same particular sequencing cycle, wherein the first and second color signals are distinguishable in the same sequencing cycle, and wherein the first color signal of any of the target barcodes in any one sequencing cycle identifies the target analyte which corresponds to its cognate target barcode.

[1077] In some embodiments, the methods for detecting and identifying target analytes comprises step (b): imaging the first and second color signals generated inside the sample(s) in the one particular sequencing cycle and identifying the target analyte that corresponds to a barcode sequence that generates the first color signal.

[1078] In some embodiments, at least two different target analytes can be simultaneously identified by imaging the first and second color signals generated in a single sequencing cycle.

[1079] In some embodiments, the plurality of sub-populations of barcoded concatemer molecules are located inside a cellular sample.

[1080] In some embodiments, all of the target barcodes can be sequenced essentially simultaneously to detect and identify target analytes using a plurality of sequencing primers comprising the same sequence.

[1081] In some embodiments, all of the target barcodes can be sequenced essentially simultaneously to detect and identify target analytes using a plurality of sequencing primers comprising different sequences.

[1082] In some embodiments, the target barcodes can be sequenced sequentially (e.g., sequenced in separate batches) to detect and identify target analytes using a plurality of sequencing primers having different sequences.

[1083] In some embodiments, only a partial length of the target barcode needs to be sequenced in order to identify the different target analytes. In some embodiments, the full length of the target barcodes can be sequenced to detect and identify the different target analytes.

[1084] In some embodiments, the target analyte is located inside the sample(s) or on the membrane of the sample(s), wherein the target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the target analyte comprises a polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the target analyte comprises a polypeptide, enzyme or lipid located in or on a cellular structure including without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

[1085] In some embodiments, simultaneously sequencing two or more sub-populations of barcoded concatemer molecules located inside a cellular sample, and imaging the color signals generated by the sequencing, can be used for simultaneously identifying two or more cellular target analytes. In some embodiments, the simultaneously sequencing and multi-color imaging can be used for cell painting.

[1086] The present disclosure provides methods for detecting and identifying target analytes comprise step (a): providing a plurality of barcoded concatemer molecules including at least a first sub-population of barcoded concatemer molecules comprising a plurality of a first target barcode which corresponds to a first target analyte wherein the first target barcode is 4-20 nucleo-bases in length, and a second sub-population of barcoded concatemer molecules comprising a plurality of a second target barcode which corresponds to a second target analyte wherein the second target barcode is 4-20 nucleo-bases in length, wherein the plurality of barcoded concatemer molecules are located inside a cellular sample.

[1087] In some embodiments, the methods for detecting and identifying target analytes comprise step (b): conducting a first sequencing cycle inside the sample(s), wherein the sequencing comprises sequencing essentially simultaneously the first nucleo-base position of the first and second target barcodes in the first and second sub-populations of barcoded concatemer molecules using a plurality of sequencing primers having the same sequence, wherein the first nucleo-base position of the first target barcode generates a first color signal and the first nucleo-base position of the second barcode generates a second color signal, wherein the first and second

color signals are distinguishable from each other in the first sequencing cycle, and wherein the first color signal identifies the first target analyte.

[1088] In some embodiments, the methods for detecting and identifying target analytes comprise step (c): conducting a second sequencing cycle inside the sample(s), wherein the sequencing comprises sequencing essentially simultaneously the second nucleo-base position of the first and second target barcodes in the first and second sub-populations of barcoded concatemer molecules, wherein the second nucleo-base position of the first target barcode generates a second color signal, wherein the second nucleo-base position of the second barcode generates a first color signal, wherein the first and second color signals are distinguishable from each other in the second sequencing cycle, and wherein the first color signal identifies the second target analyte.

[1089] In some embodiments, the methods for detecting and identifying target analytes further comprises step (d): imaging the first and second color signals generated inside the sample(s) in the first sequencing cycle and identifying the first target analyte.

[1090] In some embodiments, the methods for detecting and identifying target analytes further comprises step (e): imaging the first and second color signals generated inside the sample(s) in the second sequencing cycle and identifying the second target analyte.

[1091] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the first sequencing cycle and identifying the first target analyte which corresponds to the first color signal and identifying the second target analyte which corresponds to the second color signal.

[1092] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the second sequencing cycle and identifying the second target analyte which corresponds to the first color signal and identifying the first target analyte which corresponds to the second color signal.

[1093] In some embodiments, at least two different target analytes can be simultaneously identified by imaging the first and second color signals generated in a single sequencing cycle.

[1094] In some embodiments, the first and second target analytes can be identified by conducting no more than two sequencing cycles.

[1095] In some embodiments, the first and second target barcodes can be identified by sequencing the full length of the first and second target barcodes.

[1096] In some embodiments, in the sequencing the first nucleo-base position of the first target barcodes of step (b), and in sequencing the second nucleo-base position of the first target barcodes of step (c), wherein the first and second nucleo-base positions are consecutive nucleo-base positions in the first target barcodes.

[1097] In some embodiments, in the sequencing the first nucleo-base position of the first target barcodes of step (b), and in sequencing the second nucleo-base position of the first target barcodes of step (c), wherein the first and second nucleo-base positions are non-consecutive nucleo-base positions in the first target barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[1098] In some embodiments, in the sequencing the first nucleo-base position of the second target barcodes of step (b), and in sequencing the second nucleo-base position of the second target barcodes of step (c), wherein the first and second nucleo-base positions are consecutive nucleo-base positions in the second target barcodes.

[1099] In some embodiments, in the sequencing the first nucleo-base position of the second target barcodes of step (b), and in sequencing the second nucleo-base position of the second target barcodes of step (c), wherein the first and second nucleo-base positions are non-consecutive nucleo-base positions in the second target barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[1100] In some embodiments, the first and second target analytes are located inside the sample(s) or on the membrane of the sample(s), wherein the target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the first and second target analytes comprise a polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the first and second target analytes comprise a polypeptide, enzyme or lipid located in or on a cellular structure including without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

[1101] In some embodiments, simultaneously sequencing at least two sub-populations of barcoded concatemer molecules located inside a cellular sample, and imaging the color signals generated by the sequencing, can be used for simultaneously identifying two or more cellular target analytes. In some embodiments, the simultaneously sequencing and multi-color imaging can be used for cell painting.

[1102] In some embodiments, the first sequencing cycle of step (b) and the second sequencing cycle of step (c) can be conducted using any sequencing method including sequencing-by-binding, sequencing using chain terminator nucleotides, or sequencing using multivalent molecules.

[1103] The present disclosure provides methods for detecting and identifying target analytes comprising step (a): providing a plurality of barcoded concatemer molecules including at least a first sub-population of barcoded concatemer molecules comprising a plurality of a first target barcode which corresponds to a first target analyte wherein the first target barcode is 4-20

nucleo-bases in length, a second sub-population of barcoded concatemer molecules comprising a plurality of a second target barcode which corresponds to a second target analyte wherein the second target barcode is 4-20 nucleo-bases in length, and a third sub-population of barcoded concatemer molecules comprising a plurality of a third target barcode which corresponds to a third target analyte wherein the third target barcode is 4-20 nucleo-bases in length, and wherein the plurality of barcoded concatemer molecules are located inside a cellular sample;

[1104] In some embodiments, methods for detecting and identifying target analytes comprise step (b): conducting a first sequencing cycle inside the sample(s), wherein the sequencing comprises sequencing essentially simultaneously the first nucleo-base position of the first, second and third target barcodes in the first, second and third sub-populations of barcoded concatemer molecules using a plurality of sequencing primers having the same sequence, wherein the first nucleo-base position of the first target barcode generates a first color signal and the first nucleo-base position of the second and third barcode generates a second color signal, wherein the first and second color signals are distinguishable from each other in the first sequencing cycle, and wherein the first color signal identifies the first target analyte;

[1105] In some embodiments, methods for detecting and identifying target analytes comprise step (c): conducting a second sequencing cycle inside the sample(s), wherein the sequencing comprises sequencing essentially simultaneously the second nucleo-base position of the first, second and third target barcodes in the first, second and third sub-populations of barcoded concatemer molecules, wherein the second nucleo-base position of the first target barcode generates a second color signal, wherein the second nucleo-base position of the second barcode generates a first color signal, wherein the second nucleo-base position of the third barcode generates a second color signal, wherein the first and second color signals are distinguishable from each other in the second sequencing cycle, and wherein the first color signal identifies the second target analyte; and

[1106] In some embodiments, methods for detecting and identifying target analytes comprise step (d): conducting a third sequencing cycle inside the sample(s), wherein the sequencing comprises sequencing essentially simultaneously the third nucleo-base position of the first, second and third target barcodes in the first, second and third sub-populations of barcoded concatemer molecules, wherein the third nucleo-base position of the first target barcode generates a second color signal, wherein the third nucleo-base position of the second barcode generates a second color signal, wherein the third nucleo-base position of the third barcode generates a first color signal, wherein the first and second color signals are distinguishable from each other in the third sequencing cycle, and wherein the first color signal identifies the third target analyte.

[1107] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the first sequencing cycle and identifying the first target analyte.

[1108] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the second sequencing cycle and identifying the second target analyte.

[1109] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the third sequencing cycle and identifying the third target analyte.

[1110] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the first sequencing cycle and identifying the first target analyte which corresponds to the first color signal, identifying the second and third target analytes which correspond to the second color signal.

[1111] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the second sequencing cycle and identifying the second target analyte which corresponds to the first color signal, and identifying the first and third target analytes which correspond to the second color signal.

[1112] In some embodiments, the methods for detecting and identifying target analytes further comprises: imaging the first and second color signals generated inside the sample(s) in the third sequencing cycle and identifying the third target analyte which corresponds to the first color signal, and identifying the first and second target analytes which correspond to the second color signal.

[1113] In some embodiments, at least two different target analytes can be simultaneously identified by imaging the first and second color signals generated in a single sequencing cycle.

[1114] In some embodiments, the first, second and third target analytes can be identified by conducting no more than three sequencing cycles.

[1115] In some embodiments, the first, second and third target barcodes can be identified by sequencing the full length of the first, second and third target barcodes.

[1116] In some embodiments, in the sequencing the first nucleo-base position of the first target barcodes of step (b), and in sequencing the second nucleo-base position of the first target barcodes of step (c), wherein the first and second nucleo-base positions are consecutive nucleo-base positions in the first target barcodes.

[1117] In some embodiments, in the sequencing the first nucleo-base position of the first target barcodes of step (b), and in sequencing the second nucleo-base position of the first target barcodes of step (c), wherein the first and second nucleo-base positions are non-consecutive nucleo-base positions in the first target barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[1118] In some embodiments, in the sequencing the first nucleo-base position of the second target barcodes of step (b), and in sequencing the second nucleo-base position of the second target barcodes of step (c), wherein the first and second nucleo-base positions are consecutive nucleo-base positions in the second target barcodes.

[1119] In some embodiments, in the sequencing the first nucleo-base position of the second target barcodes of step (b), and in sequencing the second nucleo-base position of the second target barcodes of step (c), wherein the first and second nucleo-base positions are non-consecutive nucleo-base positions in the second target barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[1120] In some embodiments, in the sequencing the first nucleo-base position of the third target barcodes of step (b), and in sequencing the second nucleo-base position of the third target barcodes of step (c), wherein the first and second nucleo-base positions are consecutive nucleo-base positions in the third target barcodes.

[1121] In some embodiments, in the sequencing the first nucleo-base position of the third target barcodes of step (b), and in sequencing the second nucleo-base position of the third target barcodes of step (c), wherein the first and second nucleo-base positions are non-consecutive nucleo-base positions in the third target barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[1122] In some embodiments, the first, second and third target analytes are located inside the sample(s) and/or on the membrane of the sample(s), wherein the target analytes comprises a polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the first, second and third target analytes comprise a polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the first, second and third target analytes comprise a polypeptide, enzyme or lipid located in or on a cellular structure including without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

[1123] In some embodiments, simultaneously sequencing two or more sub-populations of barcoded concatemer molecules located inside a cellular sample, and imaging the color signals generated by the sequencing, can be used for simultaneously identifying two or more cellular

target analytes. In some embodiments, the simultaneously sequencing and multi-color imaging can be used for cell painting.

[1124] In some embodiments, the first sequencing cycle of step (b) and the second sequencing cycle of step (c) and the third sequencing cycle of step (d) can be conducted using any sequencing method including sequencing-by-binding, sequencing using chain terminator nucleotides, or sequencing using multivalent molecules.

Compaction oligonucleotides

[1125] In some embodiments, in any of the compositions or methods described herein, the rolling circle amplification reaction can be conducted inside the sample(s) in the presence of a plurality of compaction oligonucleotides which, when hybridized to a concatemer molecule, compacts the size and/or shape of the concatemer molecule to form a compact DNA nanoball. In some embodiments, the compaction oligonucleotides comprise single stranded oligonucleotides having a first region at one end that hybridizes to a portion of a concatemer molecule and a second region at the other end that hybridizes to another portion of the same concatemer molecule, where hybridization of the compaction oligonucleotide to a given concatemer molecule compacts the size and/or shape of the concatemer.

[1126] In some embodiments, the compaction oligonucleotides include a 5′ region, an optional internal region (intervening region), and a 3′ region. The 5′ and 3′ regions of the compaction oligonucleotide can hybridize to different portions of the concatemer molecule to pull together distal portions of the concatemer molecule, thereby causing compaction of the concatemer molecule to form a DNA nanoball. For example, and without limitation, the 5′ region of the compaction oligonucleotide can be designed to hybridize to a first portion of the concatemer molecule (e.g., a universal compaction oligonucleotide binding site), and the 3′ region of the compaction oligonucleotide is designed to hybridize to a second portion of the same concatemer molecule (e.g., a universal compaction oligonucleotide binding site). Inclusion of compaction oligonucleotides during RCA can promote formation of DNA nanoballs having tighter size and shape compared to concatemer molecules generated in the absence of the compaction oligonucleotides. Without wishing to be bound by theory, it is hypothesized that the compact and stable characteristics of the DNA nanoballs improves in situ sequencing accuracy by increasing signal intensity and the nanoballs retain their shape and size during multiple sequencing cycles.

[1127] In some embodiments, the compaction oligonucleotides comprise single stranded oligonucleotides comprising DNA, RNA, or a combination of DNA and RNA. The compaction oligonucleotides can be any length, including 20-150 nucleotides, or 30-100 nucleotides, or 40-80 nucleotides in length, or any range therebetween.

[1128] In some embodiments, the compaction oligonucleotides comprise a 5′ region and a 3′ region, and optionally an intervening region between the 5′ and 3′ regions. The intervening region can be any length, for example, about 2-20 nucleotides in length. In some embodiments, the intervening region comprises a homopolymer having consecutive identical bases (e.g., AAA, GGG, CCC, TTT or UUU). In some embodiments, the intervening region comprises a non-homopolymer sequence.

[1129] In some embodiments, the 5′ region of the compaction oligonucleotides can be wholly complementary or partially complementary along its length to a first portion of a concatemer molecule. In some embodiments, the 3′ region of the compaction oligonucleotides can be wholly complementary or partially complementary along its length to a second portion of a concatemer molecule. In some embodiments, the 5′ region of the compaction oligonucleotides can hybridize to a first universal sequence portion of a concatemer molecule. In some embodiments, the 3′ region of the compaction oligonucleotides can hybridize to a second universal sequence portion of the same concatemer molecule.

[1130] In some embodiments, the 5′ region of the compaction oligonucleotide can have the same sequence as the 3′ region. In some embodiments, the 5′ region of the compaction oligonucleotide can have a sequence that is different from the 3′ region. In some embodiments, the 3′ region of the compaction oligonucleotide can have a sequence that is a reverse sequence of the 5′ region. In some embodiments, the 5′ region of the compaction oligonucleotide can have a sequence that is a reverse sequence of the 3′ region.

[1131] In some embodiments, the 3′ region of any of the compaction oligonucleotides can include an additional three bases at the terminal 3′ end which comprises 2’-O-methyl RNA bases (e.g., designated mUmUmU) or the terminal 3′ end lacks additional 2’-O-methyl RNA bases.

[1132] In some embodiments, the compaction oligonucleotides comprise one or more modified bases or linkages at their 5′ or 3′ ends to confer certain functionalities. In some embodiments, the compaction oligonucleotides comprise at least one phosphorothioate linkage at their 5′ and/or 3′ ends to confer exonuclease resistance. In some embodiments, at least one nucleotide at or near the 3′ end comprises a 2’ fluoro base which confers exonuclease resistance. In some embodiments, the 3′ ends of the compaction oligonucleotides comprise at least one 2’-O-methyl RNA base which blocks polymerase-catalyzed extension. For example, the 3′ end of the compaction oligonucleotide comprises three bases comprising 2’-O-methyl RNA base (e.g., designated mUmUmU). In some embodiments, the compaction oligonucleotides comprise a 3′ inverted dT at their 3′ ends which blocks polymerase-catalyzed extension. In some embodiments, the compaction oligonucleotides comprise 3′ phosphorylation which blocks polymerase-catalyzed extension. In some embodiments, the internal region of the compaction

oligonucleotides comprises at least one locked nucleic acid (LNA) which increases the thermal stability of duplexes formed by hybridizing a compaction oligonucleotide to a concatemer molecule. In some embodiments, the compaction oligonucleotides comprise a phosphorylated 5′ end (e.g., using a polynucleotide kinase).

[1133] In some embodiments, the compaction oligonucleotides can include at least one region having consecutive guanines. For example, the compaction oligonucleotides can include at least one region having 2, 3, 4, 5, 6 or more consecutive guanines. In some embodiments, the compaction oligonucleotides comprise four consecutive guanines which can form a guanine tetrad structure (e.g., FIG. 107). The guanine tetrad structure can be stabilized via Hoogsteen hydrogen bonding. The guanine tetrad structure can be stabilized by a central cation, e.g., potassium, sodium, lithium, rubidium or cesium.

[1134] At least one compaction oligonucleotide can form a guanine tetrad (e.g., FIG. 107) and hybridize to the universal binding sequences in a concatemer which can cause the concatemer to fold to form an intramolecular G-quadruplex structure (e.g., FIG. 108). The concatemers can self-collapse, e.g., to form compact nanoballs. Formation of the guanine tetrads and G-quadruplexes in the nanoballs may increase the stability of the nanoballs to retain their compact size and shape, e.g., which can withstand changes in pH, temperature and/or repeated flows of reagents during sequencing inside the sample(s).

[1135] In some embodiments, the plurality of compaction oligonucleotides in the rolling circle amplification reaction have the same sequence. Alternatively, the plurality of compaction oligonucleotides in the rolling circle amplification reaction comprise a mixture of two or more different populations of compaction oligonucleotides having different sequences.

[1136] In some embodiment, the immobilized concatemer molecule can self-collapse into a compact nucleic acid nanoball. The nanoballs can be imaged and a FWHM measurement can be obtained to give the shape/size of the nanoballs.

[1137] In some embodiments, inclusion of compaction oligonucleotides in the rolling circle amplification reaction can promote collapsing of a concatemer molecule into a DNA nanoball. Without wishing to be bound by theory, it is hypothesized that conducting RCA with compaction oligonucleotides helps retain the compact size and shape of a DNA nanoball during multiple sequencing cycles which can improve FWHM (full width half maximum) of a spot image of the DNA nanoball inside a cellular sample. In some embodiments, the DNA nanoball does not unravel during multiple sequencing cycles. In some embodiments, the spot image of the DNA nanoball does not enlarge during multiple sequencing cycles. In some embodiments, the spot image of the DNA nanoball remains a discrete spot during multiple sequencing cycles. The spot image can be represented as a Gaussian spot and the size can be measured as a FWHM. A

smaller spot size as indicated by a smaller FWHM typically correlates with an improved image of the spot. In some embodiments, the FWHM of a nanoball spot can be about 10 μm or smaller.

Antibodies

[1138] In some embodiments, in any of the compositions or methods described herein, the antibody bridge circle complexes (1700) and bipartite complexes (1800) comprise an antibody attached to a bridge oligonucleotide (1500). In some embodiments, the antibody comprises a primary or secondary antibody. In some embodiments, the antibody comprises an intact immunoglobulin, antibody fragment, an antigen binding portion of an antibody, or single-chain antibody. The antibodies can be monoclonal or polyclonal antibodies. The antibodies are capable of binding specifically to a target analyte. In some embodiments, target analytes comprise intact polypeptides or peptide fragments. The antibodies comprises an antigen-binding region (e.g., paratope) that binds specifically to a target analyte. In some embodiments, the target analyte can be located inside the sample(s) or on the membrane of the sample(s), wherein the target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the target analyte comprises a polypeptide, enzyme or lipid located anywhere in the sample(s) including without limits the cytoplasm and nucleus. In some embodiments, the target analyte comprises a polypeptide, enzyme or lipid located in or on a cellular structure including without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome and lysosome.

[1139] An immunoglobulin is typically a tetrameric molecule comprising two identical pairs of polypeptide chains where each pair includes a light chain and a heavy chain. The amino portion of the heavy and light chains each comprise a variable region which associate with each other to form an antigen binding region (e.g., paratope). Thus, a typical immunoglobulin can bind two antigens or can bind two target analytes. The carboxyl portion of the heavy chain comprise a constant region which associate with each other to form an Fc region for effector function. The Fc portion of the heavy chains can define the class of antibody which includes IgG, IgM, IgD, IgA or IgE isotype. The heavy and/or light chains can be prepared using recombinant techniques or by immunizing an animal with an antigen of interest.

[1140] In some embodiments, the antibodies can be raised in a host species including for example rabbit, mouse, rat, rabbit, goat, sheep, guinea pig, chicken, golden Syrian hamster or horse. In some embodiments, the antibodies comprise animal-free antibodies that are produced using recombinant DNA technology.

[1141] The antibody fragment generally comprises a portion of an intact immunoglobulin that can bind an antigen. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$, and Fd. In some embodiments, an Fv fragment comprises a variable

light chain region (VL) and variable heavy chain region (VH). In some embodiments, an Fab fragment comprises a monovalent antibody fragment having a variable light chain region (VL), constant light chain region (CL), variable heavy chain region (VH), and first constant region (CH1). In some embodiments, an Fab' fragment comprises a monovalent antibody fragment having a variable light chain region (VL), constant light chain region (CL), variable heavy chain region (VH), first constant region (CH1), hinge region, and at least a portion of a second constant region (CH2). In some embodiments, an $F(ab')_2$ fragment comprises a bivalent antibody fragment having two Fab fragments linked via a disulfide bridge at the hinge region.

[1142] A single-chain antibody (scFv) typically comprises a single polypeptide chain (e.g., a monovalent antibody molecule) having a variable light chain region (VL) and variable heavy chain region (VH) joined by a polypeptide linker (see, e.g., Bird et al., 1988, Science 242:423-26 and Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-83). The amino-terminal end of the single-chain antibody comprises either the variable light chain region (VL) or the variable heavy chain region (VH). In some embodiments, the single-chain antibody comprises an scFv-Fc antibody which further comprises an antibody hinge region, and at least a portion of the Fc region including the CH2 and/or the CH3 region. In some embodiments, the single-chain antibody comprises an scFv-CH antibody which further comprises an antibody hinge region, and at least a portion of the CH3 region.

[1143] In some embodiments, antibodies can be conjugated to bridge oligonucleotides using any well-known linking chemistry. For example, antibody-bridge oligonucleotide conjugates can be prepared by cross-linking amino groups on the antibody and bridge oligonucleotide using glutaraldehyde. The lysine side chain epsilon-amide is commonly targeted to conjugate to oligonucleotides. In some embodiments, maleimide-modified antibodies can be reacted with sulfhydryl-modified oligonucleotides.

[1144] In some embodiments, homo-bifunctional or hetero-bifunctional cross-linkers can be introduced as bridges to link together the antibody and bridge oligonucleotide.

[1145] In some embodiments, an antibody can be attached to a bridge oligonucleotide using an amine-to-amine non-cleavable crosslinker comprising disuccinimidyl suberate (DSS). DSS comprises an amine-reactive NHS ester at both ends of an 8-atom spacer arm.

[1146] In some embodiments, an antibody can be attached to a bridge oligonucleotide using MCC (4-[(2,5-dioxopyrrol-1-yl)methyl]cyclohexane-1-carboxamide) which is a thioether linker which couples antibodies to oligonucleotides via a thioether bond.

[1147] In some embodiments, an antibody can be attached to a bridge oligonucleotide using copper-free click chemistry, for example using dibenzocyclooctyne (DBCO). A bridge

oligonucleotide modified with DBCO can be reacted with an azide-modified antibody to generate an antibody attached to a bridge oligonucleotide.

[1148] In some embodiments, an antibody can be attached to a bridge oligonucleotide using an amine-to-sulfhydryl cross linker succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). A thiol-modified bridge oligonucleotide can be reacted with an antibody having an amine-to-thiol linker to generate an antibody attached to a bridge oligonucleotide.

[1149] In some embodiments, an antibody can be attached to a bridge oligonucleotide using a chemically labile linker (e.g., pH sensitive linker) such as for example hydrazone linkers.

