

AMPLIFICATION-FREE PROBE COMPLEXES FOR DETECTION OF TARGET ANALYTES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to, and benefit of, U.S. Provisional Application No. 63/549,340, filed on February 2, 2024, the contents of which are incorporated by reference in their entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The contents of the electronic sequence listing (ELEM_030_001WO_SeqList_ST26.xml; Size: 5,420 bytes; and Date of Creation: December 30, 2024) are herein incorporated by reference in their entireties.

TECHNICAL FIELD

[0003] The present disclosure provides compositions, apparatuses and methods for detecting cellular target analytes using amplification-free probe complexes. In some embodiments, the amplification-free probe complexes can be used for detecting analytes on a cellular sample or inside a cellular sample.

BACKGROUND

[0004] Cells within a tissue of a subject exhibit differences in composition, morphology and function due to varied analyte levels (*e.g.*, gene and/or protein expression) within the cells. The specific position of a cell within a tissue (*e.g.*, the cell's position relative to neighboring cells or the cell's position relative to the tissue microenvironment), developmental stage, and pathological conditions can affect, *e.g.*, cell morphology, differentiation, fate, viability, proliferation, signaling, and crosstalk with other cells in the tissue, and other cellular behaviors. Spatial heterogeneity has been previously studied using techniques that only provide data for a small handful of analytes such as proteins, nucleic acids, polysaccharides or lipids, in the context of an intact tissue or a portion of a tissue. However, previous methods are limited by the number of analytes that can be identified and imaged, and the amount of time and labor involved. Thus, there exists a need for improved spatially resolved analysis methods which can be used to image multiple analytes in a cell or tissue.

SUMMARY

[0005] The disclosure provides an amplification-free probe complex comprising (i) at least one modified oligonucleotide; and (ii) at least one analyte binding moiety which can bind a target analyte, wherein the at least one modified oligonucleotide is attached to the at least one analyte binding moiety. In some embodiments, the at least one modified oligonucleotide comprises two or more tandem copies of a sequencing primer binding site. In some embodiments, the at least one modified oligonucleotide comprises canonical nucleobases located at a position that is immediately of the 5' ends of individual sequencing primer binding sites. In some embodiments, the at least one modified oligonucleotide comprises an abasic site that is located immediately 5' of the canonical nucleobase.

[0006] In some embodiments of the amplification-free probe complex of the disclosure, the canonical nucleobases are located between 1 and 10 nucleotides 5' of the 5' ends of the individual sequencing primer binding sites. In some embodiments, the canonical nucleobases are 5' and adjacent to the 5' ends of the individual sequencing primer binding sites.

[0007] The disclosure provides an amplification-free probe complex comprising (i) at least one modified oligonucleotide; and (ii) at least one analyte binding moiety which can bind a target analyte, wherein the at least one modified oligonucleotide is attached to the at least one analyte binding moiety, wherein the at least one modified oligonucleotide comprises at least one sequencing primer binding site and a barcode sequence. In some embodiments, the at least one modified oligonucleotide comprises two or more tandem copies of the sequencing primer binding site and the barcode sequence.

[0008] In some embodiments of the amplification-free probe complex of the disclosure, the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the analyte binding moiety comprises a lipid moiety, a wheat germ agglutinin, an antibody or a biotin moiety. In some embodiments, the target analyte is inside a cellular sample and/or on the surface of a cellular sample. In some embodiments, the cellular sample comprises a whole single cell, a plurality of whole cells, an intact tissue, sectioned cell or a sectioned tissue sample. In some embodiments, the cellular sample comprises a fresh cellular sample, a freshly-frozen cellular sample, or a formalin-fixed and paraffin-embedded (FFPE) cellular sample. In some embodiments, the cellular sample comprises a fixed and permeabilized cellular sample. In some embodiments, the analyte binding moiety comprises a secondary antibody or a primary antibody.

[0009] The disclosure provides a method for detecting a target analyte, comprising: (a) providing a plurality of the amplification-free probe complexes of the disclosure; (b) providing a sample comprising a plurality of analytes, including at least one target analyte, wherein the sample is deposited on a support; (c) contacting the sample with the plurality of amplification-free probe complexes, wherein the contacting is conducted under a condition suitable for binding the analyte binding moieties to the target analytes; (d) contacting the plurality of modified oligonucleotides of the plurality of amplification-free probe complexes of step (c) with a plurality of sequencing primers under a condition suitable for individual sequencing primers to bind the sequencing primer binding sites on the modified oligonucleotides to form a plurality of nucleic acid duplexes, wherein individual nucleic acid duplexes comprise a sequencing primer binding site on a modified oligonucleotide hybridized to a sequencing primer; (e) contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases and a plurality of detectably labeled multivalent molecules, wherein individual nucleic acid duplexes bind a sequencing polymerase and a nucleotide moiety of a multivalent molecule, thereby forming a plurality of detectably labeled complexes, wherein a nucleotide moiety of a detectably labeled multivalent molecule is bound to the 3' end of the sequencing primer and opposite the canonical nucleo-base in the modified oligonucleotide, wherein the contacting of step (e) is conducted under a condition suitable for inhibiting polymerase-catalyzed incorporation of the nucleotide moiety into the 3' end of the sequencing primer, and wherein the nucleotide moiety of the multivalent molecule binds opposite and is complementary to a nucleotide in the modified oligonucleotide, thereby producing a plurality of detectably labeled complexes; and (f) imaging the sample which is bound to the plurality of detectably labeled complexes thereby detecting a target analyte on a cellular sample or inside a cellular sample.