Sequencing polymerases

[1150] In some embodiments, in any of the compositions or methods described herein, a plurality of sequencing polymerases can be used for conducting any of the polymerase-catalyzed sequencing reactions described herein. In some embodiments, the sequencing polymerase(s) is/are capable of binding and incorporating a complementary nucleotide opposite a nucleotide in a concatemer template molecule. In some embodiments, the sequencing polymerase(s) is/are capable of binding a complementary nucleotide moiety of a multivalent molecule opposite a nucleotide in a concatemer template molecule. In some embodiments, the plurality of sequencing polymerases comprise recombinant mutant polymerases.

[1151] Examples of suitable polymerases for use in sequencing with nucleotides and/or multivalent molecules include but are not limited to: Klenow DNA polymerase; Thermus aquaticus DNA polymerase I (Taq polymerase); KlenTaq polymerase; Candidatus altiarchaeales archaeon; Candidatus Hadarchaeum Yellowstonense; Hadesarchaea archaeon; Euryarchaeota archaeon; Thermoplasmata archaeon; Thermococcus polymerases such as Thermococcus litoralis, bacteriophage T7 DNA polymerase; human alpha, delta and epsilon DNA polymerases; bacteriophage polymerases such as T4, RB69 and phi29 bacteriophage DNA polymerases; Pyrococcus furiosus DNA polymerase (Pfu polymerase); Bacillus subtilis DNA polymerase III; E. coli DNA polymerase III alpha and epsilon; 9 degree N polymerase; reverse transcriptases such as HIV type M or O reverse transcriptases; avian myeloblastosis virus reverse transcriptase; Moloney Murine Leukemia Virus (MMLV) reverse transcriptase; or telomerase. Further non-limiting examples of DNA polymerases include those from various Archaea genera, such as, Aeropyrum, Archaeglobus, Desulfurococcus, Pyrobaculum, Pyrococcus, Pyrolobus, Pyrodictium, Staphylothermus, Stetteria, Sulfolobus, Thermococcus, and Vulcanisaeta and the like or variants thereof, including such polymerases as are known in the art such as 9 degrees N, VENT™, DEEP VENT™, THERMINATOR™, Pfu, KOD, Pfx, Tgo and RB69 polymerases.

Sequencing-by-Binding

[1152] In some embodiments, in any of the compositions or methods described herein, the sequencing comprises conducting sequencing-by-binding (SBB) reactions inside the sample(s), where the cDNA amplicons are the concatemer molecules which serve as templates. In some embodiments, the sequencing-by-binding (SBB) procedure employs non-labeled chain-terminating nucleotides. In some embodiments, a cycle of sequencing-by-binding (SBB) comprises the steps of (a) sequentially contacting a primed concatemer molecule (e.g., a concatemer molecule annealed to a plurality of sequencing primers) with at least two separate mixtures under ternary complex stabilizing conditions, wherein the at least two separate mixtures each include a polymerase and a nucleotide, whereby the sequentially contacting results in the primed concatemer molecule being contacted, under the ternary complex stabilizing conditions, with nucleotide cognates for first, second and third base types in the concatemer molecule; (b) examining the at least two separate mixtures to determine whether a ternary complex formed; and (c) identifying the next correct nucleotide for the primed concatemer molecule, wherein the next correct nucleotide is identified as a cognate of the first, second or third base type if ternary complex is detected in step (b), and wherein the next correct nucleotide is imputed to be a nucleotide cognate of a fourth base type based on the absence of a ternary complex in step (b); (d) adding a next correct nucleotide to the primer of the primed concatemer molecule after step (b), thereby producing an extended primer; and (e) repeating steps (a) through (d) at least once on the primed concatemer molecule that comprises the extended primer. Exemplary sequencing-by-binding methods are described in U.S. Patent Nos. 10,246,744 and 10,731,141 (where the contents of both patents are hereby incorporated by reference in their entireties).

Nucleotides and chain-terminating nucleotides

[1153] In some embodiments, in any of the compositions or methods described herein, the sequencing methods described herein can employ at least one nucleotide. In some embodiments, the nucleotides can be employed to conduct polymerase-catalyzed sequencing methods, for example sequencing of steps (e1) and (e2) described above.

[1154] In some embodiments, individual nucleotides comprise a base, sugar and at least one phosphate group. In some embodiments, at least one nucleotide in the plurality comprises an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and one or more phosphate groups (e.g., 1-10 phosphate groups). The plurality of nucleotides can comprise at least one type of nucleotide selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP. The plurality of nucleotides can comprise at a mixture of any combination of two or more types of nucleotides selected from the group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP. In

some embodiments, at least one nucleotide in the plurality is not a nucleotide analog. In some embodiments, at least one nucleotide in the plurality comprises a nucleotide analog.

[1155] In some embodiments, at least one nucleotide in the plurality of nucleotides comprises a chain of one, two or three phosphorus atoms where the chain is typically attached to the 5′ carbon of the sugar moiety via an ester or phosphoramide linkage. In some embodiments, at least one nucleotide in the plurality is an analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH3. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

[1156] In some embodiments, at least one nucleotide in the plurality of nucleotides comprises a terminator nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2′ position, at the sugar 3′ position, or at the sugar 2′ and 3′ position. In some embodiments, the chain terminating moiety can inhibit polymerase-catalyzed incorporation of a subsequent nucleotide moiety or free nucleotide in a nascent strand during a primer extension reaction. In some embodiments, the chain terminating moiety is attached to the 3′ sugar hydroxyl position where the sugar comprises a ribose or deoxyribose sugar moiety. In some embodiments, the chain terminating moiety is removable/cleavable from the 3′ sugar hydroxyl position to generate a nucleotide having a 3′OH sugar group which is extendible with a subsequent nucleotide in a polymerase-catalyzed nucleotide incorporation reaction. In some embodiments, the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, silyl group or acetal group. In some embodiments, the chain terminating moiety is cleavable/removable from the nucleotide, for example by reacting the chain terminating moiety with a chemical agent, pH change, light or heat. In some embodiments, the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh3)4) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the chain terminating moieties aryl and benzyl are cleavable with H2 Pd/C. In some embodiments, the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, or disulfide are cleavable, e.g., with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). In some embodiments, the chain terminating moiety carbonate is cleavable with potassium carbonate (K2CO3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some

embodiments, the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[1157] In some embodiments, at least one nucleotide in the plurality of nucleotides comprises a terminator nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2′ position, at the sugar 3′ position, or at the sugar 2′ and 3′ position. In some embodiments, the chain terminating moiety comprises an azide, azido or azidomethyl group. In some embodiments, the chain terminating moiety comprises a 3′-O-azido or 3′-O-azidomethyl group. In some embodiments, the chain terminating moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[1158] In some embodiments, the nucleotide comprises a chain terminating moiety which is selected from a group consisting of 3′-deoxy nucleotides, 2',3′-dideoxynucleotides, 3′-methyl, 3′-azido, 3′-azidomethyl, 3′-O-azidoalkyl, 3′-O-ethynyl, 3′-O-aminoalkyl, 3′-O-fluoroalkyl, 3′-fluoromethyl, 3′-difluoromethyl, 3′-trifluoromethyl, 3′-sulfonyl, 3′-malonyl, 3′-amino, 3′-O-amino, 3′-sulfhydral, 3′-aminomethyl, 3′-ethyl, 3′butyl, 3′-tert butyl, 3′-Fluorenylmethyloxycarbonyl, 3′ tert-Butyloxycarbonyl, 3′-O-alkyl hydroxylamino group, 3′-phosphorothioate, and 3-O-benzyl, or derivatives thereof.

[1159] In some embodiments, the plurality of nucleotides comprises a plurality of nucleotides labeled with detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, the fluorophore is attached to the nucleotide base. In some embodiments, the fluorophore is attached to the nucleotide base with a linker which is cleavable/removable from the base. In some embodiments, at least one of the nucleotides in the plurality is not labeled with a detectable reporter moiety. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the nucleotide can correspond to the nucleotide base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) to permit detection and identification of the nucleotide base.

[1160] In some embodiments, the cleavable linker on the nucleotide base comprises a cleavable moiety comprising an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, silyl group or acetal group. In some

embodiments, the cleavable linker on the base is cleavable/removable from the base by reacting the cleavable moiety with a chemical agent, pH change, light or heat. In some embodiments, the cleavable moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh3)4) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the cleavable moieties aryl and benzyl are cleavable with H2 Pd/C. In some embodiments, the cleavable moieties amine, amide, keto, isocyanate, phosphate, thio, or disulfide are cleavable with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). In some embodiments, the cleavable moiety carbonate is cleavable with potassium carbonate (K2CO3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some embodiments, the cleavable moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[1161] In some embodiments, the cleavable linker on the nucleotide base comprises cleavable moiety including an azide, azido or azidomethyl group. In some embodiments, the cleavable moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or Tri(hydroxyproyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[1162] In some embodiments, the chain terminating moiety (e.g., at the sugar 2′ and/or sugar 3′ position) and the cleavable linker on the nucleotide base have the same or different cleavable moieties. In some embodiments, the chain terminating moiety (e.g., at the sugar 2′ and/or sugar 3′ position) and the detectable reporter moiety linked to the base are chemically cleavable/removable with the same chemical agent. In some embodiments, the chain terminating moiety (e.g., at the sugar 2′ and/or sugar 3′ position) and the detectable reporter moiety linked to the base are chemically cleavable/removable with different chemical agents.

Multivalent molecules comprising nucleotide moieties

[1163] In some embodiments, in any of the compositions or methods described herein, the sequencing can employ at least one multivalent molecule. In some embodiments, the multivalent molecule can be employed to conduct polymerase-catalyzed sequencing methods, for example sequencing of steps (e1) and (e2) described above.

[1164] In some embodiments, individual multivalent molecules comprise a plurality of nucleotide arms attached to a core and having any configuration including a starburst, helter skelter, or bottle brush configuration (e.g., FIG. 76). Exemplary multivalent molecules comprise:

(1) a core; and (2) a plurality of nucleotide arms which comprise (i) a core attachment moiety, (ii) a spacer comprising a PEG moiety, (iii) a linker, and (iv) a nucleotide moiety, wherein the core is attached to the plurality of nucleotide arms, wherein the spacer is attached to the linker, wherein the linker is attached to the nucleotide moiety. In some embodiments, the nucleotide moiety comprises a base, sugar and at least one phosphate group, and the linker is attached to the nucleotide moiety through the base. In some embodiments, the linker comprises an aliphatic chain or an oligo ethylene glycol chain where both linker chains having 2-6 subunits. In some embodiments, the linker also includes an aromatic moiety. An exemplary nucleotide arm is shown in FIG. 79B. Exemplary multivalent molecules are shown in FIGS. 76-79A. An exemplary spacer is shown in FIG. 81 (top) and exemplary linkers are shown in FIGS. 81 (bottom) and FIG. 82. Exemplary nucleotides attached to a linker are shown in FIGS. 83-86. An exemplary biotinylated nucleotide arm is shown in FIG. 87.

[1165] In some embodiments, a multivalent molecule comprises a core attached to multiple nucleotide arms, and wherein the multiple nucleotide arms have the same type of nucleotide moiety which is selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

[1166] In some embodiments, a multivalent molecule comprises a core attached to multiple nucleotide arms, where each arm includes a nucleotide moiety. The nucleotide moiety comprises an aromatic base, a five carbon sugar (e.g., ribose or deoxyribose), and one or more phosphate groups (e.g., 1-10 phosphate groups). The plurality of multivalent molecules can comprise one type multivalent molecule having one type of nucleotide moiety selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP. The plurality of multivalent molecules can comprise at a mixture of any combination of two or more types of multivalent molecules, where individual multivalent molecules in the mixture comprise nucleotide moieties selected from a group consisting of dATP, dGTP, dCTP, dTTP and/or dUTP.

[1167] In some embodiments, the nucleotide moiety comprises a chain of one, two or three phosphorus atoms where the chain is typically attached to the 5′ carbon of the sugar moiety via an ester or phosphoramide linkage. In some embodiments, at least one nucleotide moiety is a nucleotide analog having a phosphorus chain in which the phosphorus atoms are linked together with intervening O, S, NH, methylene or ethylene. In some embodiments, the phosphorus atoms in the chain include substituted side groups including O, S or BH3. In some embodiments, the chain includes phosphate groups substituted with analogs including phosphoramidate, phosphorothioate, phosphordithioate, and O-methylphosphoroamidite groups.

[1168] In some embodiments, the multivalent molecule comprises a core attached to multiple nucleotide arms, and wherein individual nucleotide arms comprise a nucleotide moiety which is a nucleotide analog having a chain terminating moiety (e.g., blocking moiety) at the sugar 2'

position, at the sugar 3′ position, or at the sugar 2’ and 3′ position. In some embodiments, the nucleotide moiety comprises a chain terminating moiety (e.g., blocking moiety) at the sugar 2’ position, at the sugar 3′ position, or at the sugar 2’ and 3′ position. In some embodiments, the chain terminating moiety can inhibit polymerase-catalyzed incorporation of a subsequent nucleotide moiety or free nucleotide in a nascent strand during a primer extension reaction. In some embodiments, the chain terminating moiety is attached to the 3′ sugar hydroxyl position where the sugar comprises a ribose or deoxyribose sugar moiety. In some embodiments, the chain terminating moiety is removable/cleavable from the 3′ sugar hydroxyl position to generate a nucleotide having a 3′OH sugar group which is extendible with a subsequent nucleotide in a polymerase-catalyzed nucleotide incorporation reaction. In some embodiments, the chain terminating moiety comprises an alkyl group, alkenyl group, alkynyl group, allyl group, aryl group, benzyl group, azide group, amine group, amide group, keto group, isocyanate group, phosphate group, thio group, disulfide group, carbonate group, urea group, silyl group or acetal group. In some embodiments, the chain terminating moiety is cleavable/removable from the nucleotide moiety, for example by reacting the chain terminating moiety with a chemical agent, pH change, light or heat. In some embodiments, the chain terminating moieties alkyl, alkenyl, alkynyl and allyl are cleavable with tetrakis(triphenylphosphine)palladium(0) (Pd(PPh3)4) with piperidine, or with 2,3-Dichloro-5,6-dicyano-1,4-benzo-quinone (DDQ). In some embodiments, the chain terminating moieties aryl and benzyl are cleavable with H2 Pd/C. In some embodiments, the chain terminating moieties amine, amide, keto, isocyanate, phosphate, thio, disulfide are cleavable with phosphine or with a thiol group including beta-mercaptoethanol or dithiothritol (DTT). In some embodiments, the chain terminating moiety carbonate is cleavable with potassium carbonate (K2CO3) in MeOH, with triethylamine in pyridine, or with Zn in acetic acid (AcOH). In some embodiments, the chain terminating moieties urea and silyl are cleavable with tetrabutylammonium fluoride, pyridine-HF, with ammonium fluoride, or with triethylamine trihydrofluoride.

[1169] In some embodiments, the nucleotide moiety comprises a chain terminating moiety (e.g., blocking moiety) at the sugar 2’ position, at the sugar 3′ position, or at the sugar 2’ and 3′ position. In some embodiments, the chain terminating moiety comprises an azide, azido or azidomethyl group. In some embodiments, the chain terminating moiety comprises a 3′-O-azido or 3′-O-azidomethyl group. In some embodiments, the chain terminating moieties azide, azido and azidomethyl group are cleavable/removable with a phosphine compound. In some embodiments, the phosphine compound comprises a derivatized tri-alkyl phosphine moiety or a derivatized tri-aryl phosphine moiety. In some embodiments, the phosphine compound comprises Tris(2-carboxyethyl)phosphine (TCEP) or bis-sulfo triphenyl phosphine (BS-TPP) or

Tri(hydroxyproyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[1170] In some embodiments, the nucleotide moiety comprising a chain terminating moiety which is selected from a group consisting of 3′-deoxy nucleotides, 2',3′-dideoxynucleotides, 3′-methyl, 3′-azido, 3′-azidomethyl, 3′-O-azidoalkyl, 3′-O-ethynyl, 3′-O-aminoalkyl, 3′-O-fluoroalkyl, 3′-fluoromethyl, 3′-difluoromethyl, 3′-trifluoromethyl, 3′-sulfonyl, 3′-malonyl, 3′-amino, 3′-O-amino, 3′-sulfhydral, 3′-aminomethyl, 3′-ethyl, 3′butyl, 3′-tert butyl, 3′-Fluorenylmethyloxycarbonyl, 3′ tert-Butyloxycarbonyl, 3′-O-alkyl hydroxylamino group, 3′-phosphorothioate, and 3-O-benzyl, or derivatives thereof.

[1171] In some embodiments, the multivalent molecule comprises a core attached to multiple nucleotide arms, wherein the nucleotide arms comprise a spacer, linker and nucleotide moiety, and wherein the core, linker and/or nucleotide moiety is labeled with detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the multivalent molecule can correspond to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) of the nucleotide moiety to permit detection and identification of the nucleotide base.

[1172] In some embodiments, at least one nucleotide arm of a multivalent molecule has a nucleotide moiety that is attached to a detectable reporter moiety. In some embodiments, the detectable reporter moiety is attached to the nucleotide base. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, a particular detectable reporter moiety (e.g., fluorophore) that is attached to the multivalent molecule can correspond to the base (e.g., dATP, dGTP, dCTP, dTTP or dUTP) of the nucleotide moiety to permit detection and identification of the nucleotide base.

[1173] In some embodiments, the core of a multivalent molecule comprises an avidin-like or streptavidin-like moiety and the core attachment moiety comprises biotin. In some embodiments, the core comprises a streptavidin-type or avidin-type moiety which includes an avidin protein, as well as any derivatives, analogs and other non-native forms of avidin that can bind to at least one biotin moiety. Other forms of avidin moieties include native and recombinant avidin and streptavidin as well as derivatized molecules, e.g., non-glycosylated avidin and truncated streptavidins. For example, avidin moiety includes de-glycosylated forms of avidin, bacterial streptavidin produced
by Streptomyces (e.g., Streptomyces avidinii), as well as derivatized forms, for example, N-acyl avidins, e.g., N-acetyl, N-phthalyl and N-succinyl avidin, and the commercially-available products EXTRAVIDIN™, CAPTAVIDIN™, NEUTRAVIDIN™ and NEUTRALITE AVIDIN™.

[1174] Any of the methods for sequencing nucleic acid molecules described herein can include forming a binding complex, wherein the binding complex comprises (i) a polymerase, a concatemer molecule duplexed with a primer, and a nucleotide, or the binding complex comprises (ii) a polymerase, a concatemer molecule duplexed with a primer, and a nucleotide moiety of a multivalent molecule. In some embodiments, the binding complex has a persistence time of greater than about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 or 1 second. The binding complex has a persistence time of greater than about 0.1-0.25 seconds, or about 0.25-0.5 seconds, or about 0.5-0.75 seconds, or about 0.75-1 second, or about 1-2 seconds, or about 2-3 seconds, or about 3-4 second, or about 4-5 seconds, and/or wherein the method is or may be carried out at a temperature of at or above 15 °C, at or above 20 °C, at or above 25 °C, at or above 35 °C, at or above 37 °C, at or above 42 °C at or above 55 °C at or above 60 °C, or at or above 72 °C, or at or above 80 °C, or within a range defined by any of the foregoing. The binding complex (e.g., ternary complex) remains stable until subjected to a condition that causes dissociation of interactions between any of the polymerase, concatemer molecule, primer and/or the nucleotide moiety or the nucleotide. For example, a dissociating condition comprises contacting the binding complex with any one or any combination of a detergent, EDTA and/or water. In some embodiments, the present disclosure provides methods wherein the binding complex forms inside a cellular sample where the sample(s) is deposited on a surface showing a contrast to noise ratio in the detecting step of greater than 20. In some embodiments, the present disclosure provides methods wherein the contacting is performed under a condition that stabilizes the binding complex when the nucleotide or nucleotide moiety is complementary to a next base of the template nucleic acid, and destabilizes the binding complex when the nucleotide or nucleotide moiety is not complementary to the next base of the template nucleic acid.

[1175] In some embodiments, the sequencing methods can employ multivalent molecules comprising nucleotide moieties. In some embodiments, the binding of the plurality of first complexed polymerases with the plurality of multivalent molecules can form at least one avidity complex, wherein the method comprises the steps: (a) binding a first nucleic acid primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a concatemer molecule thereby forming a first binding complex, wherein a first nucleotide moiety of the first multivalent molecule binds to the first sequencing polymerase; and (b) binding a second nucleic acid primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same concatemer molecule thereby forming a second binding complex, wherein a second nucleotide moiety of the first multivalent molecule binds to the second sequencing polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms an avidity complex. In some embodiments, the first sequencing polymerase

comprises any wild type or mutant polymerase described herein. In some embodiments, the second sequencing polymerase comprises any wild type or mutant polymerase described herein. The concatemer molecule comprises tandem repeat sequences of a sequence of interest and at least one universal sequencing primer binding site. The first and second nucleic acid primers can bind to a sequencing primer binding site along the concatemer molecule. Exemplary, non-limiting multivalent molecules are shown in FIGS. 76 - 80.

[1176] In some embodiments, the sequencing methods can employ multivalent molecules comprising nucleotide moieties. In some embodiments, the method includes binding the plurality of first complexed polymerases with the plurality of multivalent molecules to form at least one avidity complex, wherein the method comprises the steps: (a) contacting the plurality of sequencing polymerases and the plurality of nucleic acid primers with different portions of a concatemer molecule to form at least first and second complexed polymerases on the same concatemer molecule; (b) contacting a plurality of multivalent molecules to the at least first and second complexed polymerases on the same concatemer molecule, under conditions suitable to bind a single multivalent molecule from the plurality to the first and second complexed polymerases, wherein at least a first nucleotide moiety of the single multivalent molecule is bound to the first complexed polymerase which includes a first primer hybridized to a first portion of the concatemer molecule thereby forming a first binding complex (e.g., first ternary complex), and wherein at least a second nucleotide moiety of the single multivalent molecule is bound to the second complexed polymerase which includes a second primer hybridized to a second portion of the concatemer molecule thereby forming a second binding complex (e.g., second ternary complex), wherein the contacting is conducted under a condition suitable to inhibit polymerase-catalyzed incorporation of the bound first and second nucleotide moieties in the first and second binding complexes, and wherein the first and second binding complexes which are bound to the same multivalent molecule forms an avidity complex; and (c) detecting the first and second binding complexes on the same concatemer molecule, and (d) identifying the first nucleotide moiety in the first binding complex thereby determining the sequence of the first portion of the concatemer molecule, and identifying the second nucleotide moiety in the second binding complex thereby determining the sequence of the second portion of the concatemer molecule. In some embodiments, the plurality of sequencing polymerases comprise any wild type or mutant sequencing polymerase described herein. The concatemer molecule comprises tandem repeat sequences of a sequence of interest and at least one universal sequencing primer binding site. The plurality of nucleic acid primers can bind to a sequencing primer binding site along the concatemer molecule. Exemplary, non-limiting multivalent molecules are shown in FIGS. 76 - 80.

[1177] The headings provided herein are not limitations of the various aspects of the disclosure, which aspects can be understood by reference to the specification as a whole.

Certain terminology

[1178] Unless defined otherwise, technical and scientific terms used herein have meanings that are commonly understood by those of ordinary skill in the art unless defined otherwise. Generally, terminologies pertaining to techniques of molecular biology, nucleic acid chemistry, protein chemistry, genetics, microbiology, transgenic cell production, and hybridization described herein are those well-known and commonly used in the art. Techniques and procedures described herein are generally performed according to conventional methods well-known in the art and as described in various general and more specific references that are cited and discussed throughout the instant specification. For example, see Sambrook et al., Molecular Cloning: A Laboratory Manual (Third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 2000). See also Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992). The nomenclatures utilized in connection with, and the laboratory procedures and techniques described herein are those well-known and commonly used in the art.

[1179] Unless otherwise required by context herein, singular terms shall include pluralities and plural terms shall include the singular. Singular forms "a", "an" and "the", and singular use of any word, include plural referents unless expressly and unequivocally limited on one referent.

[1180] It is understood the use of the alternative term (e.g., "or") is taken to mean either one or both or any combination thereof of the alternatives.

[1181] The term "and/or" used herein is to be taken mean specific disclosure of each of the specified features or components with or without the other. For example, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include: "A and B"; "A or B"; "A" (A alone); and "B" (B alone). In a similar manner, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: "A, B, and C"; "A, B, or C"; "A or C"; "A or B"; "B or C"; "A and B"; "B and C"; "A and C"; "A" (A alone); "B" (B alone); and "C" (C alone).

[1182] As used herein and in the appended claims, terms "comprising", "including", "having" and "containing", and their grammatical variants, as used herein are intended to be non-limiting so that one item or multiple items in a list do not exclude other items that can be substituted or added to the listed items. It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[1183] As used herein, the terms "about," "approximately," and "substantially" refer to a value or composition that is within an acceptable error range for the particular value or composition as determined by one of ordinary skill in the art, which will depend in part on how the value or composition is measured or determined, i.e., the limitations of the measurement system. For example, "about," "approximately," or "substantially" can mean within one or more than one standard deviation per the practice in the art. Alternatively, "about" or "approximately" can mean a range of up to 10% (i.e., ±10%) or more depending on the limitations of the measurement system. For example, about 5 mg can include any number between 4.5 mg and 5.5 mg. Furthermore, particularly with respect to biological systems or processes, the terms can mean up to an order of magnitude or up to 5-fold of a value. When particular values or compositions are provided in the instant disclosure, unless otherwise stated, the meaning of "about," "approximately," "substantially" should be assumed to be within an acceptable error range for that particular value or composition. Also, where ranges and/or subranges of values are provided, the ranges and/or subranges can include the endpoints of the ranges and/or subranges.