[0010] The disclosure provides a method for detecting a target analyte, comprising: (a) providing a plurality of the amplification-free probe complexes of the disclosure; (b) providing a sample comprising a plurality of analytes, including at least one target analyte, wherein the sample is deposited on a support; (c) contacting the sample with the plurality of amplification-free probe complexes, wherein the contacting is conducted under a condition suitable for binding the analyte binding moieties to the target analyte; (d) contacting the plurality of modified oligonucleotides of the plurality of amplification-free probe complexes of step (c) with a plurality of sequencing primers under a condition suitable for individual sequencing primers to bind the sequencing primer binding sites on the modified oligonucleotides to form a plurality of nucleic acid duplexes, wherein individual nucleic acid

duplexes comprise a sequencing primer binding site on the modified oligonucleotide hybridized to a sequencing primer; (e) contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases and a plurality of detectably labeled multivalent molecules, wherein individual nucleic acid duplexes bind a sequencing polymerase and a nucleotide moiety of a multivalent molecule, thereby forming a plurality of detectably labeled complexes, wherein the nucleotide moiety of a detectably labeled multivalent molecule is bound to the 3' end of the sequencing primer and opposite the barcode sequence in the modified oligonucleotide, and wherein the nucleotide moiety of the multivalent molecule binds opposite and is complementary to a nucleotide in the modified oligonucleotide, thereby producing a plurality of detectably labeled complexes; and (f) imaging the sample, thereby detecting the target analyte.

[0011] In some embodiments of the method of the disclosure, the contacting of step (e) is conducted under a condition suitable for inhibiting polymerase-catalyzed incorporation of the nucleotide moiety into the 3' end of the sequencing primer.

[0012] In some embodiments of the method of the disclosure, individual multivalent molecules comprise a core attached to a plurality of nucleotide-arms, wherein each nucleotide-arm comprises a nucleotide moiety. In some embodiments, the complementary nucleotide moiety of the multivalent molecule does not incorporate into the terminal 3' end of the sequencing primer. In some embodiments, the nucleotide moiety of the multivalent molecule is complementary to a nucleotide in the modified oligonucleotide that is 5' and adjacent to the sequencing primer binding site.

[0013] In some embodiments of the method of the disclosure, the sample is a cellular sample. In some embodiments, the target analyte is on the cellular sample and/or inside the cellular sample. In some embodiments, the cellular sample comprises a whole single cell, a plurality of whole cells, an intact tissue, sectioned cell or a sectioned tissue sample. In some embodiments, the cellular sample comprises a fresh cellular sample, a freshly-frozen cellular sample, or a formalin-fixed and paraffin-embedded (FFPE) cellular sample. In some embodiments, the cellular sample comprises a fixed and permeabilized cellular sample.

[0014] The disclosure provides an amplification-free probe complex comprising: (i) at least one modified oligonucleotide; and (ii) at least one analyte binding moiety which can bind a cellular target analyte, wherein the at least one modified oligonucleotide is attached to the at least one analyte binding moiety, wherein the at least one modified oligonucleotide comprises two or more tandem copies of a sequencing primer binding site, wherein the at least one modified oligonucleotide comprises an optional barcode sequence, wherein the at

least one modified oligonucleotide comprises a canonical nucleo-base located at a position that is immediately upstream of the beginning of individual sequencing primer binding sites, and wherein the at least one modified oligonucleotide comprises an abasic site that is located immediately upstream of the canonical nucleo-base.

[0015] In some embodiments of the amplification-free probe complex of the disclosure, the analyte binding moiety can bind a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the analyte binding moiety comprises a lipid moiety, a wheat germ agglutinin, an antibody or a biotin moiety. In some embodiments, the analyte binding moiety comprises a secondary antibody or a primary antibody. In some embodiments, the analyte binding moiety comprises a polynucleotide comprising a sequence that is complementary to a target nucleic acid.

[0016] The disclosure provides a method for detecting a target analyte on a cellular sample or inside a cellular sample, comprising: (a) providing a plurality of amplification-free probe complexes of the disclosure; (b) providing a cellular sample deposited on a support wherein the cellular sample comprises a plurality of analytes including at least one target analyte; (c) contacting the cellular sample with the plurality of amplification-free probe complexes, wherein the contacting is conducted under a condition suitable for binding the analyte binding moieties of the amplification-free probe complexes to their cognate target analytes, wherein the target analytes are located on the cellular sample or the target analytes are located inside the cellular sample; (d) contacting the plurality of modified oligonucleotides of the plurality of amplification-free probe complexes of step (c) with a plurality of sequencing primers under a condition suitable for individual sequencing primers to bind their cognate sequencing primer binding sites on a modified oligonucleotide to form a plurality of nucleic acid duplexes wherein individual nucleic acid duplexes comprise a sequencing primer binding site on a modified oligonucleotide hybridized to a sequencing primer; (e) contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases and a plurality of detectably labeled multivalent molecules, wherein individual nucleic acid duplexes bind a sequencing polymerase and a nucleotide moiety of a multivalent molecule thereby forming a plurality of detectably labeled complexes, wherein a nucleotide moiety of the multivalent molecule is bound to the 3' end of the sequencing primer and opposite the canonical nucleo-base in the modified oligonucleotide, wherein individual multivalent molecules comprise a core attached to a plurality of nucleotide-arms, wherein each nucleotide-arm comprises a nucleotide moiety, wherein the contacting of step (e) is conducted under a condition suitable for inhibiting polymerase-catalyzed incorporation of a nucleotide moiety into the 3' end of

the sequencing primer, and wherein a complementary nucleotide moiety of the multivalent molecule binds opposite a nucleotide in a given modified oligonucleotide, and the complementary nucleotide moiety of the multivalent molecule does not incorporate into the terminal 3' end of the sequencing primer; and (f) imaging the cellular sample which is bound to the plurality of detectably labeled complexes thereby detecting a target analyte on a cellular sample or inside a cellular sample.