[1184] The term "polony" used herein refers to a nucleic acid library molecule that can be clonally amplified in-solution or on-support to generate an amplicon that can serve as a template molecule for sequencing. In some embodiments, a linear library molecule can be circularized to generate a circularized library molecule, and the circularized library molecule can be clonally amplified in-solution or on-support to generate a concatemer. In some embodiments, the concatemer can serve as a nucleic acid template molecule which can be sequenced. The concatemer is sometimes referred to as a polony. In some embodiments, a polony includes nucleotide strands.

[1185] As used herein, the phrases "imaging module", "imaging unit", "optical imaging module", and "optical imaging unit", are used interchangeably, and may comprise components or sub-systems of a larger system that may also include, *e.g.*, fluidics modules, temperature control modules, translation stages, robotic fluid dispensing and/or microplate handling, processor or computers, instrument control software, data analysis and display software, *etc.*

[1186] As used herein, the term "dichroic" and "dichroic filter" are used interchangeably. The "dichroic" and "dichroic filter" can comprise a dichroic mirror, dichroic reflector, dichroic beam splitter, dichroic beam combiner, or a combination there of.

[1187] As used herein, the term "detection channel" refers to an optical path (and/or the optical components therein) within an optical system that is configured to deliver an optical signal arising from a sample to a detector. In some instances, a detection channel may be configured for performing spectroscopic measurements, *e.g.*, monitoring a fluorescence signal or other

optical signal using a detector such as a photomultiplier. In some instances, a "detection channel" may be an "imaging channel", *e.g.*, an optical path (and/or the optical components therein) within an optical system that is configured to capture and deliver an image to an image sensor.

[1188] As used herein, a "detectable label" may refer to any of a variety of detectable labels or tags known to those of skill in the art. Examples include, but are not limited to, chromophores, fluorophores, quantum dots, upconverting phosphors, luminescent or chemiluminescent molecules, radioisotopes, magnetic nanoparticles, mass tags, and the like. In some instances, a preferred label may comprise a fluorophore. Fluorescent moieties which may serve as fluorescent labels or fluorophores include, but are not limited to, fluorescein and fluorescein derivatives such as carboxyfluorescein, tetrachlorofluorescein, hexachlorofluorescein, carboxynapthofluorescein, fluorescein isothiocyanate, NHS-fluorescein, iodoacetamidofluorescein, fluorescein maleimide, SAMSA-fluorescein, fluorescein thiosemicarbazide, carbohydrazinomethylthioacetyl-amino fluorescein, rhodamine and rhodamine derivatives such as TRITC, TMR, lissamine rhodamine, Texas Red, rhodamine B, rhodamine 6G, rhodamine 10, NHS-rhodamine, TMR-iodoacetamide, lissamine rhodamine B sulfonyl chloride, lissamine rhodamine B sulfonyl hydrazine, Texas Red sulfonyl chloride, Texas Red hydrazide, coumarin and coumarin derivatives such as AMCA, AMCA-NHS, AMCA-sulfo-NHS, AMCA-HPDP, DCIA, AMCE-hydrazide, BODIPY and derivatives such as BODIPY FL C3-SE, BODIPY 530/550 C3, BODIPY 530/550 C3-SE, BODIPY 530/550 C3 hydrazide, BODIPY 493/503 C3 hydrazide, BODIPY FL C3 hydrazide, BODIPY FL IA, BODIPY 530/551 IA, Br-BODIPY 493/503, Cascade Blue and derivatives such as Cascade Blue acetyl azide, Cascade Blue cadaverine, Cascade Blue ethylenediamine, Cascade Blue hydrazide, Lucifer Yellow and derivatives such as Lucifer Yellow iodoacetamide, Lucifer Yellow CH, cyanine and derivatives such as indolium based cyanine dyes, benzo-indolium based cyanine dyes, pyridium based cyanine dyes, thiozolium based cyanine dyes, quinolinium based cyanine dyes, imidazolium based cyanine dyes, Cy 3, Cy5, lanthanide chelates and derivatives such as BCPDA, TBP, TMT, BHHCT, BCOT, Europium chelates, Terbium chelates, Alexa Fluor dyes, DyLight dyes, Atto dyes, LightCycler Red dyes, CAL Flour dyes, JOE and derivatives thereof, Oregon Green dyes, WellRED dyes, IRD dyes, phycoerythrin and phycobilin dyes, Malachite green, stilbene, DEG dyes, NR dyes, near-infrared dyes and others such as those described in Haugland, Molecular Probes Handbook, (Eugene, Oreg.) 6th Edition; Lakowicz, Principles of Fluorescence Spectroscopy, 2nd Ed., Plenum Press New York (1999), or Hermanson, Bioconjugate Techniques, 2nd Edition, or derivatives thereof, or any combination thereof. Cyanine dyes may exist in either sulfonated or non-sulfonated forms, and comprise two indolenin, benzo-indolium, pyridium, thiozolium, and/or quinolinium groups separated by a

polymethine bridge between two nitrogen atoms. Commercially available cyanine fluorophores include, for example, Cy3, (which may comprise 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium or 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium-5-sulfonate), Cy5 (which may comprise 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium or 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate), and Cy7 (which may comprise 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium or 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium-5-sulfonate), where "Cy" stands for 'cyanine', and the first digit identifies the number of carbon atoms between two indolenine groups. Cy2 which is an oxazole derivative rather than indolenin, and the benzo-derivatized Cy3.5, Cy5.5 and Cy7.5 are exceptions to this rule.

[1189] As used herein, the term "excitation wavelength" refers to the wavelength of light used to excite a fluorescent indicator (*e.g.*, a fluorophore or dye molecule) and generate fluorescence. Although the excitation wavelength is typically specified as a single wavelength, *e.g.*, 620 nm, it will be understood by those of skill in the art that this specification refers to a wavelength range or excitation filter bandpass that is centered on the specified wavelength. For example, in some instances, light of the specified excitation wavelength comprises light of the specified wavelength ± 2 nm, ± 5 nm, ± 10 nm, ± 20 nm, ± 40 nm, ± 80 nm, or more. In some instances, the excitation wavelength used may or may not coincide with the absorption peak maximum of the fluorescent indicator.

[1190] As used herein, the term "emission wavelength" refers to the wavelength of light emitted by a fluorescent indicator (*e.g.*, a fluorophore or dye molecule) upon excitation by light of an appropriate wavelength. Although the emission wavelength is typically specified as a single wavelength, *e.g.*, 670 nm, it will be understood by those of skill in the art that this specification refers to a wavelength range or emission filter bandpass that is centered on the specified wavelength. In some instances, light of the specified emission wavelength comprises light of the specified wavelength ± 2 nm, ± 5 nm, ± 10 nm, ± 20 nm, ± 40 nm, ± 80 nm, or more. In some instances, the emission wavelength used may or may not coincide with the emission peak maximum of the fluorescent indicator.

[1191] As used herein, fluorescence is 'specific' if it arises from fluorophores that are annealed or otherwise tethered to the surface, such as fluorescently labeled nucleic acid sequences having a region of reverse complementarity to a corresponding segment of an oligonucleotide adapter on the surface and annealed to said corresponding segment. This fluorescence is contrasted with fluorescence arising from fluorophores not tethered to the surface through such an annealing process, or in some cases to background fluorescence of the surface.

[1192] As used herein, a "nucleic acid" (also referred to as a "nucleic acid molecule", a "polynucleotide", "oligonucleotide", ribonucleic acid (RNA), or deoxyribonucleic acid (DNA)) is a linear polymer of two or more nucleotides joined by covalent internucleosidic link ages, or variants or functional fragments thereof. In naturally occurring examples of nucleic acids, the internucleoside linkage is typically a phosphodiester bond. However, other examples optionally comprise other internucleoside linkages, such as phosphorothiolate linkages and may or may not comprise a phosphate group. Nucleic acids include double- and single-stranded DNA, as well as double- and single-stranded RNA, DNA/RNA hybrids, peptide-nucleic acids (PNAs), hybrids between PNAs and DNA or RNA, and may also include other types of nucleic acid modifications.

[1193] The term "nucleotide" as used herein refers to a molecule comprising an aromatic base, a sugar, and a phosphate. A "nucleotide moiety" as referred to here can be a nucleotide or a nucleoside that is modified, such as for example, a nucleotide moiety conjugated to a polymer core or linker (e.g., in a nucleotide conjugate, a polymer-nucleotide conjugate, or a particle-nucleotide conjugate). Canonical or non-canonical nucleotides are consistent with use of the term. The phosphate in some instances comprises a monophosphate, diphosphate, or triphosphate, or corresponding phosphate analog. In some embodiments, "nucleotide" refers to a nucleotide, nucleoside, or analog thereof. In some cases, the nucleotide is an N- or C-glycoside of a purine or pyrimidine base (*e.g.*, a deoxyribonucleoside containing 2-deoxy-D-ribose or ribonucleoside containing D-ribose). Non-limiting examples of other nucleotide analogs include, but are not limited to, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, and the like.

[1194] The term "non-flat" as it relates to a surface described herein refers to a flatness of a surface that deviates from precise flatness by greater than or equal to about 10%. The flatness of a surface may be measured using a flatness gauge. In some embodiments, the "non-flat" surface contains one or more curved portions and the curved portion(s) or the curvature(s) can be perceivable by naked eyes. In some embodiments, the "non-flat" surface contains one or more bent portions and the bent portion(s) can be perceivable by naked eyes. In some embodiments, an acute angle between tangential directions measured at two different points on the "non-flat"

surface, e.g., separated by at least 1 mm, 1 cm, or more, can be greater than 10 degrees, 15 degrees, 20 degrees, or more.

[1195] The term "support" or "sample support structure" are used interchangeable herein to include any solid or semisolid article on which reagents such as nucleic acids can be immobilized. Nucleic acids may be immobilized on the solid support by any method including but not limited to physical adsorption, by ionic or covalent bond formation, or combinations thereof. A solid support may include a polymeric, a glass, or a metallic material. Non-limiting examples of solid supports include a membrane, a planar surface, a microtiter plate, a bead, a filter, a test strip, a slide, a cover slip, and a test tube, any solid phase material upon which an oligomer is synthesized, attached, ligated or otherwise immobilized. A support may comprise a "resin", "phase", "surface," "substrate," "coating," and/or "support." A support may comprise organic polymers such as polystyrene, polyethylene, polypropylene, polyfluoroethylene, polyethyleneoxy, and polyacrylamide, as well as co-polymers and grafts thereof. A support may also be inorganic, such as glass, silica, controlled-pore-glass (CPG), or reverse-phase silica. The configuration of a support may be in the form of beads, spheres, particles, granules, a gel, or a surface. Surfaces may be planar, substantially planar, or non-planar. Supports may be porous or non-porous, and may have swelling or non-swelling characteristics. A support can be shaped to comprise one or more wells, depressions or other containers, vessels, features or locations. A plurality of supports may be configured in an array at various locations. A support may be addressable (e.g., for robotic delivery of reagents), or by detection mechanisms including scanning by laser illumination and confocal or deflective light gathering. An amplification support (e.g., a bead) can be placed within or on another support (e.g., within a well of a second support). The support may be a flow cell, such as a nucleic acid sequencing flow cell. In some embodiments, the support may have a surface that is hydrophilic due to the polymeric material of the support.

[1196] References herein to "one embodiment," "an embodiment," "an example embodiment," "some embodiments," or similar phrases, indicate that the embodiment described may include a particular feature, structure, or characteristic, but every embodiment may not necessarily include the particular feature, structure, or characteristic. Moreover, such phrases are not necessarily referring to the same embodiment. Further, when a particular feature, structure, or characteristic is described in connection with an embodiment, it would be within the knowledge of persons skilled in the relevant art(s) to incorporate such feature, structure, or characteristic into other embodiments whether or not explicitly mentioned or described herein.

[1197] It is to be appreciated that the Detailed Description section, and not any other section, is intended to be used to interpret the claims. Other sections may set forth one or more but not all

examples of embodiments as contemplated by the inventor(s), and thus, are not intended to limit this disclosure or the appended claims in any way.

[1198] While this disclosure describes examples of embodiments for some fields and applications, it should be understood that the disclosure is not limited thereto. Other embodiments and modifications thereto are possible, and are within the scope and spirit of this disclosure. For example, and without limiting the generality of this paragraph, embodiments are not limited to the software, hardware, firmware, and/or entities illustrated in the figures and/or described herein. Further, embodiments (whether or not explicitly described herein) have significant utility to fields and applications beyond the examples described herein.

[1199] Embodiments have been described herein with the aid of functional building blocks illustrating the implementation of specified functions and relationships thereof. The boundaries of these functional building blocks have been arbitrarily defined herein for the convenience of the description. Alternate boundaries may be defined as long as the specified functions and relationships (or equivalents thereof) are appropriately performed. Also, alternative embodiments may perform functional blocks, steps, operations, methods, etc. using orderings different from those described herein.

EXAMPLES

[1200] These examples are provided for illustrative purposes only and not to limit the scope of the claims provided herein.

Example 1 - Design specifications for a fluorescence imaging module for genomics applications

[1201] A non-limiting example of design specifications for a fluorescence imaging module of the present disclosure is provided in Table 2.

[1202] Table 2. Examples of design specifications for a fluorescence imaging module for genomics applications.

Design Parameter	Specification
Numerical aperture	≥ 0.3
Image quality	Diffraction limited
Field-of-view (FOV)	> 2.0 mm^2
Image plane curvature	Best focal plane within 100nm for >90% of the FOV, within 150nm for

Design Parameter	Specification
	99% of the FOV, and within 200nm for the entire FOV
Image distortion	< 0.5% across the FOV
Magnification	2x to 20x
Camera pixel size at sample plane	≥ 2 x optical system modulation transfer function (MTF) limit
Coverslip thickness	> 700 μm
Number of fluorescence imaging channels	≥ 3
Chromatic focal plane difference at camera between all imaging channels	≤ 100 nm equivalent at sample plane
Number of AF channels	1
Imaging time	≤ 2 seconds per FOV
Autofocus	Single step autofocus with error correction
Autofocus accuracy	< 100 nm
Scanning stage step and settle time	< 0.4 seconds
Channel-specific optimized tube lens	1 per imaging channel
Illumination optical path	Liquid light guide with underfilled entrance aperture

Example 2 – Fabrication of glass microfluidic flow cell devices

[1203] Wafer-scale fabrication of microfluidic devices for use as flow cells can be constructed from, for example, one, two, or three layers of glass, *e.g.*, borosilicate glass, fused-silica glass, or quartz, using one of the processed illustrated in FIGS. 36A-36C and a processing technique such as focused femtosecond laser photoablation and/or laser glass bonding.

[1204] In FIG. 36A, a first wafer is processed with a laser (*e.g.*, that produces femtosecond laser radiation) to ablate the wafer material and provide a patterned surface. The patterned wafer surface may comprise a plurality of microfluidic devices (*e.g.*, 12 devices per 210 mm diameter wafer), each of which may comprise a plurality of fluid channels. The processed wafer may then be diced to create individual microfluidic chips comprising open fluid channels that may optionally be subsequently sealed, *e.g.*, by sealing with a film or by clamping the device to another support surface.

[1205] In FIG. 36B, a first wafer is processed to create a patterned surface which may then be placed in contact with and bonded to a second wafer to seal the fluid channels. Depending on the materials used, *e.g.*, glass wafers, silicon wafers, *etc.*, the bonding may be performed using, *e.g.*, a thermal bonding process, an anodic bonding process, a laser glass bonding process, *etc.* The second wafer covers and/or seals the grooves, indentations, and/or apertures on the wafer having the patterned surface to form fluid channels and/or fluid chambers (*e.g.*, the interior portion) of the device at the interface of the two wafer components. The bonded structure may then be diced into individual microfluidic chips, *e.g.*, 12 microfluidic chips per 210 mm diameter wafer.

[1206] In FIG. 36C, the first wafer is processed to create a pattern of fluid channels that are cut or etched through the full thickness of the wafer (*e.g.*, open on either surface of the wafer). The first wafer is then sandwiched between and bonded to a second wafer on one side and a third wafer on the other side. Depending on the materials used, *e.g.*, glass wafers, silicon wafers, *etc.*, the bonding may be performed using, *e.g.*, a thermal bonding process, an anodic bonding process, a laser glass bonding process, *etc.* The second and third wafers cover and/or seal the grooves, indentations, and/or apertures in the first wafer to form fluid channels and/or fluid chambers (*e.g.*, the interior portions) of the device. The bonded structure may then be diced into individual microfluidic chips, *e.g.*, 12 microfluidic chips per 210 mm diameter wafer.

Example 3 – Coating flow cell surfaces with a hydrophilic polymer coating

[1207] Glass flow cell devices were coated by washing prepared glass channels with KOH, followed by rinsing with ethanol and then salinization for 30 minutes at 65°C. Fluid channel surfaces were activated with EDC-NHS for 30 min., followed by grafting of oligonucleotide primers by incubation of the activated surface with 5μm primer for 20 min., and then passivation with 30 μm of an amino-terminated polyethylene glycol (PEG-NH2).

[1208] Multilayer surfaces are made following the approach described above, where following the PEG-NH2 passivation step, a multi-armed PEG-NHS is flowed through the fluid channels, followed by another addition of the PEG-NH2, optionally followed by another incubation with PEG-NHS, and optionally followed by another incubation with multi-armed PEG-NH2. For these surfaces, the primer may be grafted at any step, and especially following the last addition of multi-armed PEG-NH2.

Example 4 – Flow cell devices for nucleic acid sequencing

[1209] FIG. 37A illustrates a non-limiting example of a one-piece glass microfluidic chip / flow cell design. In this design, fluid channels and inlet/outlet holes may be fabricated using, *e.g.*,

focused femtosecond laser radiation. There are two fluid channels ("lanes") in the flow cell device, and each fluid channel comprises, *e.g.*, 2 rows of 26 frames each (*e.g.*, where a "frame" is the image area equivalent to the field-of-view for a corresponding imaging module) each, such that tiling 2 x 26 = 52 images suffices to image an entire fluid channel. The fluid channel can have, *e.g.*, a depth of about 100 μm. Fluid channel 1 has an inlet hole A1 and an outlet hole A2, and fluid channel 2 has an inlet hole B1 and an outlet hole B2. The flow cell device may also comprise a 1D linear, human-readable and/or machine-readable barcode, and optionally a 2D matrix barcode.

[1210] FIG. 37B illustrates a non-limiting example of a two-piece glass microfluidic chip / flow cell design. In this design, fluid channels and inlet/outlet holes may be fabricated using, *e.g.*, focused femtosecond laser photoablation or photolithography and chemical etching processes. The 2 pieces can be bonded together using any of a variety of techniques as described above. The inlet and outlet holes may be positioned on the top layer of the structure and oriented in such a way that they are in fluid communication with at least one of the fluid channels and/or fluid chambers formed in the interior portion of the device. There are two fluid channels in the flow cell device, and as with the device illustrated in FIG. 37A, each fluid channel comprises, *e.g.*, 2 rows with 26 frames in each row. The fluid channels can have, *e.g.*, a depth of about 100 μm. Fluid channel 1 has an inlet hole A1 and an outlet hole A2, and fluid channel 2 has an inlet hole B1 and an outlet hole B2. The flow cell device may also comprise a 1D linear, human-readable and/or machine-readable barcode, and optionally a 2D matrix barcode.

[1211] FIG. 37C illustrates a non-limiting example of a three-piece glass microfluidic chip / flow cell design. In this design, fluid channels and inlet/outlet holes may be fabricated using, *e.g.*, focused femtosecond laser photoablation or photolithography and chemical etching processes. The 3 pieces can be bonded together using any of a variety of techniques as described above. The first wafer (comprising a through-pattern of fluid channels or fluid chambers) can be sandwiched between and bonded to a second wafer on one side and a third wafer on the other side. The inlet and outlet holes may be positioned on the top layer of the structure and oriented in a way such that they are in fluid communication with at least one of the fluid channels and/or fluid chambers formed in the interior portion of the device. There are two fluid channels in the flow cell device, and as with the devices illustrated in FIGS. 37A and 37B, each fluid channel has 2 rows with 26 frames in each row. The fluid channel can have a depth of, *e.g.*, about 100 μm. Fluid channel 1 has an inlet hole A1 and an outlet hole A2, and fluid channel 2 has an inlet hole B1 and an outlet hole B2. The flow cell device may also comprise a 1D linear, human-readable and/or machine-readable barcode, and optionally a 2D matrix barcode.

Example 5 – Imaging of nucleic acid clusters in a capillary flow cell

[1212] Nucleic acid clusters were established within a capillary and subjected to fluorescence imaging. A flow device having a capillary tube was used for the test. An example of the resulting cluster images is presented in FIG. 38. The figure demonstrated that nucleic acid clusters formed by amplification within the lumen of a capillary flow cell device as disclosed herein can be reliably formed and visualized.

Example 6 – Plastic sample support structures

[1213] In some instances, the disclosed sample support structures may be fabricated from a polymer. Examples of materials from which the sample support structure, *e.g.*, a capillary flow cell device, may be fabricated include, but are not limited to, polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET), or any combination thereof. Various compositions comprising both glass and plastic substrates are also contemplated.

[1214] Modification of a polymer surface for the surface coating purposes disclosed herein involves making surfaces reactive with other chemical groups (-R), including amines. When prepared on an appropriate substrate, these reactive surfaces can be stored long term at room temperature, for example, for at least 3 months or more in some instances. Such surfaces can be further grafted with R-PEG and R-primer oligomer for on-surface amplification of nucleic acids, as described elsewhere herein. Plastic surfaces, such as cyclic olefin polymer (COP), may be modified using any of a variety of methods known in the art. For example, they can be treated with Ti:Sapphire laser ablation, UV-mediated ethylene glycol methacrylate photografting, plasma treatment, or mechanical agitation (*e.g.*, sand blasting, or polishing, *etc.*) to create hydrophilic surfaces that can remain reactive for months towards a variety of chemical groups, such as amines. These groups may then allow conjugation of passivation polymers such as PEG, or biomolecules such as DNA or proteins, without loss of biochemical activity. For example, attachment of DNA primer oligomers allows DNA amplification on a passivated plastic surface while reducing or minimizing the non-specific adsorption of proteins, fluorophore molecules, or other hydrophobic molecules.

[1215] Additionally, in some instances, surface modification can be combined with, *e.g.*, laser printing or UV masking, to create patterned surfaces. This allows patterned attachment of DNA oligomers, proteins, or other moieties, providing for surface-based enzymatic activity, binding, detection, or processing. For example, DNA oligomers may be used to amplify DNA only within patterned features, or to capture amplified long DNA concatemers in a patterned fashion.

In some embodiments, enzyme islands may be generated in the patterned areas that are capable of reacting with solution-based substrates. Because plastic surfaces are especially amenable to these processing modes, in some embodiments as contemplated herein, plastic sample support surfaces or flow cell devices may be recognized as being particularly advantageous.

[1216] Furthermore, plastic can be injection molded, embossed, stamped, or 3D printed to form any shape, including microfluidic devices, much more easily than glass substrates, and thus can be used to create surfaces for the binding and analysis of biological samples in multiple configurations, *e.g.*, sample-to-result microfluidic chips for biomarker detection or DNA sequencing.

[1217] Specific and localized DNA amplification on modified plastic surfaces can be performed to produce nucleic acid spots with an ultra-high contrast to noise ratio and very low background when probed with fluorescent labels.

[1218] Hydrophilized and amine-reactive cyclic olefin polymer surface with amine-primer and amine-PEG can be prepared and has been demonstrated to support rolling circle amplification. When probed with fluorophore labeled primers, or when labeled dNTPs were added to the hybridized primers by a polymerase, bright spots of DNA amplicons were observed that exhibited signal to noise ratios greater than 100 with backgrounds that are extremely low, indicating highly specific amplification, and ultra-low levels of nonspecific protein and hydrophobic fluorophore binding, which are hallmarks of the high accuracy detection required for systems such as fluorescence-based DNA sequencers.

Example 7 – Prophetic example of the use of a structured illumination imaging system for sequencing

[1219] A structured illumination imaging system 4100 such as the non-limiting example illustrated in FIG. 41 may be used in combination with a flow cell 4187 comprising a low non-specific binding surface to perform nucleic acid sequencing. Target nucleic acid sequences are hybridized to adapter/primer sequences attached to the low non-specific binding surface 4188 on the interior of the flow cell 4187 at high surface density and clonally amplified using hybridization and amplification buffers that are specially formulated for said surface to enhance specific hybridization and amplification rates.

[1220] The flow cell 4187 is mounted in the structured illumination imaging system 4100, and a sequencing reaction cycle comprising the use of, *e.g.*, the nucleotide conjugate chemistry described above and the workflow illustrated in FIG. 40 is initiated. The fluorescently labeled nucleotide conjugate is introduced into the flow cell 4187 and contacted with the surface 4188 to form multivalent binding complexes if the nucleotide moiety of the nucleotide conjugate is

complementary to a nucleotide of the target sequence. Excess, unbound nucleotide conjugate is then rinsed away.