DESCRIPTION OF THE DRAWINGS

[0017] The features of the present disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

[0018] **FIG. 1** is a schematic of various exemplary 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 moieties are designated ‘N’, biotin is designated ‘B’, and streptavidin is designated ‘SA’.

[0019] **FIG. 2** is a schematic of an exemplary multivalent molecule comprising a generic core attached to a plurality of nucleotide-arms.

[0020] **FIG. 3** is a schematic of an exemplary multivalent molecule comprising a dendrimer core attached to a plurality of nucleotide-arms.

[0021] **FIG. 4A** shows a schematic of an exemplary multivalent molecule comprising a core attached to a plurality of nucleotide-arms, where the nucleotide arms comprise a core attachment moiety, a spacer, a linker, and a nucleotide moiety.

[0022] **FIG. 4B** is a schematic of an exemplary nucleotide-arm of a multivalent molecule, wherein the nucleotide arm comprises a core attachment moiety, a spacer, a linker, and a nucleotide moiety (*e.g.*, nucleotide unit).

[0023] **FIG. 5A** shows a schematic of an exemplary multivalent probe comprising a core attached to a plurality of nucleotide-arms, where the nucleotide arms comprise a core attachment moiety, a spacer, a linker, and a target-specific oligonucleotide probe.

[0024] FIG. 5B is a schematic of an exemplary nucleotide-arm of a multivalent probe comprising a core attachment moiety, a spacer, a linker, and a target-specific oligonucleotide probe.

[0025] FIG. 6 shows the chemical structure of an exemplary spacer (Top), and the chemical structures of various exemplary linkers, including an 11-atom Linker, 16-atom Linker, 23-atom Linker and an N3 Linker (Bottom).

[0026] FIG. 7 shows the chemical structures of various exemplary linkers, including Linkers 1-9.

[0027] FIG. 8 shows the chemical structures of various exemplary linkers joined/attached to nucleotide moieties.

[0028] FIG. 9 shows the chemical structures of various exemplary linkers joined/attached to nucleotide moieties.

[0029] FIG. 10 shows the chemical structures of various exemplary linkers joined/attached to nucleotide moieties.

[0030] FIG. 11 shows the chemical structures of various exemplary linkers joined/attached to nucleotide moieties.

[0031] FIG. 12 shows the chemical structure of an exemplary biotinylated nucleotide-arm. In this example, the nucleotide moiety 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.

[0032] FIG. 13A is a schematic of an embodiment of an amplification-free probe complex comprising an analyte binding moiety attached to a modified oligonucleotide. The modified oligonucleotide can comprise at least one modified nucleotide or modified nucleotide linkage which is designated by XXX. The modified oligonucleotide can comprise one or more abasic sites which are designated by open triangles. The modified oligonucleotide can comprise one or more canonical nucleobases, which are designated by solid arrows.

[0033] FIG. 13B is a schematic of the same amplification-free probe complex shown in FIG. 13A with a plurality of sequencing primers hybridized to the modified oligonucleotide. XXX indicates modified nucleotides or modified nucleotide linkages, the open triangle indicates an abasic site, and the solid arrow indicates a canonical nucleobase.

[0034] FIG. 14A is a schematic of an embodiment of an amplification-free probe complex comprising an analyte binding moiety (e.g., secondary antibody) attached to a modified oligonucleotide. The modified oligonucleotide can comprise at least one modified nucleotide or modified nucleotide linkage which is designated by XXX. The modified oligonucleotide can comprise one or more abasic sites which are designated by open triangles. The modified

oligonucleotide can comprise one or more canonical nucleo-bases which are designated by solid arrows.

[0035] FIG. 14B is a schematic of the same amplification-free probe complex shown in FIG. 14A with a plurality of sequencing primers hybridized to the modified oligonucleotide. XXX indicates modified nucleotides or modified nucleotide linkages, the open triangle indicates an abasic site, and the solid arrow indicates a canonical nucleo-base.

[0036] FIG. 15A is a schematic of an embodiment of an amplification-free probe complex comprising an analyte binding moiety attached to a modified oligonucleotide. The modified oligonucleotide can comprise at least one modified nucleotide or modified nucleotide linkage which is designated by XXX. The modified oligonucleotide can comprise one or more abasic sites which are designated by open triangles. The modified oligonucleotide can comprise one or more canonical nucleo-bases which are designated by solid arrows.

[0037] FIG. 15B is a schematic of the same amplification-free probe complex shown in FIG. 15A with a plurality of sequencing primers hybridized to the modified oligonucleotide. XXX indicates modified nucleotides or modified nucleotide linkages, the open triangle indicates an abasic site, and the solid arrow indicates a canonical nucleo-base.

[0038] FIG. 16 is a schematic of an embodiment of an amplification-free probe complex comprising an analyte binding moiety attached to a plurality of modified oligonucleotides. Individual modified oligonucleotides can comprise at least one modified nucleotide or modified nucleotide linkage which is designated by XXX. Individual modified oligonucleotides can comprise one or more abasic sites which are designated by open triangles. Individual modified oligonucleotides comprise one or more canonical nucleo-bases which are designated by solid arrows. The plurality of modified oligonucleotides can be hybridized to a plurality of sequencing primers, as shown.