[1221] For each detection step, a series of images of surface 4188 are captured using different orientations of a diffraction grating, *e.g.*, 4130A, in at least one branch of an illumination optical path and at several different positions of an optical phase modulator, *e.g.*, 4140A, to project illumination light fringe patterns onto the surface 4188. Following image acquisition, the series of images are processed using an image reconstruction algorithm to generate a higher resolution image than that achievable using diffraction-limited optics alone. The process may be repeated for several positions on surface 4188 to create a tiled image of the interior flow cell surface. Optionally, the focal plane may be adjusted, and the process may be repeated to generate higher resolution images of a second interior flow cell surface 4189.

[1222] The combination of high contrast-to-noise ratio images (achieved using the disclosed low-binding surfaces with multiply-labeled nucleotide conjugate sequencing chemistry) and efficient processing of a relatively small number of images acquired using a structured illumination imaging system to image flow cell surfaces at super-resolution (thus enabling the use of higher surface densities of target sequence clusters) may contribute to higher overall sequencing throughput.

Example 8 – Prophetic example of using a multiplexed read-head for multiple surface imaging

[1223] A multiplexed read-head such as that illustrated schematically in FIGS. 44A and 44B is designed to perform multiple surface imaging. The read-head comprises a plurality of microfluorometers which are assembled so that they are held in a fixed position relative to one another and may be scanned in a direction horizontal to a pair of opposed interior flow cell surfaces to acquire images of a swath of each surface. As illustrated in FIG. 44A, a first subset of the plurality of microfluorometers is configured to acquire images of a first interior flow cell surface, and a second subset of the plurality of microfluorometers is configured to acquire images of a second interior flow cell surface that faces the first interior surface and is separated from it by the thickness of an intervening fluid channel. In some cases, the horizontal direction can be parallel to the flow cell. In some cases, a vertical direction can be perpendicular or orthogonal to the flow cell.

[1224] A flow cell comprising a low non-specific binding surface coating is used to perform nucleic acid sequencing. Target nucleic acid sequences are hybridized to adapter/primer sequences attached to the low non-specific binding surfaces on the interior of the flow cell and clonally amplified using hybridization and amplification buffers that are specially formulated for said surfaces to enhance specific hybridization and amplification rates.

[1225] The flow cell is mounted in an imaging system comprising the multiplexed read-head, and a sequencing reaction cycle comprising the use of, *e.g.*, the nucleotide conjugate chemistry described above and the workflow illustrated in FIG. 40 is initiated. The fluorescently labeled nucleotide conjugate is introduced into the flow cell and contacted with the interior surfaces to form multivalent binding complexes if the nucleotide moiety of the nucleotide conjugate is complementary to a nucleotide of the target sequence. Excess, unbound nucleotide conjugate is then rinsed away.

[1226] For each detection step, the multiplexed read-head is scanned in at least one direction parallel to the interior surfaces of the flow cell (or the flow cell may be scanned relative to the multiplexed read-head) and images of both the first and second interior flow cell surfaces are acquired simultaneously, as illustrated in FIG. 44B, while an autofocus mechanism maintains the proper working distance between the objectives of the multiplexed read-head and at least one of the interior flow cell surfaces.

[1227] The ability to image both flow cell surfaces simultaneously using a single-pass scan of the flow cell (depending on the design of the read-head) may provide significant improvements in sequencing throughput.

Example 9 – Prophetic example of using an optical system

[1228] The purpose of this example is to demonstrate sequencing of a nucleic acid sequence using an optical system as described herein. Such an optical system provides additional advantages and utility for nucleic acid sequencing applications due to reduced optical components, less moving parts and higher throughput.

[1229] In this example, a sample 4515 is delivered to a hydrophobic pad 4516 of a flow cell 4521 by a liquid handling system 4514 as shown in FIG. 45. The sample 4515 is drawn into an interior channel 4517of the flow cell 4521 by a vacuum pump 4518. Nucleic acid sequences present in the sample react with primers attached to walls of the interior channel 4517 of the flow cell 4521. The nucleic acid sequences of the sample are then amplified and washed. After amplification and washing, a solution containing: (1) DAPI modified nucleotide conjugate complementary to A nucleotides; (2) FITC modified nucleotide conjugate complementary to G nucleotides; (3) TRITC modified nucleotide conjugate complementary to C nucleotides; and (4) a fourth nucleotide conjugate modified with both DAPI and TRITC that is complementary toward T nucleotides is introduced to the flow cell 4521 and allowed to react with the primed nucleic acid sequence. The sample in the flow cell 4521 is then illuminated by a 0.1 second pulse of UV-blue light via a first LED light source 4522 thus exciting the DAPI fluorophore. In synchronization with the UV-blue light pulse, the imaging sensors acquire a first image

capturing emission of light given off by any DAPI modified nucleotide conjugate bound specifically to the sample. Only light emitted by DAPI fluorescence emission is collected by the imaging sensors because the UV-blue excitation light emitted by the first light source is negligible past 405 nm. This light is blocked by a tri-band bandpass filter (Edmund Scientific stock # 87-236) with multi-band center wavelengths at 432 nm, 517 nm and 615 nm. For this filter, bandwidths are 36 @ 432 nm, 23 @ 517 nm and 61 @ 615 nm. Next, the sample is pulsed with 0.1 seconds of green light via a second LED light source 4523, capable of exciting the FITC fluorophore. In synchronization with the green light pulse, a second image is acquired capturing emission of light given off by FITC modified nucleotide conjugate bound specifically to the sample. The sample can then be pulsed with 0.1 seconds of red light via a third LED light source 4524 thus exciting the TRITC fluorophore. In synchronization with the red light pulse, a third image is acquired capturing emission of light given off by any TRITC modified nucleotide conjugate bound specifically to the sample. In this example, excitation filters are used for each LED light source to minimize fluorescence channel cross-talk, or bleed-through of the excitation light into the emission bandpasses (notches) of the tri-band bandpass filter.

[1230] In this example, the base calling process, shown schematically 4602, in FIG. 46 is as follows. The first image of the cycle is analyzed for regions of interest (ROI) showing strong fluorescence signal. ROI's showing strong fluorescence signal in the first image indicate nucleic acid amplicons with either A or T at the open position prior to exposure to the nucleotide conjugates for the following reason. Capture of the first image was synchronized with sample illumination by UV-blue light, thus exciting DAPI. Since the nucleotide conjugates complementary toward A were labeled with DAPI and nucleotide conjugates complementary toward T were labeled with both DAPI and TRITC, ROI's of the first image showing strong fluorescence indicate either an A or T. Next, the second image of the cycle is analyzed for ROI's strong fluorescence signal. Since nucleotide conjugates complementary toward G were labeled with FITC and since capture of the second image was synchronized with the green pulse capable of exciting FTIC, ROI's in the second image showing strong fluorescent signal indicate G. Next, the third image of the cycle is analyzed for ROI's strong fluorescence signal. These ROI's indicate nucleic acid amplicons with either C or T present at the open position prior to exposure to the nucleotide conjugates. This is because in synchronization with the capture of the third image, the sample is illuminated with red light, thus exciting TRITC. Nucleotide conjugates complementary to C are labeled with TRITC and nucleotide conjugates complementary toward T are labeled with both DAPI and TRITC. ROI's with strong fluorescence signal observed in both the first and third image indicate a T nucleotide at the open position prior to exposure to the nucleotide conjugates. Identification of ROI's containing T's then allows for identification

ROI's containing of A and C. The sequencing and imaging cycle is repeated until the entire nucleic acid sequence has been identified.

Example 10 –Prophetic example of using a super resolution enhanced optical system

[1231] The purpose of this example is to demonstrate sequencing of a nucleic acid sequence using a super resolution enhanced optical system as described herein. Such a system provides additional advantages and utility for nucleic acid sequencing applications due to reduced optical components, less moving parts and higher throughput, while providing for super high resolution readout.

[1232] In this example, a sample is delivered to capillary flow cell 5201 as shown in FIGS. 53A and B. Sample sites 4902 comprising nucleic acid sequences present in the sample react with primers attached to walls of the interior channel of the capillary flow cell 5201. The nucleic acid sequences of the sample are then amplified and washed. After amplification and washing, a solution containing (1) DAPI modified nucleotide conjugate complementary to A nucleotides; (2) FITC modified nucleotide conjugate complementary to G nucleotides; (3) TRITC modified nucleotide conjugate complementary to C nucleotides; and (4) a fourth nucleotide conjugate modified with both DAPI and TRITC that is complementary toward T nucleotides is introduced to the capillary flow cell 5201 and allowed to react with the primed nucleic acid sequence. The sample in the capillary flow cell 5201 is then illuminated by a 0.1 second pulse of UV-blue light via a first LED light source of the light source 4901 thus exciting the DAPI fluorophore. In synchronization with the UV-blue light pulse, the imaging sensors acquire a first image capturing emission of light given off by any DAPI modified nucleotide conjugate bound specifically to the sample. Only light emitted by DAPI fluorescence emission is collected by the imaging sensors because the UV-blue excitation light emitted by the first light source is negligible past 405 nm. This light is blocked by a tri-band band stop filter 4910. Next, the sample is pulsed with 0.1 seconds of green light via a second LED light source of the light source 4902, capable of exciting the FITC fluorophore. In synchronization with the green light pulse, a second image is acquired capturing emission of light given off by FITC modified nucleotide conjugate bound specifically to the sample. The sample is pulsed with 0.1 seconds of red light via a third LED light source of the light source 4901 thus exciting the TRITC fluorophore. In synchronization with the red light pulse, a third image is acquired capturing emission of light given off by any TRITC modified nucleotide conjugate bound specifically to the sample. In this example, excitation filters are used for each LED light source to minimize fluorescence channel cross-talk, or bleed-through of the excitation light that may not be stopped by the notches, or band stops of the tri-band band stop filter 4910.

[1233] A wedge block 4916 can be included in each optical subsystem 4914 in order to image the entire inner surface of the capillary flow cell 5201. As shown in FIG. 54A when the top-wedge piece 4907 is aligned with the bottom wedge piece 4906 the optical subsystems 4916 acquire images on the far side of the inner surface of the capillary flow cell. As shown in FIG. 54B when the top-wedge piece 4907 is moved out of alignment to increase the optical pathlength 4913 the optical subsystems 4916 acquire images on the front interior surface of the capillary flow cell 5201.

[1234] The optical system in this example is capable of super resolution imaging, wherein at least one sample site comprising clonally-amplified, sample nucleic acid molecules immobilized to a plurality of attached oligonucleotide molecules, wherein said plurality of immobilized clonally amplified sample nucleic acid molecules are present at distance less than $\lambda/(2*NA)$, wherein λ is the center wavelength of an light source and NA is the numerical aperture of an imaging system. A stochastic photo-switching chemistry is then applied to said clonally amplified sample nucleic acid molecules at the same time to cause said plurality of clonally amplified sample nucleic acid molecules to fluoresce in on and off events in up to four different colors by stochastic photo-switching; and on and off events are detected in a color channel for each color in real-time as the on and off events are occurring for said plurality of clonally amplified sample nucleic acid molecules to determine an identify of a nucleotide of said clonally amplified sample nucleic acid molecule.

[1235] In this example, the base calling process, shown schematically 4602, in FIG. 46 is as follows. The first image of the cycle is analyzed for regions of interest (ROI) showing strong fluorescence signal. ROI's showing strong fluorescence signal in the first image indicate nucleic acid amplicons with either A or T at the open position prior to exposure to the nucleotide conjugates for the following reason. Capture of the first image was synchronized with sample illumination by UV-blue light, thus exciting DAPI. Since the nucleotide conjugates complementary toward A were labeled with DAPI and nucleotide conjugates complementary toward T were labeled with both DAPI and TRITC, ROI's of the first image showing strong fluorescence indicate either an A or T. Next, the second image of the cycle is analyzed for ROI's strong fluorescence signal. Since nucleotide conjugates complementary toward G were labeled with FITC and since capture of the second image was synchronized with the green pulse capable of exciting FTIC, ROI's in the second image showing strong fluorescent signal indicate G. Next, the third image of the cycle is analyzed for ROI's strong fluorescence signal. These ROI's indicate nucleic acid amplicons with either C or T present at the open position prior to exposure to the nucleotide conjugates. This is because in synchronization with the capture of the third image, the sample is illuminated with red light, thus exciting TRITC. Nucleotide conjugates

complementary to C are labeled with TRITC and nucleotide conjugates complementary toward T are labeled with both DAPI and TRITC. ROI's with strong fluorescence signal observed in both the first and third image indicate a T nucleotide at the open position prior to exposure to the nucleotide conjugates. Identification of ROI's containing T's then allows for identification ROI's containing of A and C. The sequencing and imaging cycle is repeated until the entire nucleic acid sequence has been identified.

Example 11 –Preparation of Nucleotide-Arms

[1236] In a 1.5 mL Eppendorf tube, 320 uL of biotin-5k PEG-SVA (from Laysan Bio) was mixed with 33% DMF to produce a concentration of 25 mM of the Biotin-5k PEG-SVA. In a separate tube, add 440 uL buffer (0.2 M $NaHCO_3$ Na_2CO_3 pH 9) and 200 uL of dGTP-PA-NH_2 (10 mM stock, from MyChem), the tube was centrifuged. The dissolved biotin-5k PEG SVA was added to the second tube and incubated at room temperature for 1 hour. The reaction was purified via ion exchange chromatography.

[1237] The nucleotide-arm comprising an azido group was synthesized as follows. The FMOC N3 linker was obtained from a commercial source. An NHS ester synthesis reaction was conducted by mixing together one equivalent of the N3 linker, one equivalent of disuccinimidyl carbonate (DSC), one equivalent of 4-dimethylaminopyridine (DMAP), with anhydrous N,N-dimethylformamide (DMF), at room temperature for 1 hour. The conjugation to propargyl-amine dNTPs was conducted by reacting three equivalents of the NHS-ester solution with one equivalent of propargyl-dATP, with reaction buffer, at room temperature, for 1 hour.

Example 12 – Preparation of Streptavidin Core

[1238] Ten mg of streptavidin (Anaspec, catalog No. AS-72177) was dissolved in 525 uL of freshly-prepared 1X PBS buffer (pH 8), and centrifuged for 5 minutes at 14,000 rcf at 4 °C to aggregate the protein. The concentration of the mixture was analyzed via Nanodrop at absorption 280 nm, with ε = 179200 M-1 cm-1 for the tetramer (assuming MW = 56,000). The mixture was diluted 1:10 with water.

[1239] The fluorophore NHS ester was prepared as a 25 mM stock in DMSO. In a 5 mL Eppendorf tube, DMSO and modified 1X PBS buffer (pH8 with 0.01% Tween) and streptavidin was added. The fluorophore was added slowly, and incubated in the dark at room temperature for 7 hours. The reaction was quenched by adding 100 uL of 1M glyicine (pH 9). The mixture was centrifuged for 5 minutes at 14,000 rcf at 4 °C, and any precipitate was discarded. Unreacted fluorophore was removed using an Amicon Ultra-15 filter.

Example 13 – Preparation of Multivalent Molecules

[1240] One type of multivalent molecule was prepared by reacting propargylamine dNTPs with Biotin-PEG-NHS. This aqueous reaction was driven to completion and purified to produce a

biotin-PEG-dNTP species. In separate reactions, several different PEG lengths were used, corresponding to average molecular weights varying from 1K Da to 20K Da. The Biotin-PEG-dNTP species were mixed with either freshly prepared or commercially-sourced dye-labeled streptavidin (SA) using a Dye: SA ratio of approximately 3-5:1. Mixing of biotin-PEG-dNTP with dye-labeled streptavidin was conducted in the presence of excess biotin-PEG-dNTP to ensure saturation of the biotin binding sites on each streptavidin tetramer. Complete complexes were purified away from excess biotin-PEG-dNTP by size exclusion chromatography. Each type of multivalent nucleotide having either dATP, dGTP, dCTP or dTTP nucleotide units was conjugated and purified separately, then mixed together to create a four base mixture for nucleotide binding, nucleotide incorporation and nucleic acid sequencing reactions.

[1241] Another type of multivalent molecules was prepared in a single pot by reacting multi-arm PEG NHS with excess dye-NH_2 and propargylamine dNTPs. Various multi-arm PEG NHS variants were used ranging from 4-16 arms and ranging in molecular weight from 5K Da to 40K Da. After reacting, excess small molecule dye and dNTP were removed by size exclusion chromatography. Each type of multivalent nucleotide having either dATP, dGTP, dCTP or dTTP nucleotide units was conjugated and purified separately, then mixed together to create a four base mixture for nucleotide binding, nucleotide incorporation and nucleic acid sequencing reactions.

[1242] The single pot method is described herein. In a 2 mL Eppendorf tube, mix 914.1 uL of water, 150 uL of acetonitrile, 112.5 uL of TEAB, 51.6 uL of the biotinylated nucleotide-arms, and 271.7 uL of the labeled streptavidin core. The mixture was incubated for 15 minutes at room temperature in the dark. Unreacted biotinylated nucleotide-arms were removed with Amicon Ultra-4.

Example 14 – Trapping Assays on Plates

[1243] Trapping assays were conducted to determine the capability of a nucleotide unit (as part of a multivalent molecule) to bind a complexed polymerase. The trapping assays were conducted under conditions that permit binding of the nucleotide unit to the complexed polymerase but without incorporation. The complexed polymerase included a polymerase bound to a nucleic acid template molecule which is hybridized to a primer.

[1244] Wells of 394-well plates were coated with PEG-silane. Single-stranded polonies of template molecules (clonally-amplified) were prepared in the wells. A sequencing primer was hybridized to the polonies.

[1245] Trapping assay using nucleotides having a 3' azido-blocked moiety: The wells were pre-washed once with 20 mM TRIS pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 10 mM $MgSO_4$. Azido-blocked nucleotides were incorporated in 20 mM TRIS pH 8.8, 10 mM $(NH_4)_2SO_4$, 10

mM KCl, 10 mM $MgSO_4$, 5 uM dNTP-N3, 600 nM a polymerase at 55 °C for 5 minutes. The wells were washed six times with 50 mM TRIS pH 8.0, 1 mM EDTA pH 7.5, 750 mM NaCl, 0.02% Tween-20.

[1246] Trapping assay using multivalent molecules: The wells were washed once with 10 mM TRIS pH 8.0, 0.5 mM EDTA, 50 mM NaCl. Trap reactions were performed by adding 10 mM TRIS pH 8.0, 2 M Betaine, 1% Triton X-100, 0.48 uM polymerase, 10 mM $CaCl_2$, 0.5 mM EDTA, 100 mM NaCl, 20-160 nM fluorescently-labeled multivalent molecules, for 45 seconds at 45 °C. The wells were washed 5 times with 10 mM TRIS pH 8.0, 2 M betaine, 10 mM $CaCl_2$, 100 mM NaCl, 0.5 mM EDTA, 1% Triton X-100.

[1247] The trapping assay using the multivalent molecules were suitable for forming a plurality of avidity complexes on concatemer template molecules (e.g., polonies). For example, the trapping assays comprise: (a) binding a first nucleic acid primer, a first polymerase, and a first multivalent molecule to a first portion of a concatemer template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first polymerase; and (b) binding a second nucleic acid primer, a second polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms a first avidity complex.

[1248] The surfaces were imaged using epifluorescence and the signal intensity was determined using the 90th percentile. The data is shown in FIGS. 55 and 56.

[1249] The data in FIG 55 shows that dA multivalent molecules (dATP nucleotide unit) produce optimal signals using PA, PA11, or PA23 linkers. The dC multivalent molecules (dCTP nucleotide units) produce an optimal signal when carrying the N3 linker. It is notable that multivalent molecules carrying the PA linker produces an optimal signal when linked to a dA (dATP) nucleotide unit, however multivalent molecules carrying the same linker and Cy5 dye combination fails to produce an optimal signal when linked to a dC (dCTP) nucleotide unit.

[1250] The data in Figure 56 show that signal intensity varied as a function of linker and concentration.

Example 15 – Trapping Assays on Flow Cells

[1251] Trapping assays were conducted to determine the capability of a nucleotide unit (as part of a multivalent molecule) to bind a complexed polymerase. The trapping assays were conducted under conditions that permit binding of the nucleotide unit to the complexed polymerase but without incorporation. A complexed polymerase includes a polymerase bound to a nucleic acid template molecule which is hybridized to a primer.

[1252] Fluorescently-labeled multivalent molecules carrying Linker-6 were prepared. Labeled multivalent molecules carrying the N3-Linker, Linker-8 or 11-atom Linker (sometimes called 'PA' Linker) were also prepared. The multivalent molecules were labeled with fluorophores CF680, CF532, CF570 or AF647.

[1253] Mixes of multivalent molecules carrying two different color fluorophores were prepared. One mix contained 20 nM or 80 nM of each of dCTP-CF680 and dUTP-CF532 multivalent molecules. Another mix contained 20 nM or 80 nM of each of dATP-AF647 and dGTP-CF570 multivalent molecules. Each of these mixes were prepared for multivalent molecules having a different linker: N3-Linker; Linker-6 (A-linker); Linker-8 (mAMBA-linker); or 11 atom Linker (also called PA Linker). For example, a first mix contained 20 nM of dCTP-CF680 and dUTP-CF532 multivalent molecules carrying N3-Linkers. A second mix contained 20 nM of dCTP-CF680 and dUTP-CF532 multivalent molecules carrying Linker-6. Twelve different mixes were prepared. Each mix also contained 0.1 uM sequencing polymerase, 5 mM strontium acetate, a buffering compound, EDTA, a salt, detergent and viscosity additives. The strontium acetate was included in the mixes to promote binding of the nucleotide units of the multivalent molecules to the complexed polymerases without incorporation. Individual complexed polymerases included a polymerase bound to a template molecule which was hybridized to a primer.

[1254] Single-stranded concatemer template molecules were immobilized on a flow cell. The template molecules were hybridized with sequencing primers. The flow cell was loaded into a sequencing apparatus configured to deliver laser excitation to the flow cell and obtain fluorescent images from the flow cell.

[1255] Repeat cycles of binding reactions were conducted. Each binding cycle included the following general method: flowing a multivalent mix and incubation; washing; imaging; and washing. The flow cell was pre-washed, then flowed with a mix of labeled multivalent molecules and incubated for a different length of time (e.g., 2 – 180 seconds). The flow cell was washed. The flow cell was imaged using epifluorescence of a red and green channel, and the signal intensity was determined using the 90th percentile. The flow cell was washed. The binding cycles were repeated 62 times for the mixes containing dATP-AF647 and dGTP-CF570 multivalent molecules, and 71 times for the mixes containing dCTP-CF680 and dUTP-CF532 multivalent molecules.

[1256] The trapping assay using the multivalent molecules were suitable for forming a plurality of avidity complexes on concatemer template molecules (e.g., polonies). For example, the trapping assays comprise: (a) binding a first nucleic acid primer, a first polymerase, and a first multivalent molecule to a first portion of a concatemer template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the

first polymerase; and (b) binding a second nucleic acid primer, a second polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms a first avidity complex.

[1257] In FIGS. 57 and 58, the data for N3-Linker molecules are in green, Linker-molecules are in blue, Linker-8 molecules are in red, and 11 atom Linker molecules are in purple.

[1258] The data in Figure 57 generally shows that multivalent molecules at a concentration of 20 nM or 80 nM, and having dCTP or dUTP nucleotide units, and labeled with CF680 or CF532, the N3-Linker generated the highest signal intensities at all binding times tested, the Linker-6 molecules generated the next highest signal intensities, Linker-8 molecules generated lower signal intensities, and the 11 atom Linker molecules generated the lowest signal intensities.

[1259] The data in Figure 58 generally shows that multivalent molecules at a concentration of 20 nM or 80 nM, and having dATP nucleotide units, and labeled with AF647, the N3-Linker generated the highest signal intensities at all binding times tested, the Linker-6 molecules generated the next highest signal intensities, Linker-8 molecules generated lower signal intensities, and the 11 atom Linker molecules generated the lowest signal intensities.

[1260] The data in Figure 58 generally shows that multivalent molecules at a concentration of 20 nM or 80 nM, and having dGTP nucleotide units, and labeled with CF570, the N3-Linker generated the highest signal intensities at all binding times tested, and the Linker-8 molecules generated the lowest signal intensities. The Linker-6 and 11 atom Linker molecules generated similar signal intensities that were lower than the intensities of the N3-Linker molecules and higher than the Linker-8 molecules.

[1261] The data in FIGS. 57 and 58 indicate that signal intensities generated by labeled multivalent molecules binding to complexed polymerases may be impacted by the linker structure, the nucleotide unit, the fluorophore dye, or a combination thereof.

Example 16 – Real-Time Imaging of Trapping on a Microscope

[1262] Real-time trapping assays were conducted to determine the binding kinetics of a nucleotide unit (as part of a multivalent molecule) to bind a complexed polymerase. The real-time trapping assays were conducted under conditions that permit binding of the nucleotide unit to the complexed polymerase but without incorporation. The complexed polymerase included a polymerase bound to a nucleic acid template molecule which is hybridized to a primer.