[0039] FIG. 17 is a schematic of an embodiment of an amplification-free probe complex comprising an analyte binding moiety attached to a plurality of modified oligonucleotides in branched form. Individual modified oligonucleotides can comprise at least one modified nucleotide or modified nucleotide linkage which is designated by XXX. Individual modified oligonucleotides can comprise one or more abasic sites which are designated by open triangles. Individual modified oligonucleotides can comprise one or more canonical nucleo-bases which are designated by solid arrows. The plurality of modified oligonucleotides can be hybridized to a plurality of sequencing primers, as shown.

[0040] FIG. 18A is a schematic of an embodiment of a modified oligonucleotide comprising at least one sequencing primer binding site and an optional barcode sequence.

The modified oligonucleotide can comprise a plurality of sequencing primer binding sites. The modified oligonucleotide can comprise a conjugation moiety for attachment to the analyte binding moiety. The modified oligonucleotide can comprise a linker moiety, *e.g.* to link one end of the modified oligonucleotide to the conjugation moiety. The modified oligonucleotide can comprise a canonical nucleo-base located at a position that is upstream of the beginning of individual sequencing primer binding sites. For example, the canonical nucleo-base can be located at a position that is immediately upstream of the beginning of individual sequencing primer binding sites. The canonical nucleo-base in the modified oligonucleotide is designated with a solid arrow. The at least one modified oligonucleotide can comprise an abasic site located upstream of the canonical nucleo-base. For example, the abasic site can be located immediately upstream of the canonical nucleo-base. The abasic site in the modified oligonucleotide is designated with an open triangle.

[0041] FIG. 18B is a schematic of the same modified oligonucleotide and sequencing primer as shown in Fig. 18A, with a sequencing polymerase and a detectably labeled multivalent molecule. The sequencing primer can be hybridized to the modified oligonucleotide, thereby forming a nucleic acid duplex. The sequencing polymerase can bind the nucleic acid duplex and can bind a complementary nucleotide moiety (NT) of a multivalent molecule opposite (*e.g.*, complementary to) a nucleotide in the modified oligonucleotide, thereby forming a detectably labeled complex. The detectably labeled complex can emit a detectable signal. The modified oligonucleotide can include at least one abasic site, which can inhibit a second binding event by another detectably labeled multivalent molecule, so that one nucleotide moiety of a detectably labeled multivalent molecule binds one nucleic acid duplex and one sequencing polymerase to emit a signal that is associated with one detectably labeled complex.

[0042] FIG. 19A is a schematic of an embodiment of an amplification-free probe complex comprising an analyte binding moiety attached to a modified oligonucleotide. The modified oligonucleotide can comprise at least one modified nucleotide or modified nucleotide linkage which is designated by XXX. The modified oligonucleotide can comprise one or more target barcode sequences.

[0043] FIG. 19B a schematic of an embodiment of a portion of a modified oligonucleotide comprising at least one sequencing primer binding site, a target barcode sequence and linker and conjugation moieties. XXX designates one or more modified nucleotides or modified nucleotide linkages.

[0044] FIG. 20 is a schematic of an embodiment of a plurality of amplification-free probe complexes binding a cellular sample. The plurality of amplification-free probe complexes can bind an outer cell membrane as shown in FIG. 20. Alternately, or in addition, the plurality of amplification-free probe complexes can enter a cell and can bind a cell membrane of a cellular organelle located inside the cellular sample, including for example a nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome or lysosome.

[0045] FIG. 21 is a table showing several embodiments of 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 binding cycle and employing multi-color imaging. In some embodiments, the barcode sequences listed in the table in FIG. 21 can be used for cell painting. The sequences, in FIG. 21, from top to bottom, are:

CTACCCGTGGTG (SEQ ID NO: 1); TCAAAATGGGGT (SEQ ID NO: 2);
CATCACTGTGGG (SEQ ID NO: 3); CCCTCAGTGTGG (SEQ ID NO: 4);
AAACCTTGTTT (SEQ ID NO: 5).

DETAILED DESCRIPTION

Definitions

[0046] 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.

[0047] 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.

[0048] 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.

[0049] 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.

[0050] 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).

[0051] 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.

[0052] As used herein, the terms “about” and “approximately” 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” or “approximately” 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.*, $\pm 10\%$) 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” or “approximately” 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.

[0053] As used herein, “corresponding to” or “corresponds to” refers to two or more entities whose identities are sufficiently related such that the identity of one entity can be used to determine the identity, position and/or other properties of the other entity. As non-limiting example, a barcode sequence can be said to correspond to a particular target analyte or fluorophore color if the fluorophore color can be used to determine the identity of the barcode sequence, and similarly, the barcode sequence can be used to determine the identity of the target analyte.

[0054] The term “polymerase” and its variants, as used herein, comprises an enzyme comprising a domain that binds a nucleotide (or nucleoside) where the polymerase can form a complex having a template nucleic acid and a complementary nucleotide. The polymerase can have one or more activities including, but not limited to, base analog detection activities, DNA polymerization activity, reverse transcriptase activity, DNA binding, strand displacement activity, and nucleotide binding and recognition. A polymerase can be any enzyme that can catalyze polymerization of nucleotides (including analogs thereof) into a nucleic acid strand. Typically, but not necessarily, such nucleotide polymerization can occur in a template-dependent fashion. Typically, a polymerase comprises one or more active sites at which nucleotide binding and/or catalysis of nucleotide polymerization can occur. In some embodiments, a polymerase includes other enzymatic activities, such as for example, 3' to 5' exonuclease activity or 5' to 3' exonuclease activity. In some embodiments, a polymerase has strand displacing activity. A polymerase can include, without limitation, naturally occurring polymerases and any functional subunits and truncations thereof, mutant polymerases, variant polymerases, recombinant, fusion or otherwise engineered polymerases, chemically modified polymerases, synthetic molecules or assemblies, and any analogs, derivatives or fragments thereof that retain the ability to catalyze nucleotide polymerization (*e.g.*, catalytically active fragment). Polymerases can include catalytically inactive polymerases, catalytically active polymerases, reverse transcriptases, and other enzymes comprising a nucleotide binding domain. In some embodiments, a polymerase can be isolated from a cell, or generated using recombinant DNA technology or chemical synthesis methods. In some embodiments, a polymerase can be expressed in prokaryote, eukaryote, viral, or phage organisms. In some embodiments, a polymerase can be post-translationally modified proteins, or functional fragments thereof. A polymerase can be derived from a prokaryote, eukaryote, virus, or phage. A polymerase can comprise a DNA-directed DNA polymerase and RNA-directed DNA polymerase.

[0055] The terms “nucleic acid”, “polynucleotide” and “oligonucleotide” and other related terms used herein are used interchangeably and refer to polymers of nucleotides and are not limited to any particular length. Nucleic acids can include recombinant and chemically-synthesized forms. Nucleic acids can be isolated. Nucleic acids can include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs (*e.g.*, peptide nucleic acids (PNA) and non-naturally occurring nucleotide analogs), chimeric forms containing DNA and RNA, and combinations thereof. Nucleic acids can be single-stranded or double-stranded. Nucleic acids can comprise polymers of nucleotides, where the nucleotides include natural or non-natural bases and/or sugars. Nucleic acids comprise naturally-occurring internucleosidic linkages, for example and without limitation, phosphodiester linkages. Nucleic acids can lack a phosphate group. Nucleic acids can comprise non-natural internucleoside linkages, including phosphorothioate, phosphorothiolate, or peptide nucleic acid (PNA) linkages. In some embodiments, nucleic acids comprise one type of polynucleotide, or a mixture of two or more different types of polynucleotides.

[0056] The term “operably linked” and “operably joined” or related terms as used herein refers to juxtaposition of components. The juxtapositioned components can be linked together covalently. For example, and without limitation, two nucleic acid components can be enzymatically ligated together where the linkage that joins together the two components comprises phosphodiester linkage. A first and second nucleic acid component can be linked together, where the first nucleic acid component can confer a function on a second nucleic acid component. For example, and without limitation, linkage between a primer binding sequence and a sequence of interest forms a nucleic acid library molecule having a portion that can bind to a primer. In another non-limiting example, a transgene (*e.g.*, a nucleic acid encoding a polypeptide or a nucleic acid sequence of interest) can be ligated to a vector where the linkage permits expression or functioning of the transgene sequence contained in the vector. In some embodiments, a transgene is operably linked to a host cell regulatory sequence (*e.g.*, a promoter sequence) that affects expression of the transgene. In some embodiments, the vector comprises at least one host cell regulatory sequence, including a promoter sequence, enhancer, transcription and/or translation initiation sequence, transcription and/or translation termination sequence, polypeptide secretion signal sequences, and the like. In some embodiments, the host cell regulatory sequence controls expression of the level, timing and/or location of the transgene.

[0057] The terms “linked”, “joined”, “attached”, “appended” and variants thereof as used herein comprise any type of fusion, bond, adherence or association between any combination of compounds or molecules that is of sufficient stability to withstand use in the particular procedure. The procedures can include but are not limited to: nucleotide binding; nucleotide incorporation; de-blocking (*e.g.*, removal of chain-terminating moiety); washing; removing; flowing; detecting; imaging and/or identifying. Such linkage can comprise, for example, and without limitation, covalent, ionic, hydrogen, dipole-dipole, hydrophilic, hydrophobic, or affinity bonding, bonds or associations involving van der Waals forces, mechanical bonding, and the like. In some embodiments, such linkage occurs intramolecularly, for example, by linking together the ends of a single-stranded or double-stranded linear nucleic acid molecule to form a circular molecule. In some embodiments, such linkage can occur between a combination of different molecules, or between a molecule and a non-molecule, including but not limited to: linkage between a nucleic acid molecule and a solid surface; linkage between a protein and a detectable reporter moiety; linkage between a nucleotide and detectable reporter moiety; and the like. Suitable linkages are known in the art and some examples of linkages can be found, for example and without limitation, in Hermanson, G., “Bioconjugate Techniques”, Second Edition (2008); Aslam, M., Dent, A., “Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences”, London: Macmillan (1998); Aslam, M., Dent, A., “Bioconjugation: Protein Coupling Techniques for the Biomedical Sciences”, London: Macmillan (1998).

[0058] The term “primer” and related terms used herein refers to an oligonucleotide that is capable of hybridizing with a DNA and/or RNA polynucleotide template to form a duplex molecule. Primers can comprise natural nucleotides and/or nucleotide analogs. Primers can be recombinant nucleic acid molecules. Primers may have any length, but typically range from about 4-50 nucleotides. A typical primer comprises a 5' end and 3' end. The 3' end of the primer can include a 3' OH moiety which serves as a nucleotide polymerization initiation site in a polymerase-catalyzed primer extension reaction. Alternatively, the 3' end of the primer can lack a 3' OH moiety, or can include a terminal 3' blocking group that inhibits nucleotide polymerization in a polymerase-catalyzed reaction. Any one nucleotide, or more than one nucleotide, along the length of the primer can be labeled with a detectable reporter moiety (*e.g.*, a fluorophore). A primer can be in solution (*e.g.*, a soluble primer) or can be immobilized to a support (*e.g.*, a capture primer).