[1263] A trap mix with quencher was prepared, which included: Tris HCl (pH 8.8), EDTA (pH 7.5), NaCl, Triton X-100, strontium acetate, sucrose, and a combination of reagents that can act

as singlet oxygen quenchers. Sequencing polymerase was added to the trap/quencher mix to generate a trap/quencher/enzyme mix. The trap/quencher/enzyme mix was split into twelve separate aliquots, and each aliquot was mixed with one type of multivalent molecule at a concentration of 2.5 nM, 7.5 nM or 15 nM (e.g., the multivalent molecules included nucleotide units dATP, dGTP, dCTP or dUTP) to generate twelve separate enzyme/multivalent molecule mixes. The multivalent molecules in each of the twelve separate mixes were labeled with either a red or green fluorophore. Different enzyme/multivalent molecule mixes were prepared to test and compare multivalent molecules carrying a different linker, including Linker 6, 10, 11, 12, 13, 14, 15 or 16.

[1264] A flow cell having immobilized concatemer template molecules was prepared. The flow cell was loaded into a sequencing apparatus configured to deliver laser excitation to the flow cell and obtain fluorescent images from the flow cell (e.g., a flow cell as described elsewhere herein, a flow cell coupled to a microscope as described elsewhere herein, etc.). The enzyme/multivalent molecule mixes were flowed onto the flow cell. Images were obtained for 0.25 second exposure time (e.g., 400 images were obtained for 100 seconds). The signal intensities of the images were plotted and fitted to a single-phase exponential curve to determine the K value, and upper and lower limits. The results are shown in FIGS. 59, 60 and 61. The legend shown in FIG. 60 is also applicable to the date in FIG. 59.

Example 17 – Sequencing by avidity system

[1265] Figure 62 generally shows an example of a combined sequencing by avidity system, according to some embodiments. A system can comprise a flow cell 5901. The flow cell can be as described elsewhere herein (e.g., a flow cell of Example 16). The flow cell can be configured with a plurality of immobilized concatemer template molecules 5902 on a substrate 5904 as described elsewhere herein. The template molecules can be configured to form a concatemer template molecule (e.g., polony) to which the multivalent molecules 5903 can be configured to bind. The multivalent molecules may be as described elsewhere herein. The system may comprise an optical system 5905. The optical system may be as described elsewhere herein. For example, the optical system can be configured with a light source, filter, and sensor as described elsewhere herein. Additional elements may also be present in the system (e.g., reagent storage, fluidic systems, pumps, etc.). In some cases, the system can be configured as described elsewhere herein to implement the methods described elsewhere herein.

Example 18- Sequencing using multivalent molecules

[1266] A two-stage sequencing reaction was conducted on a flow cell having a plurality of concatemer template molecules immobilized thereon.

[1267] The first-stage sequencing reaction was conducted by hybridizing a plurality of soluble sequencing primer(s) to the immobilized concatemers to form immobilized primer-concatemer duplexes. A plurality of a first sequencing polymerase was flowed onto the flow cell (e.g., contacting the immobilized primer-concatemer duplexes) and incubated under a condition suitable to bind the sequencing polymerase to the duplexes to form complexed polymerases. A mixture of fluorescently labeled multivalent molecules (e.g., at a concentration of about 20-100 nM) was flowed onto the flow cell in the presence of a buffer that included a non-catalytic cation (e.g., strontium, barium, calcium, or combination thereof) and incubated under conditions suitable to bind complementary nucleotide units of the multivalent molecules to the complexed polymerases to form avidity complexes without polymerase-catalyzed incorporation of the nucleotide units. The complexed polymerases were washed. An image was obtained of the fluorescently labeled multivalent molecules that remained bound to the complexed polymerases. The first sequencing polymerases and multivalent molecules were removed, while retaining the sequencing primers hybridized to the immobilized concatemers (retained duplexes), by washing with a buffer comprising a detergent.

[1268] The first stage sequencing reaction was suitable for forming a plurality of avidity complexes on concatemer template molecules (e.g., polonies). For example, the first stage sequencing reaction comprises: (a) binding a first nucleic acid primer, a first polymerase, and a first multivalent molecule to a first portion of a concatemer template molecule thereby forming a first binding complex, wherein a first nucleotide unit of the first multivalent molecule binds to the first polymerase; and (b) binding a second nucleic acid primer, a second polymerase, and the first multivalent molecule to a second portion of the same concatemer template molecule thereby forming a second binding complex, wherein a second nucleotide unit of the first multivalent molecule binds to the second polymerase, wherein the first and second binding complexes which include the same multivalent molecule forms a first avidity complex.

[1269] The second-stage sequencing reaction was conducted by contacting the retained duplexes with a plurality of second sequencing polymerases to form complexed polymerases. A mixture of fluorescently labeled nucleotide analogs (e.g., 3'O-methylazido nucleotides) (e.g., about 1-5 uM) was added to the complexed polymerases in the presence of a buffer that included a catalytic cation (e.g., magnesium, manganese, or a combination of magnesium and manganese) and incubated under conditions suitable to bind complementary nucleotides to the complexed polymerases and promote polymerase-catalyzed incorporation of the nucleotides to generate a nascent extended sequencing primer. The complexed polymerases were washed. An image was obtained of the incorporated fluorescently labeled nucleotide analogs as a part of the complexed polymerases. The incorporated fluorescently labeled nucleotide analogs were reacted with a

cleaving reagent that removes the 3' O-methylazido group and generates an extendible 3'OH group.

[1270] In an alternative second stage sequencing reaction, a mixture of non-labeled nucleotide analogs (e.g., 3'O-methylazido nucleotides) (e.g., about 1-5 uM) was added to the complexed polymerases in the presence of a buffer that included a catalytic cation (e.g., magnesium, manganese, or a combination of magnesium and manganese) and incubated under conditions suitable to bind complementary nucleotides to the complexed polymerases and promote polymerase-catalyzed incorporation of the nucleotides to generate a nascent extended sequencing primer. The complexed polymerases were washed. No image was obtained. The incorporated non-labeled nucleotide analogs were reacted with a cleaving reagent that removes the 3' O-methylazido group and generates an extendible 3'OH group.

[1271] The second sequencing polymerases were removed, while retaining the nascent extended sequencing primers hybridized to the concatemers (retained duplexes), by washing with a buffer comprising a detergent. Recurring sequencing reactions were conducted by performing multiple cycles of first-stage and second-stage sequencing reactions to generate extended forward sequencing primer strands.

Example 19 - Sequencing in situ samples using an optical system with wide FOV

[1272] AN optical system as described herein which is capable of providing ultrawide FOV with homogenous illumination (e.g., less than 10% illumination energy variance) is used to image in situ sample(s) for detecting morphological features, RNAs, and proteins within the cells. The in situ sample(s) are immobilized on a flow cell device. The in situ sample(s) are volumetric and require sequencing on at least two different z levels that are displaced from each other along the z direction. The in situ sample(s) include 16 different types of cells. Two sequencing cycles are used to decode the probes for labeing the sample(s), each probe uniquely identify a different morphology, RNA, or protein target. The 16 different probes at the first z-level in two consecutive sequencing cycles are: AA, AC, AT, AG, CA, CC, CT, CG, TT, TA, TC, TG, GA, GG, CG, and CT. Each of the 16 different probes at the second z-level is used to identify different target as the corresponding probe at the first z-level, thereby allowing unique identification of 32 different targets. The optical system is used to acquire images for sequencing the sample at two different z-levels with a spatial resolution of 0.4 um in x,y, and/or z directions.

[1273] Such an optical system provides additional advantages and utility for nucleic acid sequencing applications by providing reduced optical components, less moving parts and higher sequencing throughput, while providing for high resolution readout which includes a resolution of 0.5um or higher. Sequencing using the optical system advantageously allow unique

identification of different cellular features or targets with their corresponding spatial relationship within the cells.

[1274] In this example, a cellular sample is delivered to capillary flow cell 5201 as shown in FIGS. 53A and B. Sample sites 4902 of the flow cell devices comprising nucleic acid sequences (e.g., barcode sequences corresponding to mRNAs or proteins) present in the sample react with primers attached to walls of the interior channel of the capillary flow cell 5201. The nucleic acid sequences of the sample are then amplified and washed. In a first sequencing cycle, after amplification and washing, a solution containing (1) DAPI modified nucleotide conjugate complementary to A nucleotides; (2) FITC modified nucleotide conjugate complementary to G nucleotides; (3) TRITC modified nucleotide conjugate complementary to C nucleotides; and (4) a fourth nucleotide conjugate modified with both DAPI and TRITC that is complementary toward T nucleotides is introduced to the capillary flow cell 5201 and allowed to react with the primed nucleic acid sequence. The sample in the capillary flow cell 5201 is then illuminated by a 0.1 second pulse of UV-blue light via a first LED light source of the light source 4901 thus exciting the DAPI fluorophore. In synchronization with the UV-blue light pulse, the imaging sensors acquire a first image capturing emission of light given off by any DAPI modified nucleotide conjugate bound specifically to the sample. Only light emitted by DAPI fluorescence emission is collected by the imaging sensors because the UV-blue excitation light emitted by the first light source is negligible past 405 nm. This light is blocked by a tri-band band stop filter 4910. Next, the sample is pulsed with 0.1 seconds of green light via a second LED light source of the light source 4902, capable of exciting the FITC fluorophore. In synchronization with the green light pulse, a second image is acquired capturing emission of light given off by FITC modified nucleotide conjugate bound specifically to the sample. The sample is pulsed with 0.1 seconds of red light via a third LED light source of the light source 4901 thus exciting the TRITC fluorophore. In synchronization with the red light pulse, a third image is acquired capturing emission of light given off by any TRITC modified nucleotide conjugate bound specifically to the sample. In this example, excitation filters are used for each LED light source to minimize fluorescence channel cross-talk, or bleed-through of the excitation light that may not be stopped by the notches, or band stops of the tri-band band stop filter 4910.

[1275] A wedge block 4916 can be included in each optical subsystem 4914 in order to image the entire inner surface of the capillary flow cell 5201. As shown in FIG. 54A when the top-wedge piece 4907 is aligned with the bottom wedge piece 4906 the optical subsystems 4916 acquire images on the far side of the inner surface of the capillary flow cell. As shown in FIG. 54B when the top-wedge piece 4907 is moved out of alignment to increase the optical

pathlength 4913 the optical subsystems 4916 acquire images on the front interior surface of the capillary flow cell 5201.

[1276] The light sources used in this example, e.g., the blue, red, and green light source illuminate a field of view of greater than 10 mm^2 at each z-level with an image sensor size of greater than 10 mm^2, so that the flow cell images can be acquired with a field of view of greater than 10 mm^2. The optical system in this example is capable of super resolution imaging, wherein at least one sample site comprising clonally-amplified, sample nucleic acid molecules immobilized to a plurality of attached oligonucleotide molecules, wherein said plurality of immobilized clonally amplified sample nucleic acid molecules are present at distance less than $\lambda/(2*NA)$, wherein λ is the center wavelength of a light source and NA is the numerical aperture of an imaging system. A stochastic photo-switching chemistry is then applied to said clonally amplified sample nucleic acid molecules at the same time to cause said plurality of clonally amplified sample nucleic acid molecules to fluoresce in on and off events in up to four different colors by stochastic photo-switching; and on and off events are detected in a color channel for each color in real-time as the on and off events are occurring for said plurality of clonally amplified sample nucleic acid molecules to determine an identify of a nucleotide of said clonally amplified sample nucleic acid molecule.

[1277] The non-cycle dependent time in a sequencing run includes time duration for amplification, hybridization/de-hybridization, pair end turn between Read 1 and Read 2, system washing time after the entire sequence is completed, which may be in a range from 1 hour to 5 hours.

[1278] In each cycle, the total time duration for administering reagents to the flow cell device, performing sequencing reactions before imaging and other sequencing reactions and washing after imaging may be in a range from 1 second to 5 minutes.

[1279] The imaging cycle time for each cycle includes: time duration for positioning the sample relative to the optical system (e.g., objective of the optical system); time duration for autofocusing of the sample at a first z-level, time duration for light excitation of the sample(s) and acquiring flow cell images from different color channels in parallel, time for autofocusing of the sample at a second z level, time duration for light excitation of the sample(s) and acquiring flow cell images from different color channels in parallel. The imaging cycle time for each cycle is less than 5 minutes.

[1280] In some embodiments, the imaging time in each cycle is for a FOV of greater than 10mm^2, and an image resolution of greater than 1 um or 0.6 um.

[1281] In this example, the base calling process, shown schematically 4602, in FIG. 46 is as follows. The first image of the cycle is analyzed for regions of interest (ROI) showing strong

fluorescence signal. ROI's showing strong fluorescence signal in the first image indicate nucleic acid amplicons with either A or T at the open position prior to exposure to the nucleotide conjugates for the following reason. Capture of the first image was synchronized with sample illumination by UV-blue light, thus exciting DAPI. Since the nucleotide conjugates complementary toward A were labeled with DAPI and nucleotide conjugates complementary toward T were labeled with both DAPI and TRITC, ROI's of the first image showing strong fluorescence indicate either an A or T. Next, the second image of the cycle is analyzed for ROI's strong fluorescence signal. Since nucleotide conjugates complementary toward G were labeled with FITC and since capture of the second image was synchronized with the green pulse capable of exciting FTIC, ROI's in the second image showing strong fluorescent signal indicate G. Next, the third image of the cycle is analyzed for ROI's strong fluorescence signal. These ROI's indicate nucleic acid amplicons with either C or T present at the open position prior to exposure to the nucleotide conjugates. This is because in synchronization with the capture of the third image, the sample is illuminated with red light, thus exciting TRITC. Nucleotide conjugates complementary to C are labeled with TRITC and nucleotide conjugates complementary toward T are labeled with both DAPI and TRITC. ROI's with strong fluorescence signal observed in both the first and third image indicate a T nucleotide at the open position prior to exposure to the nucleotide conjugates. Identification of ROI's containing T's then allows for identification ROI's containing of A and C. The sequencing and imaging cycle is repeated until the entire nucleic acid sequence has been identified.

Example 20 – Optical system efficiency

[1282] FIGS. 96A – 96B provide diffraction modulation transfer functions (MTFs) for optical systems, according to some embodiments. FIG. 96A shows the MTF of an objective based system with a p = 480 nm, while FIG. 96B shows the MTF of an optical system of the present disclosure with a p=500 nm. As can be seen, the overall effectiveness of the optical system of the present disclosure is higher at all spatial frequencies than the objective based system, and the system of the present disclosure provides a much larger field of view (9 mm versus the 2 mm of the objective based system). FIGS. 97A – 97B show wavefront analysis calculations for an optical system of the present disclosure, according to some embodiments. In both cases, over a wide field of view (e.g., 9 mm), the optical systems demonstrated low composite root-mean-square errors of about 24 mλ and 26 mλ, respectively.

[1283] FIGS. 98 - 99 show a top and bottom, respectively, surface optical performance curve, according to some embodiments. In both cases, the longitudinal spherical aberration, astigmatic

field curve, and distortion are low, indicating improved optical (e.g., imaging) performance. FIG. 100 shows a plot of an MTF of an optical system, according to some embodiments. The MTF plot can show a wide depth of field (e.g., ± 1 micrometer) with low field curvature, which can show the ability of the optical system to provide wide area, large depth of field imaging capable of imaging many samples on a solid support at a same time. In some cases, the wide depth of field can enable multi-surface imaging (e.g., imaging a plurality of surfaces of a solid support at a same time). FIG. 101 shows a plot of a cumulative probability of achieving a given wavefront error, according to some embodiments. The plot shows data regarding the low wavefront errors that the systems of the present disclosure can achieve.

EXAMPLE 21 In Situ Sequencing – Cell Painting

Cell fixation and permeabilization

[1284] HeLa cells were washed with DPBS or PBS and then fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in PBS and water for about 30 minutes at room temperature. The cells were washed twice with DPBS or PBS. The fixed cells were permeabilized with 70% ethanol for about 30 minutes at room temperature, then washed with DPBS or PBS. The fixed and permeabilized cells remained whole and were not embedded in paraffin or sectioned into slices.

Preparing bipartite complexes (1800)

[1285] Bipartite complexes (1800) were prepared by conjugating bridge oligonucleotides (1500) with secondary antibodies using DSS linking chemistry. Briefly, amine-modified bridge oligonucleotides were mixed with DSS linkers (disuccinimidyl suberate), acetonitrile and triethylamine, and the mixture was incubated at room temperature for 30 minutes. The mixture was ethanol precipitated by adding sodium acetate (pH 5.2) and pure ethanol, vortexed, and stored at -80 degrees C for at least 2 hours. The mixture was centrifuged at 14,000 RPM at 4 degrees C for 30 minutes. The supernatant was removed and the remaining pellet was air dried at room temperature for at least 10 minutes. The dried pellet was resuspended in a resuspension buffer comprising HEPES (pH 7.4) and sodium chloride.

[1286] Secondary antibody (goat anti-rabbit IgG Fc, from Abcam, catalog No. ab97196) was prepared by buffer exchange in 1X PBS buffer using a desalting column (Zeba Spin desalting column, 7 kDa MWCO, from Thermo Fisher Scientific, catalog No. 89883) at 4 degrees C. The conjugation reaction was conducted by mixing the prepared secondary antibody with the prepared bridge oligonucleotides at a ratio of 1:3 (antibody to oligonucleotide) and incubated at room temperature overnight. The conjugate mixture was loaded onto a spin column (50 kDa MWCO) with PBS buffer and the column was spun at 4000 RPM for 7 minutes at 4 degrees C. The column was re-wetted with PBS buffer and the spinning was repeated five times. The

resulting antibody-oligonucleotide conjugate was prepared for hybridizing with pre-circularized barcoded oligonucleotides (1400).

[1287] The antibody-oligonucleotide conjugate was mixed with pre-circularized barcoded oligonucleotides (1400) in 1X PBS buffer and incubated at room temperature for at least one hour for hybridization to generate secondary antibodies attached to bridge oligonucleotides (1500), and the bridge oligonucleotides were hybridized to the pre-circularized barcoded oligonucleotides (1400). The conjugated secondary antibodies were mixed with primary antibodies in 1X PBS buffer to generate bipartite complexes (1800). Examples of primary antibodies used to prepare bipartite complexes included anti-alpha tubulin antibody (e.g., from Abcam, catalog No. ab52866), anti-histone antibody (e.g., from Abcam, catalog No. ab176842), anti-c-myc antibody (e.g., from Abcam, catalog No. ab185656), anti-HMOX-1 (heme oxygenase-1) antibody (e.g., from Abcam, catalog No. ab189491), anti-claudin-18 antibody (e.g., from Abcam, catalog No. ab203563) and anti-GM130 (e.g., from Abcam, catalog No. ab52649).

In situ Rolling Circle Amplification

[1288] The HeLa cells were placed in multi-well plates, and washed with 1X PBS buffer. The bipartite complexes (1800) were added to the fixed and permeabilized cells, and incubated at room temperature for one hour to permit the bipartite complexes (1800) to bind their cognate target polypeptides inside the cells. Each type of bipartite complex (1800) comprised a primary antibody that binds a specific target analyte and a secondary antibody attached to a circularized barcoded oligonucleotide carrying a batch-specific sequencing primer binding site and a 12-mer target barcode sequence that corresponded with the target analyte. The batch-specific sequencing primer binding site and target barcode sequences were unique for each type of bipartite complex (1800) to permit batch-specific sequencing. The table shown in FIG. 121 lists several examples of the target barcode sequences. The cells were incubated with one type (e.g., single-plex) and up to eight different types (e.g., 8-plex) of bipartite complexes wherein each type was designed to bind a specific target analyte. The cells were washed with 1X PBS, three times, and each wash was 10 minutes.

[1289] A rolling circle amplification reagent was prepared which included a strand-displacing DNA polymerase and a mixture of nucleotides including dATP, dGTP, dCTP, dTTP and dUTP. The rolling circle amplification reagent was added to the cells and the cells were incubated at 35 degrees C for 20 minutes to generate barcoded concatemers that corresponded to target polypeptides. In some experiments, one type of bipartite complex (1800) was amplified by rolling circle amplification to generate cells harboring one type of barcoded concatemer molecules which corresponded to one type of target protein. In some experiments, multiple types

of bipartite complexes (1800) were simultaneously amplified by rolling circle amplification to generate cells harboring multiple types of barcoded concatemer molecules which corresponded to multiple types of target proteins.

In situ sequencing using 2-stage sequencing method using multivalent molecules

[1290] The flow cells were washed with sequencing primer reagent which included 2X SSC, 20% formamide, and 1 μM universal sequencing primer. The sequence of the sequencing primer is listed in Table 1 above. The flow cells were washed twice with a wash buffer which included 10 mM Tris (pH 8), 100 mM NaCl, 0.4 mM EDTA and 0.3% Tween-20.

[1291] A two-stage sequencing method was used to sequence the concatemer molecules inside the cells. Each sequencing cycle included a first stage and a second stage. The first stage employed a first sequencing polymerase and fluorescently labeled multivalent molecules. The second stage employed a second sequencing polymerase and non-labeled nucleotide analogs.

[1292] The first stage sequencing was conducted with fluorescently labeled multivalent molecules. An image of the cells on the flow cells was obtained prior to the start of sequencing. A solution of a first sequencing polymerase was flowed onto the flow cells and incubated at 42 °C for about 10 minutes to form complexed polymerases on the concatemers inside the cells. A trap reagent was flowed onto the flow cells. The trap reagent included a mixture of fluorescently labeled multivalent molecules (e.g., about 40-100 nM) (see FIGS. 76 – 78, 79A and 79B) and a non-catalytic cation (e.g., strontium, barium or calcium). The mixture of fluorescently labeled multivalent molecules included dATP, dGTP, dCTP and dUTP. The flow cells were incubated at 42 °C for about 10 minutes to permit the multivalent molecules to bind the complexed polymerases, without incorporation of the nucleotide moieties, and form avidity complexes on the concatemers inside the cells. The flow cells were washed at room temperature with a trap reagent that lacked the fluorescently labeled multivalent molecules. The flow cells were washed with an imaging buffer. An image was obtained using a fluorescent microscope: 1 minute/FOV, 4 channels, 200 MS/frame, using Z-stack imaging. The multivalent molecules and first sequencing polymerases were removed by washing the flow cells twice at 42 °C with a removal reagent. The flow cells were washed four times at 52 °C with a wash buffer.

[1293] The second stage sequencing was conducted with non-labeled nucleotide analogs. A solution of a second sequencing polymerase, a mixture of non-labeled nucleotide analogs and a catalytic divalent cation (e.g., magnesium) was flowed onto the flow cells and incubated at 52 °C for about 2-5 minutes to permit incorporation of the nucleotide analogs. The mixture of non-labeled nucleotide analogs included 3′O-methylazido nucleotides with dATP, dGTP, dCTP and dTTP. The flow cells were washed twice with a removal reagent at 51 °C for about 20 seconds. The 3′ blocking moieties were removed from the incorporated nucleotide by washing the flow

cells twice with a cleaving reagent at 51 °C for about 40 seconds. The flow cells were washed twice with a wash buffer.

[1294] The next sequencing cycle was conducted by repeating at least once the two-stage sequencing method described above.

In situ detection and identification of target proteins

[1295] Cellular target proteins were detected and identified by sequencing the barcoded concatemers and imaging the color fluorescent signals at each sequencing cycle. The two-stage sequencing workflow was conducted using fluorescently labeled multivalent molecules for the first stage and non-labeled nucleotide analogs for the second stage. The two-stage sequencing workflow is described above.

[1296] In some experiments, one type of sequencing primer was used to sequence one type of barcoded concatemer molecules, where the barcoded concatemer molecules corresponded to one type of target protein. Images of the fluorescent signals at each sequencing cycle was matched with that target barcode sequence which identified the target protein. For example, the images shown in FIG. 27 represent five consecutive sequencing cycles of the same cells and the same field-of-view using sequencing primers specific for the tubulin barcoded concatemers. The images are rendered in false color. The fluorescent signals emitted during the five sequencing cycles detected and identified tubulin structures inside the cells. The target barcode sequence that corresponds with tubulin is listed in the table.

[1297] In some experiments, two or more types of sequencing primers were used to simultaneously sequence multiple types of barcoded concatemer molecules where the different types of barcoded concatemers corresponded to different types of target proteins. The image shown in FIG. 123A TOP represents cells harboring two types of barcoded concatemer molecules corresponding to histone or tubulin where each type of barcoded concatemer molecules carried a target barcode sequence that was unique to the target protein histone or tubulin. The barcoded concatemers were simultaneously sequenced using a mixture of sequencing primers specific for the histone concatemers or the tubulin concatemers. Images of multiple sequencing cycles were obtained. The image shown in FIG. 123A TOP shows fluorescent signals emitted from a single sequencing cycle in which the tubulin barcode emits a green signal and the histone barcode emits a red signal. The top image of FIG. 123A shows histone (red) and tubulin (green) structures inside the cells. The top image is not a merged image.