[0059] The term “template nucleic acid”, “template polynucleotide”, “template strand” and other variations as used herein refers to a nucleic acid strand that serves as the basis

nucleic acid molecule for a polymerase chain reaction or sequencing method employing a templated strand extension step or the like. The template nucleic acid can be single-stranded or double-stranded, or the template nucleic acid can have single-stranded or double-stranded portions. The template nucleic acid can be obtained from a naturally-occurring source, recombinant form, or chemically synthesized to include any type of nucleic acid analog. The template nucleic acid can be linear, circular, or other suitable forms. In some embodiments, the template nucleic acid can be in a concatemer form. The template nucleic acids can include an insert portion having an insert sequence. The template nucleic acids can also include at least one adaptor sequence. The insert portion can be isolated in any form, including chromosomal, genomic, organellar (*e.g.*, mitochondrial, chloroplast or ribosomal), recombinant molecules, cloned, amplified, cDNA, RNA such as precursor mRNA or mRNA, oligonucleotides, whole genomic DNA, obtained from fresh frozen paraffin embedded tissue (FFPE), needle biopsies, circulating tumor cells, cell free circulating DNA, or any type of nucleic acid library. The template nucleic acid can be subjected to nucleic acid analysis, including sequencing and composition analysis.

[0060] In some embodiments, any of the amplification primer sequences, sequencing primer sequences, barcode sequences or spatial barcode sequences can be about 3-50 nucleotides in length, or about 5-40 nucleotides in length, or about 5-25 nucleotides in length, or any range therebetween. In some embodiments, any of the aforementioned sequences may be about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 nucleotides in length.

[0061] The term “universal sequence” and related terms as used herein refers to a sequence in a nucleic acid molecule that is common among two or more polynucleotide molecules. For example, and without limitation, an adaptor having a universal sequence can be operably joined to a plurality of polynucleotides so that the population of co-joined molecules carry the same universal adaptor sequence. Non-limiting examples of universal adaptor sequences can include an amplification primer sequence, a sequencing primer sequence or a capture primer sequence (*e.g.*, soluble primers, or immobilized capture primers).

[0062] As used herein, the term “selectively binds” in the context of any binding agent, *e.g.*, an oligonucleotide or oligonucleotide complex of the disclosure, refers to a binding agent that binds specifically to a target, *e.g.*, a target sequence, such as with a high affinity, and does not significantly bind other unrelated targets or sequences. The person of ordinary skill in the art will appreciate that a binding agent that binds specifically to a target with high

affinity, but binds to non-targets (off-targets) with suitably low affinity can still be said to selectively bind to the target.

[0063] As used herein, the term “target polynucleotide” refers to any nucleic acid inside a cellular sample having a sequence that can bind at least one amplification-free probe complex. Target polynucleotides include without limitation RNA, cDNA and DNA. In some embodiments, target polynucleotides comprise polynucleotides expressed inside a cellular sample including, without limitation, naturally occurring polynucleotides and recombinant polynucleotides.

[0064] As used herein, the term “target analyte” refers to any analyte that can be bound by an analyte binding moiety of the amplification-free probe complexes disclosed herein. Exemplary target analytes can be on the surface of or inside a cell. Exemplary target analytes include, but are not limited to polynucleotides, proteins, lipids, polysaccharides and the like. When used with respect to a primary antibody, the term “target analyte” refers to any analyte that can be bound by the primary antibody.

[0065] The term “binds specifically” or “specific binding activity” and related terms, refers to two molecules, such as an analyte binding moiety and target analyte, that form a complex that is relatively stable under assay conditions. The term is also applicable where an antigen-binding domain of an antibody is specific for a particular epitope, which is carried by a number of antigens, in which case the antibody carrying the antigen-binding domain will be able to bind to the various antigens carrying the epitope. Specific binding is characterized by a high affinity and a low to moderate capacity. As an example, the binding is considered specific when the affinity constant is about 1×10^{-6} M, generally at least about 1×10^{-7} M, usually at least about 1×10^{-8} M, and preferably at least about 1×10^{-9} M or 1×10^{-10} M or less.

[0066] As used herein, the term “target sequence” refers to a sequence in a modified oligonucleotide or concatemer molecule which can bind a probe arm of a multivalent probe. In some embodiments, a multivalent probe comprises a core attached to a plurality of probe arms wherein individual probe arms comprise a polymer linked to a target-specific oligonucleotide probe. In some embodiments, a target sequence comprises a barcode sequence, a sample index sequence and/or a batch barcode sequence.

[0067] As used herein, with respect to a nucleotide sequence, “upstream” refers to sequence located 5' of a particular reference feature in the nucleotide sequence. Similarly, “downstream” refers to sequence located 3' of the particular reference feature. “Immediately” upstream or downstream refers to a nucleotide or sequence that is approximately 1-10

nucleotides (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10) upstream or downstream of the reference feature.

[0068] As used herein, the term “sequencing read product” refers to a primer extension product generated by conducting a sequencing reaction using a sequencing primer hybridized to a template molecule to be sequenced (*e.g.*, a modified oligonucleotide or a concatemer molecule), a sequencing polymerase and a plurality of nucleotides. In some embodiments, the sequencing polymerase catalyzes nucleotide incorporation using the 3' end of the sequencing primer as an initiation site and generates an extension product comprising a sequence that is complementary to the template molecule. In some embodiments, a nucleotide incorporation reaction extends the sequencing primer by one nucleotide. In some embodiments, the number of nucleotide incorporation reactions that are conducted will dictate the length of the sequencing read product. For example, conducting eleven nucleotide incorporation reactions will generate a sequencing primer that is extended by eleven nucleotides. In some embodiments, one cycle of a sequencing reaction comprises: conducting one nucleotide incorporation reaction using a sequencing polymerase and a nucleotide thereby extending the sequencing primer by one nucleotide. In some embodiments, one cycle of a sequencing reaction comprises: (i) binding a multivalent molecule to the 3' end of a first sequencing primer and a sequencing polymerase under a condition that inhibits polymerase-catalyzed nucleotide incorporation where the multivalent molecule comprises a plurality of nucleotide arms attached to a core; (ii) removing the multivalent molecule and the sequencing polymerase while retaining the template molecule hybridized to the sequencing primer; and (iii) conducting one nucleotide incorporation reaction using a second sequencing polymerase and a nucleotide thereby extending the sequencing primer by one nucleotide.