[1298] The image shown in FIG. 123A demonstrates that a set of two or more different bipartite complexes each carrying a unique target barcode sequence can be used to generate different types of barcoded concatemer molecules which correspond to different target analytes, and the

different types of barcoded concatemer molecules can be used to simultaneously detect and identify two or more cellular structures by conducting a single sequencing cycle and employing multi-color imaging. In some embodiments, this sequencing-based workflow can be used for cell painting.

[1299] After the sequencing was completed, the sequencing read products were dissociated from the barcoded concatemers and removed via washing. The barcoded concatemers remained inside the cells. The cells were subjected to multiple cycles of re-sequencing using sequencing primers specific for the barcoded concatemers that correspond to histones (e.g., first batch sequencing). The image shown in FIG. 123A BOTTOM shows fluorescent signals emitted from a single sequencing cycle in which the histone barcode emits a red signal. The bottom image shows histone (red) structures inside the cells. In FIG. 123A the top and bottom images represent the same cells and the same field-of-view. After sequencing the histone concatemers was completed, the sequencing read products were dissociated from the barcoded concatemers and removed via washing. The barcoded concatemers remained inside the cells. The cells were again subjected to multiple cycles of re-sequencing using sequencing primers specific for the barcoded concatemers that correspond to tubulin (e.g., second batch sequencing). The image shown in FIG. 123B BOTTOM shows fluorescent signals emitted from a single sequencing cycle in which the tubulin barcode emits a green signal. The bottom image shows tubulin (green) structures inside the cells. The top and bottom images represent the same cells and the same field-of-view.

[1300] The image shown in FIG. 124 shows cells harboring two types of barcoded concatemer molecules corresponding to histone or tubulin where each type of barcoded concatemer molecules carried a target barcode sequence that was unique to the target protein histone or tubulin. The barcoded concatemers were simultaneously sequenced using a mixture of sequencing primers specific for the histone concatemers or the tubulin concatemers. Images of multiple sequencing cycles were obtained. The image shown in FIG. 124 shows fluorescent signals emitted from a single sequencing cycle in which the tubulin barcode emits a green signal and the histone barcode emits a red signal. The image of FIG. 124 shows histone (red) and tubulin (green) structures inside a cell undergoing cell division. The image of FIG. 124 is not a merged image.

[1301] In some experiments, a first type of sequencing primer was used to sequence a first type of barcoded concatemer molecules (e.g., first batch sequencing) and the sequencing read products were removed while retaining the barcoded concatemers inside the cells, and a second type of sequencing primer was used to sequence a second type of barcoded concatemer molecules (e.g., second batch sequencing).

Numbered embodiments of the disclosure

1. An optical system, comprising:

a stage configured to hold one or more samples immobilized on a solid support;

a light source configured to illuminate the one or more samples; and

an optical assembly disposed at least partly within an optical path from said stage to said light source, wherein said optical assembly is configured to provide an illumination over an area of the one or more samples that is greater than about 20 square millimeters (mm^2) with a peak-to-valley energy variation of no more than 5% across said area.

2. The optical system of any one of the preceding embodiments, wherein said optical assembly does not comprise an objective.
3. The optical system of any one of the preceding embodiments, wherein said optical system does not comprise said objective.
4. The optical system of any one of the preceding embodiments, wherein said optical assembly does not comprise a tube lens.
5. The optical system of any one of the preceding embodiments, wherein said optical system does not comprise said tube lens.
6. The optical system of any one of the preceding embodiments, wherein said stage does not adjust in an optical axis of said system.
7. The optical system of any one of the preceding embodiments, wherein said illumination has an irradiance of at least about 40 milliwatts per square millimeter.
8. The optical system of any one of the preceding embodiments, wherein said optical assembly is configured to receive an emission light from said solid support.
9. The optical system of any one of the preceding embodiments, wherein said optical assembly has a numerical aperture (NA) of at least about 0.3.
10. The optical system of any one of the preceding embodiments, wherein said emission light has a wavelength of about 500 nanometers to about 750 nanometers.
11. The optical system of any one of the preceding embodiments, wherein said optical assembly has a working distance of at least about 1mm to 25 mm.
12. The optical system of any one of the preceding embodiments, further comprising a motion coil housed within said optical assembly configured to move a focusing element within said optical path of said optical system.

13. The optical system of any one of the preceding embodiments, wherein a motor external to the optical system is configured to move a focusing element along the optical axis in one or both directions.
14. The optical system of any one of the preceding embodiments, wherein said motor is coupled directly with a piece of a first, second, or third housing of the optical assembly, and the piece of the first, second, or third housing of the optical assembly is coupled directly with the focusing element.
15. The optical system of any one of the preceding embodiments, wherein said light source is a pulsed light source.
16. The optical system of any one of the preceding embodiments, wherein said optical system has a composite root mean square error of less than about 0.05.
17. The optical system of any one of the preceding embodiments, wherein said optical assembly has an illumination efficiency of at least about 90%.
18. The optical system of any one of the preceding embodiments, wherein said area is greater than 30 mm^2.
19. The optical system of any one of the preceding embodiments, wherein said area is greater than 50 mm^2 or 60 mm^2.
20. The optical system of any one of the preceding embodiments, further comprising said solid support within said stage.
21. The optical system of any one of the preceding embodiments, wherein said solid support comprises two or more surfaces having one or more samples immobilized thereon which are imaged by said optical system.
22. The optical system of any one of the preceding embodiments, wherein said solid support comprises three or more surfaces having one or more samples immobilized thereon imaged by said optical system.
23. The optical system of any one of the preceding embodiments, wherein said three or more surfaces are axially displaced from each other at least along an optical axis of the optical system.
24. The optical system of any one of the preceding embodiments, wherein said solid support comprises a probe configured to bind a nucleic acid molecule.
25. The optical system of any one of the preceding embodiments, wherein said probe is bound to a surface of said solid support.
26. The optical system of any one of the preceding embodiments, wherein said light source is a laser light source.

27. The optical system of any one of the preceding embodiments, wherein said optical assembly comprises a dichroic filter configured to transmit said illumination.
28. The optical system of any one of the preceding embodiments, wherein said optical assembly comprises a first segment comprising a first housing comprising a first plurality of lenses, a second segment comprising a second housing, and a third segment comprising a third housing comprising a second plurality of lenses.
29. The optical system of any one of the preceding embodiments, wherein said first segment and said third segment are optically aligned.
30. The optical system of any one of the preceding embodiments, wherein said first segment is positioned between said third segment and said stage.
31. The optical system of any one of the preceding embodiments, wherein said third segment is positioned between said first segment and an image sensor of the optical system.
32. The optical system of any one of the preceding embodiments, wherein said first plurality of lenses are movable along said optical path with a range of about 0 to about 2 millimeters.
33. The optical system of any one of the preceding embodiments, wherein said first plurality of lenses comprises an asymmetric convex-convex lens.
34. The optical system of any one of the preceding embodiments, wherein said second plurality of lenses comprises an asymmetric concave-concave lens.
35. The optical system of any one of the preceding embodiments, wherein said asymmetric concave-concave lens is an aspheric asymmetric concave-concave lens.
36. The optical system of any one of the preceding embodiments, wherein said optical system is configured to acquire images of the solid support without moving an optical compensator into the optical path between the solid support and a detector of the optical system.
37. The optical system of any one of the preceding embodiments, wherein said optical system is configured to acquire images of the solid support without moving an optical compensator out from the optical path between the sample and a detector of the optical system.
38. The optical system of any one of the preceding embodiments, wherein said solid support comprises a flow cell.
39. The optical system of any one of the preceding embodiments, wherein said optical assembly is configured to generate one or more spatial constrictions lateral to said optical path of light which travels therethrough.
40. The optical system of any one of the preceding embodiments, wherein said optical assembly is configured to generate one or more field curvature corrections lateral to said optical path of light which travels therethrough.

41. The optical system of any one of the preceding embodiments, wherein said optical assembly is configured to generate at least one field curvature correction lateral to the optical path of light travels therethrough in a first segment, second segment, or third segment.
42. A method of analyzing a biological molecule, comprising:
    (a) providing a solid support comprising said biological molecule comprising a label;
    (b) using an optical system comprising a light source to provide illumination to said biological molecule comprising said label, thereby generating a signal light or a change thereof, wherein said illumination is provided over an area of said solid support that is greater than about 20 square millimeters (mm^2) with a peak-to-valley variation of at most about 5%;
    (c) detecting, using a detector of said optical system, said signal light or said change thereof; and
    (d) processing at least in part said signal light or said change thereof to analyze said biological molecule.
43. The method of any one of the preceding embodiments, wherein said biological molecule is a nucleic acid molecule, a protein, or a polypeptide.
44. The method of any one of the preceding embodiments, wherein said biological molecule is a nucleic acid.
45. The method of any one of the preceding embodiments, further comprising, prior to (a), binding said biological molecule to a probe bound to said solid support, and coupling said label to said biological molecule.
46. The method of any one of the preceding embodiments, wherein said label is coupled to said biological molecule by hybridization.
47. The method of any one of the preceding embodiments, wherein said optical system does not comprise an objective.
48. The method of any one of the preceding embodiments, wherein said solid support is not moved in an optical axis of said optical system.
49. The method of any one of the preceding embodiments, wherein a plurality of images of said solid support are acquired without moving said solid support in said optical axis.
50. The method of any one of the preceding embodiments, wherein said illumination has an irradiance of at least about 40 milliwatts per square millimeter.
51. The method of any one of the preceding embodiments, wherein said signal light has a wavelength of about 500 nanometers to about 750 nanometers.
52. The method of any one of the preceding embodiments, wherein said detecting of (c) is performed using an optical element with a numerical aperture of at least about 0.3.

53. The method of any one of the preceding embodiments, further comprising, in (b), using a motion coil within said optical system to move a focusing element within an optical path of said optical system, thereby changing a focus of said optical system on said solid support.
54. The method of any one of the preceding embodiments, wherein said light source is a pulsed light source.
55. The method of any one of the preceding embodiments, wherein said illumination is provided with an efficiency of at least about 90%.
56. The method of any one of the preceding embodiments, further comprising repeating (b) – (d) for an additional biological molecule coupled to an additional surface of said solid support.
57. The method of any one of the preceding embodiments, further comprising, subsequent to (c), removing said label from said biological molecule.
58. The method of any one of the preceding embodiments, further comprising repeating (a) – (d) for an additional label that binds to another portion of the biological molecule.
59. The method of any one of the preceding embodiments, wherein said optical assembly is configured to generate one or more spatial constrictions lateral to said optical path of light that travels therethrough.
60. The method of any one of the preceding embodiments, wherein said optical assembly is configured to generate one or more field curvature corrections lateral to said optical path of light that travels therethrough.
61. The method of any one of the preceding embodiments, wherein said optical assembly is configured to generate at least one field curvature correction lateral to the optical path of light that travels therethrough in a first segment, second segment, or third segment.
62. The method of any one of the preceding embodiments, wherein (d) comprises processing at least in part said signal light or said change thereof to generate one or more solid support images and analyze said one more solid support images to generate base calls of the sample.
63. The method of any one of the preceding embodiments, wherein each of said solid support images comprises a field-of-view (FOV) that is greater than 20 square millimeters (mm^2).
64. The method of any one of the preceding embodiments, wherein said solid support is a flow cell.
65. A method for analyzing a biological molecule, comprising:
    (a) providing a solid support comprising a biological sample comprising a label;
    (b) using an optical system comprising a light source to provide illumination to said biological sample comprising said label, thereby generating a signal light or a change thereof, wherein said illumination is provided through a despeckler in an optical path of said optical system;

(c) detecting, using a detector of said optical system, said signal light or said change thereof; and

(d) processing at least in part said signal light or said change thereof to analyze said biological molecule.

66. The method of any one of the preceding embodiments, further comprising repeating (b) – (d) for an additional biological sample coupled to an additional surface of said solid support.

67. The method of any one of the preceding embodiments, further comprising, subsequent to (c), removing said label from said biological sample.

68. The method of any one of the preceding embodiments, further comprising repeating (a) – (d) for an additional label that binds to the biological sample.

69. The method of any one of the preceding embodiments, wherein said despeckler uses vibration to despeckle said illumination.

70. The method of any one of the preceding embodiments, further comprising using an additional light source to illuminate said solid support.

71. The method of any one of the preceding embodiments, wherein said additional light source provides a different wavelength of light to said solid support.

72. The method of any one of the preceding embodiments, wherein said additional light source is optically coupled to said despeckler.

73. The method of any one of the preceding embodiments, wherein said biological sample comprises a nucleic acid molecule, a protein, or a polypeptide.

74. The method of any one of the preceding embodiments, wherein said biological sample comprises a nucleic acid.

75. An illumination system for a multi-channel fluorescence imaging module, comprising:

an illumination subsystem, comprising:

a light source;

a despeckler; and

a light beam delivery subsystem optically coupled to the illumination system, comprising:

a collimator; and

one or more optical lens elements.

76. The illumination system of any one of the preceding embodiments, wherein said illumination system is configured to provide an illumination field that is no less than 10 mm^2, 20 mm^2, 30 mm^2, 40 mm^2, or 50 mm^2 at a sample plane.
77. The illumination system of any one of the preceding embodiments, wherein said illumination system is configured to provide a power density that is no less than 40, 50, or 60 milliwatts/mm^2 at a sample plane.
78. The illumination system of any one of the preceding embodiments, wherein said sample plane is orthogonal to a z-axis.
79. The illumination system of any one of the preceding embodiments, wherein said light source comprises one or more lasers.
80. The illumination system of any one of the preceding embodiments, wherein said one or more lasers comprises one or more laser diodes.
81. The illumination system of any one of the preceding embodiments, wherein said one or more lasers emit light of multiple wavelengths.
82. The illumination system of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more optical fibers.
83. The illumination system of any one of the preceding embodiments, wherein at least one of said optical fibers comprises a length of 0.5 m to 5 m.
84. The illumination system of any one of the preceding embodiments, wherein at least one of said optical fibers comprises a core with a maximum dimension of 50 um to 1500 um in a cross-section of the core.
85. The illumination system of any one of the preceding embodiments, wherein said cross-section of the core is circular.
86. The illumination system of any one of the preceding embodiments, wherein the power efficiency of the illumination system is no less than 65%, 70%, 75%, 80%, or 90%.
87. The illumination system of any one of the preceding embodiments, wherein the illumination field is of a rectangular or square shape.
88. The illumination system of any one of the preceding embodiments, wherein said one or more optical lens elements comprise one or more multi-lens arrays.
89. The illumination system of any one of the preceding embodiments, wherein each of said one or more multi-lens arrays comprises one or more of: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens.

90. The illumination system of any one of the preceding embodiments, wherein each of said one or more multi-lens arrays comprises multiple lens elements at least in a direction that is orthogonal to a z-axis.
91. The illumination system of any one of the preceding embodiments, wherein said illumination subsystem further comprises an optical fiber that is optically coupled to the laser diode and mitigates speckle of the light source.
92. The illumination system of any one of the preceding embodiments, wherein said despeckler comprises an optical fiber that is optically coupled to the laser diode and mitigates speckle of the light source.
93. The illumination system of any one of the preceding embodiments, wherein said one or more optical lens elements comprises: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens, or a combination thereof.
94. The illumination system of any one of the preceding embodiments, wherein said one or more optical lens elements comprises: a first multi-lens array and a second multi-lens array that are positioned along a z-axis between the collimator and an entrance pupil of the illumination system.
95. The illumination system of any one of the preceding embodiments, wherein said illumination system is configured to generate an illumination field at a sample stage that is greater than 50 mm^2 with less than ± 2%, 5%, 8%, 10%, or 12% variance in illumination power density across the illumination field.
96. The illumination system of any one of the preceding embodiments, wherein said despeckler comprises a mechanical vibration source.
97. The illumination system of any one of the preceding embodiments, wherein said despeckler comprises a vibration source that produces vibration at a predetermined frequency range.
98. The illumination system of any one of the preceding embodiments, wherein said mechanical vibration source is configured to vibrate at one or more frequencies in an audible sound range, a ultrasound range, or both.
99. The illumination system of any one of the preceding embodiments, wherein said mechanical vibration source is configured to generate vibrating motions in one, two, or three dimensions.
100. The illumination system of any one of the preceding embodiments, wherein at least a portion of each of said optical fibers or single optical fiber is wound or coiled for one or more rounds.

101. The illumination system of any one of the preceding embodiments, wherein at least a portion of each of said optical fibers or single optical fiber is fixedly or loosely attached to said mechanical vibration source.
102. The illumination system of any one of the preceding embodiments, wherein said despeckler is physically isolated from the sample stage, an objective lens, and said one or more image sensors so that mechanical motion of the despeckler is independent from the sample stage, the objective lens, and the one or more image sensors.
103. The illumination system of any one of the preceding embodiments, wherein said despeckler is configured to reduce speckle noise to be no more than 4%, 4.5%, 5%, or 5.5%.
104. The illumination system of any one of the preceding embodiments, wherein said light source comprises a multi-color laser array.
105. The illumination system of any one of the preceding embodiments, wherein said multi-color laser array comprises an array of laser diodes that emits laser lights at 2, 3, 4, 5, or 6 wavelengths or in 2, 3, 4, 5, or 6 wavelength ranges.
106. The illumination system of any one of the preceding embodiments, wherein said multi-color laser array comprise lasers that emit light of 2, 3, or 4 color wavelengths or wavelength ranges at least in a direction that is orthogonal to a z-axis.
107. The illumination system of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more coupling lens.
108. The illumination system of any one of the preceding embodiments, wherein said illumination subsystem comprises a single optical fiber.
109. The illumination system of any one of the preceding embodiments, wherein said single optical fiber comprises a core with a maximum dimension of 500 um to 1500 um in a cross-section of the core.
110. The illumination system of any one of the preceding embodiments, wherein a power in the light beam delivery subsystem is greater than 1, 2, 5, 8, 10, 12, or 14 Watts for one or more wavelengths or wavelength ranges.
111. The illumination system of any one of the preceding embodiments, wherein a power at a sample plane is greater than 1, 2, 5, 8, 10, 12, or 14 Watts for one or more wavelengths or wavelength ranges.
112. The illumination system of any one of the preceding embodiments, wherein the illumination subsystem further comprises a plurality of optical fibers, each optical fiber optically coupled to one or more corresponding lasers of the light source, wherein the one or more corresponding lasers emit light of a same wavelength or wavelength range as the light source.

113. The illumination system of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more dichroic filters.
114. The illumination system of any one of the preceding embodiments, wherein said light source comprises multiple light beam combiners.
115. The illumination system of any one of the preceding embodiments, wherein said light source comprises multiple polarization light beam combiners.
116. The illumination system of any one of the preceding embodiments, wherein said light source comprises two or more lasers that emit light at a same wavelength or in a same wavelength range.
117. The illumination system of any one of the preceding embodiments, wherein each of said polarization light beam combiners is configured to combine light emitted from two or more lasers at the same light wavelength or in the same light wavelength range.
118. The illumination system of any one of the preceding embodiments, wherein said despeckler is positioned in an optical path between a collimator and the sample plane.
119. The illumination system of any one of the preceding embodiments, wherein said despeckler is positioned where a diameter of an optical beam is greater than 5 mm, 10 mm, or 20 mm, and wherein the diameter of the optical beam is orthogonal to a z-axis.
120. The illumination system of any one of the preceding embodiments, wherein said optical fiber comprises a core with a rectangular or square cross-section.
121. The illumination system of any one of the preceding embodiments, wherein said optical fiber comprises a core with a non-circular cross-section.
122. The illumination system of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more liquid light guides.
123. The illumination system of any one of the preceding embodiments, wherein said one or more liquid light guides are optically coupled to the light source in the absence of an optical fiber.
124. The illumination system of any one of the preceding embodiments, wherein said one or more liquid light guides comprise a liquid core with a maximum dimension of 0.5 mm to 10 mm in a cross-section of the liquid core, and wherein the cross-section is orthogonal to a z-axis.
125. The illumination system of any one of the preceding embodiments, wherein said liquid core comprises a cross-section that is circular.
126. The illumination system of any one of the preceding embodiments, wherein said liquid core comprises a cross-section that is non-circular.
127. An imaging module for multi-channel fluorescence imaging, comprising:

the illumination system in any one of the preceding embodiments; and

an image acquisition system configured to acquire flow cell images of a sample immobilized on a sample stage at a sample plane.

128. The imaging module of any one of the preceding embodiments, wherein each of said flow cell images comprises a field-of-view (FOV) that is greater than 20 mm^2, 30 mm^2, 40 mm^2, or 50 mm^2.

129. The imaging module of any one of the preceding embodiments, wherein each of said flow cell images comprises a field-of-view (FOV) that overlaps with an illumination field generated by the illumination system at the sample plane.

130. The imaging module of any one of the preceding embodiments, wherein each of said flow cell images comprises a field-of-view (FOV) that overlaps with at least 80% , 85%, 90%, 95% of an illumination field generated by the illumination system at the sample plane.

131. The imaging module of any one of the preceding embodiments, wherein each of said flow cell images comprises a field-of-view (FOV) with a size that is at least 80% , 85%, 90%, 95% of an illumination field generated by the illumination system at the sample plane.

132. The imaging module of any one of the preceding embodiments, wherein said imaging module comprises:

one or more image sensors; and

an objective lens.

133. The imaging module of any one of the preceding embodiments, wherein the numerical aperture (NA) of the imaging module is greater than 0.4, 0.5, or 0.6.

134. The imaging module of any one of the preceding embodiments, wherein said imaging module comprise an optical axis that is parallel with a z-axis.

135. The imaging module of any one of the preceding embodiments, wherein said flow cell images are of the sample immobilized on one or more surfaces of a solid support.

136. The imaging module of any one of the preceding embodiments, wherein said one or more surfaces comprises 2 or more surfaces that are axially displaced from each other at least along a z-axis.

137. The imaging module of any one of the preceding embodiments, wherein said flow cell images are acquired without moving an optical compensator into an optical path between the objective lens and the one or more image sensors.

138. The imaging module of any one of the preceding embodiments, wherein said flow cell images of one or more surfaces are acquired without moving an optical compensator out from an optical path between the objective lens and the at least one image sensor.

139. The imaging module of any one of the preceding embodiments, wherein each of said flow cell images comprises a contrast to noise ratio (CNR) of at least 5 when: nucleic acid polonies disposed on the three or more surfaces are labeled with cyanine dye 3 (Cy3); the dichroic mirror and bandpass filter set are optimized for Cy3 emission; and the flow cell image is acquired by the optical system under non-signal saturating conditions while one or more of the surfaces is immersed in 25 mM ACES, pH 7.4 buffer.

140. The imaging module of any one of the preceding embodiments, wherein said imaging module is configured for determining nucleotide in the nucleic acid molecules in the sample.

141. The imaging module of any one of the preceding embodiments, wherein said imaging module is configured for performing sequencing-by-avidity, sequencing-by-nucleotide base-pairing, sequencing-by-nucleotide binding, or sequencing-by-nucleotide incorporation reactions on at least one of said one or more surfaces.

142. The imaging module of any one of the preceding embodiments, wherein each of said one or more surfaces comprises a plurality of primed target nucleic acid sequences coupled thereto, wherein a primed target nucleic acid sequence of the plurality of primed target nucleic acid sequences has a polymerase bound thereto.

143. A method for sequencing nucleic acid molecules comprising:

providing a flow cell comprising one or more surfaces, wherein each surface comprises:

at least one hydrophilic polymer coating layer;

a plurality of oligonucleotide molecules attached to the at least one hydrophilic polymer coating layer; and

at least one discrete region of each surface that comprises a plurality of clonally-amplified, sample nucleic acid molecules immobilized to the plurality of attached oligonucleotide molecules;

causing, by an illumination system, the plurality of clonally amplified sample nucleic acid molecules in an illumination field to fluoresce in on and off events in different colors; and

detecting, by the one or more image sensors, fluorescent signal emitted from the one or more samples in one or more color channels for the plurality of clonally amplified sample nucleic acid molecules to determine an identity of a nucleotide of the clonally amplified sample nucleic acid molecule.

144. The method of any one of the preceding embodiments, wherein said method further comprises:

adjusting the NA of an imaging module.

145. The method of any one of the preceding embodiments, wherein said method further comprises:

changing the NA of an imaging module in a range from 0.4 to 0.6 by changing an adjustable size of an optical aperture.

146. The method of any one of the preceding embodiments, wherein said illumination system is configured to provide an illumination field that is no less than 10 mm^2, 20 mm^2, 30 mm^2, 40 mm^2, or 50 mm^2 at a sample plane.

147. The method of any one of the preceding embodiments, wherein said illumination system is configured to provide a power density that is no less than 40, 50, or 60 milliwatts/mm^2 at a sample plane.

148. The method of any one of the preceding embodiments, wherein said illumination system comprises:

an illumination subsystem, comprising:

a light source;

a despeckler; and

a light beam delivery subsystem optically coupled to the illumination system, comprising:

a collimator; and

one or more optical lens elements.

149. The method of any one of the preceding embodiments, wherein the sample plane is orthogonal to a z-axis.

150. The method of any one of the preceding embodiments, wherein said light source comprises one or more lasers.

151. The method of any one of the preceding embodiments, wherein said one or more lasers comprises one or more laser diodes.

152. The method of any one of the preceding embodiments, wherein said one or more lasers emit light of multiple wavelengths.