[0069] When used herein in reference to nucleic acid molecules, the terms “hybridize” or “hybridizing” or “hybridization” or other related terms refers to hydrogen bonding between two different nucleic acids to form a duplex nucleic acid. Hybridization also can include hydrogen bonding between two different regions of a single nucleic acid molecule to form a self-hybridizing molecule having a duplex region. Hybridization can comprise Watson-Crick or Hoogsteen binding to form a duplex double-stranded nucleic acid, or a double-stranded region within a nucleic acid molecule. The double-stranded nucleic acid, or the two different regions of a single nucleic acid, may be wholly complementary, or may be partially complementary. Complementary nucleic acid strands need not hybridize with each other across their entire length. The complementary base pairing can be the standard A-T or C-G

base pairing, or can be other forms of base-pairing interactions. Duplex nucleic acids can include mismatched base-paired nucleotides.

[0070] When used herein in reference to nucleic acids, the terms “extend”, “extending”, “extension” and other variants, refer to incorporation of one or more nucleotides into a nucleic acid molecule. Nucleotide incorporation comprises polymerization of one or more nucleotides into the terminal 3' OH end of a nucleic acid strand, resulting in extension of the nucleic acid strand. Nucleotide incorporation can be conducted with natural nucleotides and/or nucleotide analogs. Typically, but not necessarily, nucleotide incorporation occurs in a template-dependent fashion. Any suitable method of extending a nucleic acid molecule known in the art may be used, including, but not limited to, primer extension catalyzed by a DNA polymerase or RNA polymerase.

[0071] As used herein, the term “nucleotides” and related terms refers to a molecule comprising an aromatic base, a five carbon sugar (*e.g.*, ribose or deoxyribose), and at least one phosphate group. Canonical or non-canonical nucleotides are consistent with use of the term. The phosphate in some embodiments comprises a monophosphate, diphosphate, or triphosphate, or corresponding phosphate analog. The term “nucleoside” as used herein refers to a molecule comprising an aromatic base and a sugar. Nucleotides and nucleosides can be non-labeled or labeled with a detectable reporter moiety.

[0072] Nucleotides (and nucleosides) typically comprise a hetero cyclic base including substituted or unsubstituted nitrogen-containing parent heteroaromatic ring which are commonly found in nucleic acids, including naturally-occurring, substituted, modified, or engineered variants, or analogs of the same. The base of a nucleotide (or nucleoside) is capable of forming Watson-Crick and/or Hoogsteen hydrogen bonds with an appropriate complementary base. Exemplary bases include, but are not limited to, purines and pyrimidines, such as: 2-aminopurine, 2,6-diaminopurine, adenine (A), ethenoadenine, N⁶-Δ²-isopentenyladenine (6iA), N⁶-Δ²-isopentenyl-2-methylthioadenine (2ms6iA), N⁶-methyladenine, guanine (G), isoguanine, N²-dimethylguanine (dmG), 7-methylguanine (7mG), 2-thiopyrimidine, 6-thioguanine (6sG), hypoxanthine and O⁶-methylguanine; 7-deaza-purines such as 7-deazaadenine (7-deaza-A) and 7-deazaguanine (7-deaza-G); pyrimidines such as cytosine (C), 5-propynylcytosine, isocytosine, thymine (T), 4-thiothymine (4sT), 5,6-dihydrothymine, O⁴-methylthymine, uracil (U), 4-thiouracil (4sU) and 5,6-dihydrouracil (dihydrouracil; D); indoles such as nitroindole and 4-methylindole; pyrroles such as nitropyrrole; nebularine; inosines; hydroxymethylcytosines; 5-methylcytosines; base (Y); as well as methylated, glycosylated, and acylated base moieties; and the like. Additional

exemplary bases can be found in Fasman, 1989, in "Practical Handbook of Biochemistry and Molecular Biology", pp. 385-394, CRC Press, Boca Raton, Fla.

[0073] Nucleotides (and nucleosides) typically comprise a sugar moiety, such as carbocyclic moiety (Ferraro and Gotor 2000 Chem. Rev. 100: 4319-48), acyclic moieties (Martinez, et al., 1999 Nucleic Acids Research 27: 1271-1274; Martinez, et al., 1997 Bioorganic & Medicinal Chemistry Letters vol. 7: 3013-3016), and other sugar moieties (Joeng, et al., 1993 J. Med. Chem. 36: 2627-2638; Kim, et al., 1993 J. Med. Chem. 36: 30-7; Eschenmosser 1999 Science 284:2118-2124; and U.S. Pat. No. 5,558,991). In some embodiments, the sugar moiety comprises ribosyl; 2'-deoxyribosyl; 3'-deoxyribosyl; 2',3'-dideoxyribosyl; 2',3'-didehydrodideoxyribosyl; 2'-alkoxyribosyl; 2'-azidoribosyl; 2'-aminoribosyl; 2'-fluororibosyl; 2'-mercaptoriboxyl; 2'-alkylthioribosyl; 3'-alkoxyribosyl; 3'-azidoribosyl; 3'-aminoribosyl; 3'-fluororibosyl; 3'-mercaptoriboxyl; 3'-alkylthioribosyl carbocyclic; acyclic or other modified sugars.