153. The method of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more optical fibers.

154. The method of any one of the preceding embodiments, wherein at least one of said optical fibers comprises a length of 0.5 m to 5 m.

155. The method of any one of the preceding embodiments, wherein at least one of said optical fibers comprises a core with a maximum dimension of 50 um to 1500 um in a cross-section of the core.

156. The method of any one of the preceding embodiments, wherein said cross-section of the core is circular.

157. The method of any one of the preceding embodiments, wherein the power efficiency of the illumination system is no less than 65%, 70%, 75%, 80%, or 90%.

158. The method of any one of the preceding embodiments, wherein the illumination field is of a rectangular or square shape.

159. The method of any one of the preceding embodiments, wherein said one or more optical lens elements comprise one or more multi-lens arrays.

160. The method of any one of the preceding embodiments, wherein each multi-lens array of said multi-lens arrays comprises one or more of: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens.

161. The method of any one of the preceding embodiments, wherein each multi-lens array of said multi-lens arrays comprises multiple lens elements at least in a direction that is orthogonal to a z axis.

162. The method of any one of the preceding embodiments, wherein said illumination subsystem further comprises an optical fiber that is optically coupled to the laser diode and mitigates speckle of the light source.

163. The method of any one of the preceding embodiments, wherein said despeckler is comprised of an optical fiber that is optically coupled to the laser diode and mitigates speckle of the light source.

164. The method of any one of the preceding embodiments, wherein said one or more optical lens elements comprise: an asymmetric convex-convex lens, a convex-plano lens, a concave-plano lens, an asymmetric concave-concave lens, and an asymmetric convex-concave lens, or a combination thereof.

165. The method of any one of the preceding embodiments, wherein said one or more optical lens elements comprise: a first multi-lens array and a second multi-lens array that are positioned along a z-axis between the collimator and an entrance pupil of said illumination system.

166. The method of any one of the preceding embodiments, wherein said illumination system is configured to generate an illumination field at the sample stage that is greater than 50 mm^2

with less than ± 2%, 5%, 8%, 10%, or 12% variance in illumination power density across the illumination field.

167. The method of any one of the preceding embodiments, wherein said despeckler comprises a mechanical vibration source.

168. The method of any one of the preceding embodiments, wherein said despeckler comprises a vibration source that produces vibration at a predetermined frequency range.

169. The method of any one of the preceding embodiments, wherein said mechanical vibration source is configured to vibrate at one or more frequencies in an audible sound range, a ultrasound range, or both.

170. The method of any one of the preceding embodiments, wherein said mechanical vibration source is configured to generate vibrating motions in one, two, or three dimensions.

171. The method of any one of the preceding embodiments, wherein at least a portion of each of said optical fibers or single optical fiber is wound or coiled for one or more rounds.

172. The method of any one of the preceding embodiments, wherein at least a portion of each of said optical fibers or single optical fiber is fixedly or loosely attached to the mechanical vibration source.

173. The method of any one of the preceding embodiments, wherein said despeckler is physically isolated from the sample stage, the objective lens, and the one or more image sensors so that mechanical motion of the despeckler is independent from the sample stage, the objective lens, and the one or more image sensors.

174. The method of any one of the preceding embodiments, wherein said despeckler is configured to reduce speckle noise to be no more than 4%, 4.5%, 5%, or 5.5%.

175. The method of any one of the preceding embodiments, wherein said light source comprises a multi-color laser array.

176. The method of any one of the preceding embodiments, wherein said multi-color laser array comprises an array of laser diodes that emits laser lights at 2, 3, 4, 5, or 6 wavelengths or in 2, 3, 4, 5, or 6 wavelength ranges.

177. The method of any one of the preceding embodiments, wherein said multi-color laser array comprise lasers that emit light of 2, 3, or 4 color wavelengths or wavelength ranges at least in a direction that is orthogonal to a z axis.

178. The method of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more coupling lens.

179. The method of any one of the preceding embodiments, wherein said illumination subsystem comprises a single optical fiber.

180. The method of any one of the preceding embodiments, wherein said single optical fiber comprises a core with a maximum dimension of 500 um to 1500 um in a cross-section of the core.

181. The method of any one of the preceding embodiments, wherein a power in the light beam delivery subsystem is greater than 5, 8, 10, 12, or 14 Watts for one or more wavelengths or wavelength ranges.

182. The method of any one of the preceding embodiments, wherein a power at a sample plane is greater than 5, 8, 10, 12, or 14 Watts for one or more wavelengths or wavelength ranges.

183. The method of any one of the preceding embodiments, wherein said illumination subsystem further comprises a plurality of optical fibers, each optical fiber optically coupled to one or more corresponding lasers of the light source, the one or more corresponding lasers emitting light of a same wavelength or wavelength range.

184. The method of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more dichroic filters.

185. The method of any one of the preceding embodiments, wherein said light source comprises multiple light beam combiners.

186. The method of any one of the preceding embodiments, wherein said light source comprises multiple polarization light beam combiners.

187. The method of any one of the preceding embodiments, wherein said light source comprises two or more lasers that emit light at a same wavelength or in a same wavelength range.

188. The method of any one of the preceding embodiments, wherein each of said polarization light beam combiners is configured to combine light emitted from two or more lasers at the same light wavelength or in the same light wavelength range.

189. The method of any one of the preceding embodiments, wherein said despeckler is positioned in an optical path between a collimator and the sample plane.

190. The method of any one of the preceding embodiments, wherein said despeckler is positioned where a diameter of an optical beam is greater than 5 mm, 10 mm, or 20 mm, and wherein the diameter of the optical beam is orthogonal to a z-axis.

191. The method of any one of the preceding embodiments, wherein said optical fiber comprises a core with a rectangular or square cross-section.

192. The method of any one of the preceding embodiments, wherein said optical fiber comprises a core with a non-circular cross-section.

193. The method of any one of the preceding embodiments, wherein said illumination subsystem further comprises one or more liquid light guides.

194. The method of any one of the preceding embodiments, wherein said one or more liquid light guides are optically coupled to the light source in the absence of an optical fiber.

195. The method of any one of the preceding embodiments, wherein said one or more liquid light guides comprise a liquid core with a maximum dimension of 0.5 mm to 10 mm in a cross-section of the liquid core, and wherein the cross-section is orthogonal to a z-axis.

196. The method of any one of the preceding embodiments, wherein said liquid core comprises a circular cross-section.

197. The method of any one of the preceding embodiments, wherein said liquid core comprises a non-circular cross-section.

198. A sample stage for holding DNA samples for DNA sequencing reactions and imaging, comprising:

a base stage comprising a top surface, wherein the base stage is rotatable about a z-axis relative to an optical system of a sequencing system;

one or more top stages positioned on the top surface of the base stage, wherein each of the one or more top stages is configured to receive and secure one or more flow cell devices thereon, and wherein said each of the one or more top stages are movable relative to the base stage;

a first motor configured to actuate the base stage to rotate with a first resolution.

199. The sample stage of any one of the preceding embodiments, Wherein the top surface is of a circular shape.

200. The sample stage of any one of the preceding embodiments, wherein the first resolution is angular resolution and less than 0.1 degrees, 0.2 degrees 0.5 degrees, 1 degree, 2 degrees, 3 degrees, 4 degrees, 5 degrees, 10 degrees, 20 degrees, 30 degrees, or 50 degrees.

201. The sample stage of any one of the preceding embodiments, wherein each of the flow cell devices comprises one or more samples immobilized thereon to be sequenced.

202. The sample stage of any one of the preceding embodiments, wherein at least one of the flow cell devices comprises an in situ sample immobilized thereon.

203. The sample stage of any one of the preceding embodiments, wherein the sample stage further comprises one or more second motors configured to acuate the one or more top stages relative to the base stage at a second resolution individually.

204. The sample stage of any one of the preceding embodiments, wherein the sample stage further comprises a second motor configured to acuate the one or more top stages relative to the base stage at a second resolution simultaneously.

205. The sample stage of any one of the preceding embodiments, wherein the second resolution is less than 0.01 mm, 0.015 mm, 0.02 mm, 0.03 mm, 0.04 mm, 0.05 mm, 0.08 mm, 0.1 mm, 0.2 mm, or 1 mm.

206. The sample stage of any one of the preceding embodiments, Wherein the sequencing system comprises a fluidic control device in fluidic communication with the flow cell devices positioned on the sample stage.

207. The sample stage of any one of the preceding embodiments, wherein said each of the one or more top stages are movable within a sample plane relative to the base stage.

208. The sample stage of any one of the preceding embodiments, wherein a first top stage of the one or more top stages is movable independently relative to a second top stage of the one or more top stages.

209. The sample stage of any one of the preceding embodiments, wherein a first top stage of the one or more top stages is movable simultaneously with a second top stage of the one or more top stages relative to the base stage.

210. The sample stage of any one of the preceding embodiments, wherein said each of the one or more top stages are movable along a radius of the top surface of the base stage relative to the base stage.

211. The sample stage of any one of the preceding embodiments, wherein said each of the one or more top stages are movable orthogonal to a radius of the top surface of the base stage relative to the base stage.

212. A method of sequencing multiple DNA samples positioned on a rotary sample stage, comprising:

obtaining a sample stage comprising a base stage and one or more top stages positioned on a top surface of the base stage, wherein the base stage is rotatable about a z-axis relative to an optical system of a sequencing system;

positioning and securing a first flow cell device relative to a first top stage of the one or more top stages;

positioning and securing a second flow cell device relative to a second top stage of the one or more top stages;

dispensing, by a first fluidic control device, one or more sequencing reagents to the first flow cell device;

imaging a first sample region of the first flow cell device using the optical system of the sequencing system;

moving the first top stage within the x-y plane relative to the optical system while preventing the second flow cell device from moving relative to the optical system;

imaging a second sample region of the first flow cell device using the optical system of the sequencing system;

rotating the sample stage with a predetermined angular resolution to position the second flow cell device in a predetermined position relative to the optical system; and

imaging a first sample region of the second flow cell device using the optical system of the sequencing system.

213. The method of any one of the preceding embodiments, Wherein moving the first top stage within the x-y plane relative to the optical system while preventing the second flow cell device from moving relative to the optical system comprises:

moving the first top stage along a radius of the top surface of the base stage with a predetermined distance relative to the optical system independently while preventing the second flow cell device from moving relative to the optical system.

214. The method of any one of the preceding embodiments, wherein moving the first top stage within the x-y plane relative to the optical system while preventing the second flow cell device from moving relative to the optical system comprises:

moving the first top stage along a direction orthogonal to a radius of the top surface of the base stage with a predetermined distance relative to the optical system independently while preventing the second flow cell device from moving relative to the optical system.

215. The method of any one of the preceding embodiments, wherein the method further comprises:

moving the first fluidic control device or a second fluidic control device to position the second fluidic cell device in a predetermined position relative to the first fluidic control device or the second fluidic control device.

216. The method of any one of the preceding embodiments, wherein the first sample region or the second sample region comprises a tile.

217. The method of any one of the preceding embodiments, wherein each of the one or more top stages comprises a motion range of greater than 15 mm and less than 80 mm, along a radius or orthogonal to the radius of the top surface of the base stage.

218. The method of any one of the preceding embodiments, wherein each of the one or more top stages comprises a motion range of greater than 25 mm and less than 100 mm, along a radius or orthogonal to the radius of the top surface of the base stage.

219. A system for sequencing nucleic acids, the system comprising:

an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 1 mm^2 or 10 mm^2 wherein the one or more surfaces are axially displaced from each other, the optical system comprising:

a light source;

an objective lens;

at least one image sensor; and

wherein the light source comprises a center wavelength λ with a root-mean-square (RMS) wavefront error of less than 0.09λ, 0.08λ, 0.07λ, 0.06λ, or 0.02λ.

220. A system for sequencing nucleic acids, the system comprising:

an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 1 mm^2 or 10 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising:

a light source;

an objective lens; and

at least one image sensor,

wherein the optical resolution of the optical system is at least 0.4 um, 0.5 um, 0.8 um, or 1 um in a plane orthogonal to an axial axis.

221. A system for sequencing nucleic acids, the system comprising:

an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2, 20 mm^2, or 50 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising:

a light source; and

an objective lens; and

at least one image sensor, wherein the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV.

222. A system for sequencing nucleic acids, the system comprising:

an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2 or 50 mm^2, wherein the one or more surfaces are axially displaced from each other, the optical system comprising:

a light source;

an objective lens; and

at least one image sensor,

wherein the optical resolution of the optical system is at least 0.4 um, 0.5 um, 0.8 um, or 1 um in a plane orthogonal to an axial axis, and

wherein the system is configured to complete a sequencing cycle in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds.

223. A system for sequencing nucleic acids, the system comprising:

an optical system configured to acquire flow cell images of one or more samples immobilized on one or more surfaces with a field-of-view (FOV) of greater than 10 mm^2 or 50 mm^2 wherein the one or more surfaces are axially displaced from each other, the optical system comprising:

a light source;

an imaging module that lacks an objective lens; and

at least one image sensor,

wherein the optical resolution of the optical system is at least 0.4 um, 0.5 um, 0.8 um, or 1 um in a plane orthogonal to an axial axis, and

wherein the system is configured to complete a sequencing cycle in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds.

224. The system of any one of the preceding embodiments, wherein the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV within a wavelength range from 420 nm to 800 nm.

225. The system of any one of the preceding embodiments, wherein the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV within a wavelength range from 500 nm to 750 nm.

226. The system of any one of the preceding embodiments, wherein the light source illuminates the FOV with less than 15%, 10%, or 5% energy variation across the FOV with at least two different colors.

227. The system of any one of the preceding embodiments, wherein the system is configured to complete a sequencing cycle by acquiring flow cell images from at least two different color channels in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds.

228. The system of any one of the preceding embodiments, wherein the system is configured to complete a sequencing cycle by acquiring flow cell images from at least two different color channels and from at least two different z levels in less than 5 minutes, 3 minutes, 2 minutes, 60 seconds, 30 seconds, or 10 seconds.

229. The system of any one of the preceding embodiments, wherein the light source comprises one or more lasers.

230. The system of any one of the preceding embodiments, wherein the light source comprises a single laser.

231. The system of any one of the preceding embodiments, wherein the energy variation across the FOV comprises a root mean square of energy differences.

232. The system of any one of the preceding embodiments, wherein the energy variation across the FOV comprises a ratio of the root mean square of energy differences to an average energy level, and wherein the ratio is less than 5%, 6%, 7%, 8%, 9%, 10% or 15%.

233. The system of any one of the preceding embodiments, wherein the RMS wavefront error is for a field of view (FOV) of 1.5 mm, 2 mm, 2.5 mm, 3 mm, 3.5 mm, 4 mm, 5 mm, or 8 mm in x or y direction that is orthogonal to an axial axis.

234. The system of any one of the preceding embodiments, wherein the one or more surfaces comprises four surfaces, and the RMS wavefront error is greater for a fourth surface than a first, second, or third surface of the one or more surfaces.

235. The system of any one of the preceding embodiments, wherein the one or more surfaces comprises three or more surfaces.

236. The system of any one of the preceding embodiments, wherein the RMS wavefront error of the optical system is less than a diffraction limit of the optical system.

237. The system of any one of the preceding embodiments, wherein the RMS wavefront error is less than 2%, 5%, 7%, 9%, 10%, 12% or 15% of a predetermined wavelength.

238. The system of any one of the preceding embodiments, wherein the predetermined wavelength is a central wavelength of the light source.

239. The system of any one of the preceding embodiments, wherein the optical system further comprising a numerical aperture (NA) of less than 0.4, 0.5, 0.6, or 0.7.

240. The system of any one of the preceding embodiments, wherein the optical system further comprising a numerical aperture (NA) of greater than 0.25.

241. The system of any one of the preceding embodiments, wherein the optical system further comprising a numerical aperture (NA) in a range from 0.25 to 0.5.

242. The system of any one of the preceding embodiments, wherein the resolution of the optical system is less than 1000, 800, or 500 nm.

243. The system of any one of the preceding embodiments, wherein the optical system is configured to complete a sequencing cycle within less than 10 minutes and wherein the FOV of the flow cell images is greater than 10 mm^2, 20 mm^2, 40 mm^2, 60 mm^2, or 100 mm^2.

244. The system of any one of the preceding embodiments, wherein the optical system is configured to complete a sequencing cycle within less than 60 seconds, 30 seconds, or 10 seconds per mm^2 per cycle.

245. The system of any one of the preceding embodiments, wherein the flow cell images are from two or more different color channels.

246. The system of any one of the preceding embodiments, wherein the flow cell images are from 4 different color channels.

247. The system of any one of the preceding embodiments, wherein the one or more color channels comprises 1, 2, 3, or 4 color channels.

248. The system of any one of the preceding embodiments, wherein the flow cell images comprises a FOV that is greater than 10 mm^2, 20 mm^2, 40 mm^2, 60 mm^2, or 100 mm^2.

249. The system of any one of the preceding embodiments, wherein the flow cell images are of the one or more samples of at least two z-levels.

250. The system of any one of the preceding embodiments, wherein the optical system is configured to enable a sequencing cycle time of less than 2 mins, 3 mins, or 6 mins.

251. The system of any one of the preceding embodiments, wherein the optical system is configured to enable an imaging cycle time of less than 2 mins, 3 mins, or 6 mins.

252. The system of any one of the preceding embodiments, wherein the flow cell images comprises optical signals emitting from a sample immobilized on a support.

253. The system of any one of the preceding embodiments, wherein the sample comprises a volumetric sample.

254. The system of any one of the preceding embodiments, wherein the volumetric sample comprises a thickness that is greater than 0.5 um, 1 um, 1.5 um, 2 um, 2.5 um, or 5 um.

255. The system of any one of the preceding embodiments, wherein the sample comprises an in situ sample of a cell, tissue, or both.

256. The system of any one of the preceding embodiments, wherein the light source is configured to uniformly illuminate an area of a sample that is greater than 1 mm^2 , 10 mm^2, 50 mm^2 or 100 mm^2 with less than 10% variance in illumination power across the illuminated area.

257. The system of any one of the preceding embodiments, wherein the system further comprising: a processor configured to process the flow cell images to correct for optical aberration.

258. The system of any one of the preceding embodiments, wherein the optical system further comprising: a processor configured to process the flow cell images to generate an optical resolution that is about identical in the flow cell images of the one or more surfaces.

259. The system of any one of the preceding embodiments, wherein the objective lens comprises an optical aperture stop with an adjustable size configured to change the NA of the optical system.

260. The system of any one of the preceding embodiments, wherein changing the adjustable size of the optical aperture is configured to change the NA of the optical system in the range from 0.4 to 0.6.

261. The system of any one of the preceding embodiments, wherein changing the adjustable size of the optical aperture is configured to change the NA of the optical system in the range from 0.25 to 0.85.

262. The system of any one of the preceding embodiments, wherein the processor is configured to detect a fluorescent-labeled composition comprising nucleic acids and disposed on the one or more surfaces to determine an identity of a nucleotide.

263. The system of any one of the preceding embodiments, wherein the at least one image sensor comprising pixels having a pixel size such that a spatial sampling frequency for the optical system is at least twice an optical resolution of the optical system.

264. The system of any one of the preceding embodiments, wherein the flow cell images of the one or more surfaces are acquired without moving an optical compensator into an optical path between the objective lens and the at least one image sensor.

265. The system of any one of the preceding embodiments, wherein the optical system is configured to acquire the flow cell images of the one or more surfaces with the optical solution without moving an optical compensator into an optical path between the objective lens and the at least one image sensor.

266. The system of any one of the preceding embodiments, wherein the flow cell images of one or more surfaces are acquired without moving an optical compensator out from an optical path between the objective lens and the at least one image sensor.

267. The system of any one of the preceding embodiments, wherein the optical system is configured to acquire the flow cell images of the one or more surfaces with the optical solution without moving an optical compensator out from an optical path between the objective lens and the at least one image sensor.

268. The system of any one of the preceding embodiments, wherein the flow cell images are acquired after refocusing the optical system for each of the one or more surfaces.

269. The system of any one of the preceding embodiments, wherein the one or more surfaces comprise at least three surfaces that are interior surfaces of two fluidic channels of the flow cell.

270. The system of any one of the preceding embodiments, wherein the two fluidic channels are displaced from each other along an axial direction.

271. The system of any one of the preceding embodiments, wherein the hydrophilic coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density of greater than 10,000 nucleic acid polonies/mm^2.

272. The system of any one of the preceding embodiments, wherein the hydrophilic coating layer comprises labeled nucleic acid polonies disposed thereon at a surface density of greater than 100,000 nucleic acid polonies/mm^2.

273. The system of any one of the preceding embodiments, wherein the flow cell has a total thickness of about 0.33, 0.335, 0.34, 0.345, 0.35, 0.355, or 0.36 mm along a direction orthogonal to the image plane.

274. The system of any one of the preceding embodiments, wherein the flow cell has a top or bottom wall thickness of at least 700 um along an axial direction orthogonal to the image plane.

275. The system of any one of the preceding embodiments, wherein the flow cell has a gap of at least 50 um along an axial direction orthogonal to the image plane in a first or second fluidic channel.

276. The system of any one of the preceding embodiments, wherein the flow cell has an interposer of at least 500 um along an axial direction orthogonal to the image plane between a first and second fluidic channel.

277. The system of any one of the preceding embodiments, wherein the flow cell has a top wall thickness of about 0.9 mm, a gap defined by a first fluidic channel of about 0.1 mm, an interposer wall thickness of about 0.145mm, a second gap defined by a second fluidic channel of about 0.1 mm, and a bottom wall thickness of about 0.9 mm.

278. The system of any one of the preceding embodiments, wherein the flow cell has a total thickness of about 0.22, 0.225, 0.23, 0.235, 0.24, 0.245, 0.25, or 0.255 mm along a direction orthogonal to the one or more surfaces.

279. The system of any one of the preceding embodiments, wherein the flow cell has a top wall thickness of about 0.93 mm, a gap defined by a first fluidic channel of about 0.07 mm, an interposer wall thickness of about 0.1 mm, a second gap defined by a second fluidic channel of about 0.07 mm, and a bottom wall thickness of about 0.93 mm.

280. The system of any one of the preceding embodiments, wherein the flow cell has a total thickness of about 0.2 to 0.39 mm along an axial direction orthogonal to the image plane and wherein each of a first and second fluidic channel has a gap of about 50 um to 100 um.

281. The system of any one of the preceding embodiments, wherein the optical system comprises 1, 2, 3, or 4 channels configured to detect nucleic acid ponies disposed on at least one of the one or more surfaces and have been labeled with 1, 2, 3, or 4 distinct detectable labels.

282. The system of any one of the preceding embodiments, wherein the optical system further comprises a focusing mechanism configured to refocus the optical system between acquiring the flow images of two different surfaces of the one or more surfaces.

283. The system of any one of the preceding embodiments, wherein the focusing mechanism comprises an autofocus laser and an autofocus sensor.

284. The system of any one of the preceding embodiments, wherein the optical system is configured to image two or more FOVs on at least one surface of the one or more surfaces.

285. The system of any one of the preceding embodiments, wherein the optical resolution of the flow cell images is diffraction-limited across the entire FOV.

286. The system of any one of the preceding embodiments, wherein the at least one image sensor comprises an active area with a diagonal of greater than or equal to about 15 mm.

287. The system of any one of the preceding embodiments, wherein the field-of-view (FOV) is greater than 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, or 6 mm^2.

288. The system of any one of the preceding embodiments, wherein the objective lens has a magnifying power sufficient to image the two or more FOVs.

289. The system of any one of the preceding embodiments, wherein the optical system further comprises a dichroic mirror and bandpass filter set.

290. The system of any one of the preceding embodiments, wherein the determining of the nucleotide in the nucleic acid molecule comprises performing sequencing-by-avidity, sequencing-by-nucleotide base-pairing, sequencing-by-nucleotide binding, or sequencing-by-nucleotide incorporation reaction on at least one of the one or more surfaces.

291. The system of any one of the preceding embodiments, wherein a first surface of the one or more surfaces is disposed in an optical path between the objective lens and a second surface of the one or more surfaces, and wherein the second surface is disposed in the optical path between the first surface and a third surface of the one or more surfaces.

292. The system of any one of the preceding embodiments, wherein the system further comprises: a fluid flow controller configured to control sequential and iterative delivery of a reagent to the one or more surfaces.

293. The system of any one of the preceding embodiments, wherein the light source comprises a illumination system.

294. The system of any one of the preceding embodiments, wherein the processor is programed to instruct the system to iteratively perform a sequencing method comprising:

contacting the plurality of primed target nucleic acid sequences coupled to the one or more surfaces with a nucleotide composition to form a transient binding complex between the plurality of primed target nucleic acid sequences and a plurality of nucleotide moieties when a nucleotide moiety of the nucleotide composition is complementary to a nucleotide of the primed target nucleic acid sequence; and

imaging the one or more surfaces of the flow cell to detect the transient binding complex and thereby determine an identity of the nucleotide of the primed target nucleic acid sequence.

295. The system of any one of the preceding embodiments, wherein the illumination system comprises an optical system designed to project periodic patterns of light on each of the one or more surfaces of the flow cell, and wherein a relative orientation or phase shift of a plurality of the periodic patterns of light is adjustable.

296. The system of any one of the preceding embodiments, wherein the illumination system comprises a first optical arm comprising a first light emitter to emit light and a first beam splitter to split light emitted by the first light emitter to project a first plurality of fringes on the one or more surfaces.

297. The system of any one of the preceding embodiments, wherein the illumination system further comprises a second optical arm comprising a second light emitter to emit light and a second beam splitter to split light emitted by the second light emitter to project a second plurality of fringes on the one or more surfaces.

298. The system of any one of the preceding embodiments, wherein the illumination system further comprises an optical element to combine a first optical path of the first arm and a second path of the second arm.

299. The system of any one of the preceding embodiments, wherein the first beam splitter comprises a first transmissive diffraction grating and the second beam splitter comprises a second transmissive diffraction grating.

300. The system of any one of the preceding embodiments, wherein the first and second light emitters emit unpolarized light, and wherein the first and second transmissive diffraction gratings are to diffract unpolarized light emitted by a respective one of the first and second light emitters.

301. The system of any one of the preceding embodiments, wherein the optical element to combine an optical path of the first plurality of fringes and the second plurality of fringes comprises a mirror with holes, with the mirror arranged to reflect light diffracted by the first

transmissive diffraction grating and with the holes arranged to pass through at least first orders of light diffracted by the second transmissive diffraction grating.

302. The system of any one of the preceding embodiments further comprising: one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes.

303. The system of any one of the preceding embodiments, wherein the one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes comprise a first rotating optical window to phase shift the first plurality of fringes and a second rotating optical window to phase shift the second plurality of optical fringes.

304. The system of any one of the preceding embodiments, wherein the one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes comprise a first linear motion stage to translate the first diffraction grating and a second linear motion stage to translate the second diffraction grating.

305. The system of any one of the preceding embodiments, wherein the one or more optical elements to phase shift the first plurality of fringes and the second plurality of fringes comprise a single rotating optical window, wherein the single rotating optical window is positioned after the mirror with holes in an optical path to the sample.

306. The system of any one of the preceding embodiments, wherein an axis of rotation of the single rotating optical window is offset by about 45 degrees from an optical axis of each of the gratings.

307. The system of any one of the preceding embodiments, wherein the first plurality of fringes is angularly offset from the second plurality of fringes on the sample plane by about 90 degrees.

308. The system of any one of the preceding embodiments, wherein the sample comprises a plurality of features regularly patterned in a rectangular array or hexagonal array.

309. The system of any one of the preceding embodiments, wherein the objective lens is configured to project each of the first plurality of fringes and the second plurality of fringes on the sample.

310. The system of any one of the preceding embodiments, the system further comprising: one or more optical beam blockers for blocking zero orders of light emitted by each of the first and second diffraction gratings.

311. The system of any one of the preceding embodiments, wherein the optical element to combine an optical path of the first arm and the second arm comprises a polarizing beam splitter, wherein the first diffraction grating diffracts vertically polarized light and wherein the second diffraction grating diffracts horizontally polarized light.

312. The system of any one of the preceding embodiments, wherein the first and second beam splitters each comprise a beam splitter cube or plate.

313. The system of any one of the preceding embodiments, wherein the first beam splitter comprises a first reflective diffraction grating and the second beam splitter comprises a second reflective diffraction grating.

314. The system of any one of the preceding embodiments, wherein the illumination system comprises a multiple beam splitter slide comprising a plurality of beam splitters mounted on a linear translation stage.

315. The system of any one of the preceding embodiments, wherein the plurality of beam splitters has fixed orientations with respect to the system's optical axis.

316. The system of any one of the preceding embodiments, wherein the plurality of beam splitters comprises a plurality of diffraction gratings.

317. The system of any one of the preceding embodiments, wherein the plurality of diffraction gratings comprises two diffraction gratings.

318. The system of any one of the preceding embodiments, wherein the illumination system comprises a fixed two-dimensional diffraction grating used in combination with a spatial filter wheel to project one-dimensional diffraction patterns on the one or more surfaces of the flow cell.

319. The system of any one of the preceding embodiments, wherein the one or more samples are at least uni-plex, 4-plex, 8-plex, 16-plex, 32-plex, 64-plex, 128-plex, 256-plex, or 512-plex.

320. The system of any one of the preceding embodiments, wherein the system is configured to sequence the one or more samples to uniquely identify a first number of morphological features, a second number of RNA features, and a third number of protein features within no more than two sequencing cycles and at two different z levels.

321. The system of any one of the preceding embodiments, wherein a sum of the first, second, and third number is greater than 10, 20, 32, 64, or 128.

322. The system of any one of the preceding embodiments, wherein each of the first, second, and third number is a non-zero integer

323. The system of any one of the preceding embodiments, wherein the one or more samples comprises more than 2, 4, 8, 16, 32 or 64 types of cells.

324. A method for sequencing nucleic acid molecules comprising:

providing a flow cell comprising one or more surfaces, wherein each surface comprises, immobilized thereon:

a plurality of clonally amplified sample nucleic acid molecules; or

a cellular sample comprising a plurality of DNA amplicons;

adjusting a NA of the optical system;

causing, by an illumination system, the plurality of clonally amplified sample nucleic acid molecules to fluoresce in on and off events in different colors, wherein the illumination system generate a center wavelength λ of excitation with a root-mean-square (RMS) wavefront error of less than 0.09λ, 0.08λ, 0.07λ, 0.06λ, or 0.02λ; and

detecting, at least by an image sensor, fluorescent signal emitted from the one or more samples in a color channel for each color for said each surface of the one or more surfaces to determine an identify of a nucleotide of the sample nucleic acid molecule or the DNA amplicons.

325. A method for sequencing nucleic acid molecules comprising:

providing a flow cell comprising one or more surfaces, wherein each surface comprises, immobilized thereon:

a plurality of clonally amplified sample nucleic acid molecules; or

a cellular sample comprising a plurality of DNA amplicons;

adjusting a NA of the optical system;

causing, by an illumination system, the plurality of clonally amplified sample nucleic acid molecules to fluoresce in on and off events in different colors; and

detecting, at least by an image sensor and in a field of view of greater than 10 mm^2 or 50 mm^2, fluorescent signal emitted from the one or more samples in one or more color channels for each color for said each surface of the one or more surfaces to determine an identify of a nucleotide of the sample nucleic acid molecule or the DNA amplicons.

326. A method for sequencing nucleic acid molecules comprising:

providing a flow cell comprising one or more surfaces, wherein each surface comprises, immobilized thereon:

a plurality of clonally amplified sample nucleic acid molecules; or

a cellular sample comprising a plurality of DNA amplicons;

causing, by an illumination system, the plurality of clonally amplified sample nucleic acid molecules to fluoresce in different colors in a first sequencing cycle; and

detecting, at least by an image sensor and in a field of view of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in one or more color channels for each color at two different z-levels of the one or more surfaces;

causing, by the illumination system, the plurality of clonally amplified sample nucleic acid molecules to fluoresce in on and off events in different colors in a second sequencing cycle; and

detecting, at least by the image sensor and in a field of view of greater than 10 mm^2, fluorescent signal emitted from the one or more samples in the one or more color channels for each color at two different z-levels of the one or more surfaces; and

uniquely identifying a first number of morphological features, a second number of RNA features, and a third number of protein features of the one or more samples based on the detection in the first and second sequencing cycle.

327. A method of imaging a sample, comprising:

providing the system of any one of the preceding embodiments;

performing, for each cycle of a sequence run and within a predetermined sequence cycle time, one or more of the following operations:

moving a sample stage relative to an objective lens of the optical system to a predetermined location in a plane orthogonal to an axial direction;

flowing a first reagent to a first surface of the one or more surfaces;

autofocusing the objective lens along the axial direction;

illuminating the first surface of the one or more surfaces with light from a light source;

obtaining flow cell images of the first surface with the at least one image sensor from one or more channels at a first axial level in less than 1, 2, 3, or 5 minutes;

obtaining flow cell images of the second surface with the at least one image sensor from the one or more channels at a second axial level different from the first axial level in less than 1, 2, 3, or 5 minutes;

flowing a second reagent to the first and second surfaces of the one or more surfaces; and

flowing a washing solution to the first and second surfaces of the one or more surfaces.

328. A method of imaging a sample, comprising:

providing the system of any one of the preceding embodiments;

performing, for a first cycle of a sequence run and within a predetermined sequence cycle time, one or more of the operations:

moving a sample stage relative to the optical system to a predetermined location in a plane orthogonal to an axial direction;

flowing a first reagent to a first surface of the one or more surfaces;

autofocusing the optical system along the axial direction;

illuminating the first surface of the one or more surfaces with light from a light source;

obtaining flow cell images of the first surface with the at least one image sensor from one or more channels at a first axial level;

obtaining flow cell images of the second surface with the at least one image sensor from one or more channels at a second axial level different from the first axial level;

flowing a second reagent to the first surface of the one or more surfaces; and

flowing a washing solution to the first surface of the one or more surfaces.

329. The method of any one of the preceding embodiments further comprising repeat performing, for a second cycle of a sequence run and within a predetermined sequence cycle time, one or more of the operations:

moving a sample stage relative to the optical system to a predetermined location in a plane orthogonal to an axial direction;

flowing a first reagent to a first surface of the one or more surfaces;

autofocusing the optical system along the axial direction;

illuminating a FOV of at least 10 mm^2 or 50 mm^2 on the first surface of the one or more surfaces with light from a light source;

obtaining flow cell images of the first surface with the at least one image sensor from one or more channels at a first axial level;

obtaining flow cell images of the second surface with the at least one image sensor from one or more channels at a second axial level different from the first axial level;

flowing a second reagent to the first surface of the one or more surfaces; and

flowing a washing solution to the first surface of the one or more surfaces.

330. The method of any one of the preceding embodiments, wherein moving a sample stage relative to the optical system to a predetermined location in a plane orthogonal to an axial direction comprises:

keeping the sample state still relative to a housing of the system; and

moving, by an actuator, a focusing element of the imaging module of the optical system relative to the housing of the system, wherein the optical system lacks an objective lens.

331. The method of any one of the preceding embodiments, wherein a RMS wavefront error of the optical system is for a field of view (FOV) of 1.5 mm, 2 mm, 2.5 mm, 3 mm, 3.5 mm, or 4 mm in x or y direction that is orthogonal to an axial axis.

332. The method of any one of the preceding embodiments, wherein the one or more surfaces comprises four surfaces, and the RMS wavefront error is greater for a fourth surface than a first, second, or third surface of the four surfaces.

333. The method of any one of the preceding embodiments, wherein the RMS wavefront error of the optical system is less than a diffraction limit of the optical system.

334. The method of any one of the preceding embodiments, wherein the optical system further comprising a numerical aperture (NA) of less than 0.4, 0.5, or 0.6, or 0.7.

335. The method of any one of the preceding embodiments, wherein the optical resolution of the optical system is less than 1000, 800, or 500 nm.

336. The method of any one of the preceding embodiments, wherein the optical system is configured to acquire the flow cell images per 100 mm^2, including sequencing time, within less than 20 mins, 15 mins, 10 mins, 5 mins, or 2 mins.

337. The method of any one of the preceding embodiments, wherein the flow cell images are from two or more different color channels.

338. The method of any one of the preceding embodiments, wherein the flow cell images are from 4 different color channels.

339. The method of any one of the preceding embodiments, wherein the predetermined sequence cycle time is less than 1 mins, 2 mins, 3 mins, 5 mins, or 6 mins.

340. The method of any one of the preceding embodiments, wherein the optical system is configured to enable an imaging cycle time of less than 2 mins, 3 mins, or 6 mins.

341. The method of any one of the preceding embodiments, wherein the flow cell images comprises optical signals emitting from a sample immobilized on a support.

342. The method of any one of the preceding embodiments, wherein the sample comprises a volumetric sample.

343. The method of any one of the preceding embodiments, wherein the volumetric sample comprises a thickness that is greater than 0.4 um, 0.5 um, 1 um, 1.5 um, 2 um, 2.5 um, or 5 um.

344. The method of any one of the preceding embodiments, wherein the sample comprises an in situ sample of a cell, tissue, or both.

345. The method of any one of the preceding embodiments, wherein the light source is configured to uniformly illuminate an area of a sample that is greater than 1 mm^2 with less than 10% variance in illumination power across the illuminated area.

346. The method of any one of the preceding embodiments, wherein the system further comprising: a processor configured to process the flow cell images to correct for optical aberration.

347. The method of any one of the preceding embodiments, wherein the optical system further comprising: a processor configured to process the flow cell images to generate an optical resolution that is about identical in the flow cell images of the one or more surfaces.

348. The method of any one of the preceding embodiments, wherein the objective lens comprises an optical aperture stop with an adjustable size configured to change the NA of the optical system.

349. The method of any one of the preceding embodiments, wherein changing the adjustable size of the optical aperture is configured to change the NA of the optical system in the range from 0.4 to 0.6.

350. The method of any one of the preceding embodiments, wherein changing the adjustable size of the optical aperture is configured to change the NA of the optical system in the range from 0.25 to 0.85.

351. The method of any one of the preceding embodiments, wherein the processor is configured to detect a fluorescent-labeled composition comprising nucleic acids and disposed on the one or more surfaces to determine an identity of a nucleotide.

352. The method of any one of the preceding embodiments, wherein the at least one image sensor comprising pixels having a pixel size such that a spatial sampling frequency for the optical system is at least twice an optical resolution of the optical system.

353. The method of any one of the preceding embodiments, wherein the one or more samples are uni-plex, 4-plex, 8-plex, 16-plex, 32-plex, 64-plex, 128-plex, 256-plex, or 512-plex.

354. The method of any one of the preceding embodiments, wherein the one or more samples comprises more than 2, 4, 8, 16, 32 or 64 types of cells.

355. The method of any one of the preceding embodiments, wherein a sum of the first, second, and third number is greater than 10, 20, 32, 64, or 128.

356. The method of any one of the preceding embodiments, wherein each of the first, second, and third number is a non-zero integer.

357. The method of any one of the preceding claims, wherein the first number of morphological features, the second number of RNA features, the third number of protein features, or their combinations are located inside cells or tissue.

CLAIMS

What is claimed is:

1. A system for in situ biomolecule analysis, the system comprising:
   an imaging system comprising:
      a flow cell configured to hold a cell or a tissue immobilized thereon, wherein said cell or said tissue comprises a plurality of analytes that differ in type from each other;
      a light source configured to illuminate said cell or said tissue, thereby generating a plurality of signals corresponding to the plurality of analytes; and
      a detector configured to image said plurality of signals; and
      one or more processors communicatively coupled to said imaging system, wherein said one or more processors is individually or collectively programed to
         (a) illuminate, using said light source, said cell or said tissue, thereby generating said plurality of signals corresponding to said plurality of analytes;
         (b) detect, using said detector, said plurality of signals; and
         (c) determine, using said one or more computer processors, an identity or sequence of said plurality of analytes using said plurality of signals.
2. The system of claim 1, wherein said cell or tissue is an in situ cell or tissue sample.
3. The system of claim 1, wherein said light source is configured to illuminate greater than about 20 square millimeters (mm^2) of said flow cell and said cell or said tissue with a peak-to-valley energy or power variation of at most about 5%.
4. The system of claim 1, wherein said light source is configured to illuminate greater than about mm^2 of said flow cell and said cell or said tissue with a RMS wavefront error of at most about 0.09λ.
5. The system of claim 1, wherein said imaging system has a composite root mean square error of less than about 0.05.
6. The system of claim 1, wherein said cell or said tissue is a whole cell or whole tissue.
7. The system of claim 1, wherein said imaging system does not comprise an objective disposed within an optical path of said light source or said detector.
8. The system of claim 7, wherein said imaging system does not comprise an objective.
9. The system of claim 1, wherein said imaging system does not comprise a tube lens.
10. The system of claim 1, wherein said illumination has an irradiance of at least about 40 milliwatts per square meter.
11. The system of claim 1, wherein said cell or said tissue has been permeabilized.
12. The system of claim 1, wherein said plurality of signals are a plurality of fluorescent signals.

13. The system of claim 1, wherein said plurality of signals are detected with a Q-score of at least 30, 40, or 50.
14. The system of claim 1, wherein said cell or said tissue is illuminated with 10 illumination fields in one or more planes perpendicular to an optical axis of said imaging system.
15. The system of claim 1, wherein a field of view of said detector is at least about 10 mm^2.
16. The system of claim 1, wherein said cell or said tissue is imaged with a resolution of at least about 1 micrometer.
17. The system of claim 1, wherein said flow cell is configured to permit the flow of one or more reagents into contact with said cell or said tissue.
18. The system of claim 1, wherein said cell or said tissue is a cultured cell or a cultured tissue.
19. The system of claim 1, wherein said cell or said tissue is an isolated cell or an isolated tissue.
20. The system of claim 1, wherein a fidelity of imaging of said cell or said tissue is at least about 0.1 micrometers.
21. The system of claim 1, wherein said plurality of analytes comprise a nucleic acid molecule.
22. The system of claim 21, wherein said nucleic acid molecule is a deoxyribonucleic acid molecule.
23. The system of claim 21, wherein said nucleic acid molecule is a ribonucleic acid molecule.
24. The system of claim 1, wherein said plurality of analytes comprise a protein.
25. The system of claim 1, wherein said plurality of analytes comprise a carbohydrate.
26. A method for imaging an in situ sample, comprising:
    (a) providing said in situ sample comprising a plurality of different types of analytes;
    (b) illuminating said plurality of different types of analytes to generate a plurality of signals related to said plurality of analytes; and
    (c) imaging said plurality of signals.
27. The method of claim 26, wherein said illuminating said plurality of different types of analytes is a sequential illumination of said plurality of different types of analytes.
28. The method of claim 26, wherein said illuminating said plurality of different types of analytes is a simultaneous illumination of said plurality of different types of analytes.
29. The method of claim 26, wherein said plurality of different types of analytes are selected from the group consisting of deoxyribonucleic acid molecules, ribonucleic acid molecules, proteins, morphological features, and phosphorylated proteins.
30. The method of claim 26, further comprising applying one or more sequencing reagents on said in situ sample configured to sequence said plurality of different types of analytes.

31. The method of claim 26, wherein said illuminating is over an area of said flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%.
32. The method of claim 26, wherein said illuminating is over at least about 1 mm^2 of said flow cell with a RMS wavefront error of at most about 0.09λ.
33. The method of claim 26, wherein said illuminating is over an area of said flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%.
34. The method of claim 26, further comprising (d) using a computer processor operatively coupled to said detector to analyze said plurality of signals.
35. The method of claim 34, wherein said analyzing said plurality of signals comprises determining a sequence of a nucleic acid molecule within said in situ sample.
36. The method of claim 35, wherein said sequence of said nucleic acid molecule is determined with an accuracy, sensitivity, or specific of at least about 95%.
37. The method of claim 36, wherein said sequence of said nucleic acid molecule is determined in an absence of altering a spatial relationship of said nucleic acid within said in situ sample.
38. The method of claim 26, wherein said in situ sample has a length, width, or height of at least about 10 micrometers.
39. The method of claim 26, wherein said in situ sample comprises a tissue.
40. The method of claim 26, wherein said in situ sample comprises a plurality of cultured cells.
41. The method of claim 26, wherein said in situ sample comprises a plurality of isolated cells.
42. The method of claim 26, wherein said in situ sample is imaged with about 10 images in a plane perpendicular to an optical axis of said optical assembly.
43. The method of claim 26, wherein said plurality of signals are a plurality of fluorescent signals.
44. The method of claim 26, wherein said plurality of signals are detected with a Q-score of at least 30, 40, or 50.
45. The method of claim 26, wherein said in situ sample comprises a nucleic acid molecule.
46. The method of claim 45, wherein said nucleic acid molecule is a deoxyribonucleic acid molecule.
47. The method of claim 45, wherein said nucleic acid molecule is a ribonucleic acid molecule.
48. The method of claim 26, wherein a field of view of said optical assembly is at least about 10 mm^2.
49. The method of claim 26, wherein said in situ sample is imaged at a resolution of at least about 1 micrometer.

50. The method of claim 26, wherein said in situ sample is imaged within at most about 24 hours.
51. The method of claim 26, wherein a fidelity of imaging a plurality of images of said in situ sample is at least about 0.1 micrometers.
52. An optical assembly for in situ imaging, comprising:

    a flow cell configured to contain an in situ sample;

    a light source configured to illuminate said in situ sample in said flow cell, thereby generating a signal related to a property of said in situ sample; and

    a detector configured to image said signal.
53. The optical assembly of claim 52, wherein said illumination over an area of said flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley energy or power variation of at most about 5%.
54. The optical assembly of claim 52, wherein said illumination has a root-mean-square (RMS) wavefront error of at most about 0.09λ over an area of at least about 1 square millimeter (mm^2).
55. The optical assembly of claim 52, further comprising a processor configured to analyze said signal to determine said property of said in situ sample.
56. The optical assembly of claim 52, wherein said in situ sample has a length, width, or height of at least about 10 micrometers.
57. The optical assembly of claim 52, wherein said optical assembly does not comprise an objective.
58. The optical assembly of claim 57, wherein said system does not comprise an objective.
59. The optical assembly of claim 52, wherein said optical assembly does not comprise a tube lens.
60. The optical assembly of claim 59, wherein said system does not comprise an objective.
61. The optical assembly of claim 52, wherein said in situ sample comprises a tissue.
62. The optical assembly of claim 52, wherein said in situ sample comprises a plurality of cultured cells.
63. The optical assembly of claim 52, wherein said in situ sample comprises a plurality of isolated cells.
64. The optical assembly of claim 52, wherein said in situ sample is imaged with at most about 10 images in a plane perpendicular to an optical axis of said optical assembly.
65. The optical assembly of claim 52, wherein said signal is a fluorescent signal.

66. The optical assembly of claim 52, wherein said signal is detected with a Q-score of at least about 30.
67. The optical assembly of claim 52, wherein said in situ sample comprises a nucleic acid molecule.
68. The optical assembly of claim 67, wherein said nucleic acid molecule is a deoxyribonucleic acid molecule.
69. The optical assembly of claim 67, wherein said nucleic acid molecule is a ribonucleic acid molecule.
70. The optical assembly of claim 52, wherein a field of view of said optical assembly is at least about 10 mm^2.
71. The optical assembly of claim 52, wherein said in situ sample is imaged at a resolution of at least about 1 micrometer.
72. The optical assembly of claim 52, wherein said in situ sample is imaged within at most about 24 hours.
73. The optical assembly of claim 52, wherein a fidelity of imaging a plurality of images of said in situ sample is at least about 0.1 micrometers.
74. A method for imaging an in situ sample, comprising:
    (a) providing said in situ sample in a flow cell comprised within a system comprising an optical assembly comprising a light source and a detector;
    (b) illuminating said in situ sample and generating a signal related to an analyte of said in situ sample; and
    (c) imaging, using said detector, said signal.
75. The method of claim 74, wherein said illuminating is over an area of said flow cell that is greater than about 20 square millimeters (mm^2) has a peak-to-valley variation of at most about 5%.
76. The method of claim 74, wherein said illuminating is over at least about 1 mm^2 of said flow cell with a RMS wavefront error of at most about 0.09λ.
77. The method of claim 74, further comprising (d) using a computer processor operatively coupled to said detector to analyze said signal.
78. The method of claim 77, wherein said analyzing said signal comprises determining a sequence of a nucleic acid molecule within said in situ sample.
79. The method of claim 78, wherein said sequence of said nucleic acid molecule is determined with an accuracy, sensitivity, or specific of at least about 95%.
80. The method of claim 79, wherein said sequence of said nucleic acid molecule is determined in an absence of destroying said in situ sample.

81. The method of claim 74, wherein said in situ sample has a length, width, or height of at least about 10 micrometers.
82. The method of claim 74, wherein said optical assembly does not comprise an objective.
83. The method of claim 82, wherein said system does not comprise an objective.
84. The method of claim 74, wherein said optical assembly does not comprise a tube lens.
85. The method of claim 84, wherein said system does not comprise an objective.
86. The method of claim 74, wherein said in situ sample comprises a tissue.
87. The method of claim 74, wherein said in situ sample comprises a plurality of cultured cells.
88. The method of claim 74, wherein said in situ sample comprises a plurality of isolated cells.
89. The method of claim 74, wherein said in situ sample is imaged with at most about 10 images in a plane perpendicular to an optical axis of said optical assembly.
90. The method of claim 74, wherein said signal is a fluorescent signal.
91. The method of claim 74, wherein said signal is detected with a Q-score of at least about 30.
92. The method of claim 74, wherein said in situ sample comprises a nucleic acid molecule.
93. The method of claim 92, wherein said nucleic acid molecule is a deoxyribonucleic acid molecule.
94. The method of claim 92, wherein said nucleic acid molecule is a ribonucleic acid molecule.
95. The method of claim 74, wherein a field of view of said optical assembly is at least about 10 mm^2.
96. The method of claim 74, wherein said in situ sample is imaged at a resolution of at least about 1 micrometer.
97. The method of claim 74, wherein said in situ sample is imaged within at most about 24 hours.
98. The method of claim 74, wherein a fidelity of imaging a plurality of images of said in situ sample is at least about 0.1 micrometers.