[0074] In some embodiments, nucleotides comprise 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 phosphoramidate linkage. In some embodiments, the nucleotide 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.

[0075] The term "reporter moiety", "reporter moieties" or related terms as used herein refers to a compound that generates, or causes to generate, a detectable signal. A reporter moiety is sometimes called a "label". Any suitable reporter moiety known in the art may be used, including, but not limited to, luminescent, photoluminescent, electroluminescent, bioluminescent, chemiluminescent, fluorescent, phosphorescent, chromophore, radioisotope, electrochemical, mass spectrometry, Raman, hapten, affinity tag, atom, or an enzyme reporter moiety. A reporter moiety can generate a detectable signal resulting from a chemical or physical change (*e.g.*, heat, light, electrical, pH, salt concentration, enzymatic activity, or proximity events). A proximity event can include two reporter moieties approaching each other, or associating with each other, or binding to each other. It is well known to one skilled in the art to select reporter moieties so that each absorbs excitation radiation and/or emits fluorescence at a wavelength distinguishable from the other reporter moieties to permit monitoring the presence of different reporter moieties in the same reaction or in different

reactions. Two or more different reporter moieties can be selected having spectrally distinct emission profiles, or having minimal overlapping spectral emission profiles. Reporter moieties can be linked (*e.g.*, operably linked) to nucleotides, nucleosides, nucleic acids, enzymes (*e.g.*, polymerases or reverse transcriptases), or supports (*e.g.*, surfaces).

[0076] In some embodiments, a reporter moiety (or label) comprises a fluorescent label or a fluorophore. Exemplary 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, carboxynaphthofluorescein, 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 1A, BODIPY 530/551 1A, 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, thiazolium 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 consist of two indolenin, benzo-indolium, pyridium, thiazolium, 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. Additional fluorophores are described in WO 2024/124008, the contents of which are incorporated by reference in their entirety herein.

[0077] In some embodiments, the reporter moiety can be a fluorescence resonance energy transfer (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.

[0078] When used herein in reference to nucleic acids, the terms “amplify”, “amplifying”, “amplification”, and other related terms include producing multiple copies of an original polynucleotide template molecule, where the copies comprise a sequence that is complementary to the template sequence, or the copies comprise a sequence that is the same as the template sequence. In some embodiments, the copies comprise a sequence that is substantially identical to a template sequence, or is substantially identical to a sequence that is complementary to the template sequence.

[0079] The term “support” as used herein refers to a substrate that is designed for deposition of biological molecules or cellular samples for assays and/or analyses. Examples of biological molecules to be deposited onto a support include nucleic acids (*e.g.*, DNA, RNA), polypeptides, saccharides, lipids, a single cell or multiple cells. Examples of cellular

samples include, but are not limited to saliva, phlegm, mucus, blood, plasma, serum, urine, stool, sweat, tears, smears, fluids from tissues or organs, as well as tissue samples (*e.g.*, biopsy samples).

[0080] In some embodiments, the support is solid, semi-solid, or a combination of both. In some embodiments, the support is porous, semi-porous, non-porous, or any combination of porosity. In some embodiments, the support can be substantially planar, concave, convex, or any combination thereof. In some embodiments, the support can be cylindrical, for example comprising a capillary or interior surface of a capillary.

[0081] In some embodiments, the surface of the support can be substantially smooth. In some embodiments, the support can be regularly or irregularly textured, including bumps, etched, pores, three-dimensional scaffolds, or any combination thereof.

[0082] In some embodiments, the support comprises a bead having any shape, including spherical, hemi-spherical, cylindrical, barrel-shaped, toroidal, disc-shaped, rod-like, conical, triangular, cubical, polygonal, tubular or wire-like.

[0083] The support can be fabricated from any material, including but 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 substrates are contemplated.

[0084] The term “persistence time” and related terms as used herein refers to the length of time that a binding complex, which is formed between the binding components of a target nucleic acid, a polymerase, and a conjugated or unconjugated nucleotide, remains stable without any binding component dissociating from the binding complex. The persistence time is indicative of the stability of the binding complex and strength of the binding interactions. Persistence time can be measured by observing the onset and/or duration of a binding complex, such as by observing a signal from a labeled component of the binding complex. For example, and without limitation, a labeled nucleotide or a labeled reagent comprising one or more nucleotides may be present in a binding complex, thus allowing the signal from the label to be detected during the persistence time of the binding complex. One exemplary non-limiting label is a fluorescent label. Such labels for detection may also be referred to as “detectable labels” herein.

Amplification-Free Probe Complexes

[0085] The present disclosure provides compositions comprising at least one amplification-free probe complex and methods that employ the at least one amplification-free probe complex for detection of target analytes. In some embodiments, the target analyte can be located inside a cellular sample and/or on the membrane of a cellular sample. In some embodiments, the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the target analyte is located anywhere in the cellular sample, including without limits the cytoplasm and nucleus. In some embodiments, the target analyte is located, without limits, on any cellular membrane, or the nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, peroxisome or lysosome. In some embodiments, the cellular sample, comprises, for example and without limitation, a single cell, multiple cells, a tissue, an organ, a tumor, or portions thereof. In some embodiments, the amplification-free probe complex does not undergo amplification, for example rolling circle amplification. In some embodiments, the amplification-free probe complex does not generate an amplicon (*e.g.*, concatemer molecule).

[0086] The present disclosure provides an amplification-free probe complex comprising: (i) at least one modified oligonucleotide; and (ii) at least one analyte binding moiety which can bind a target analyte. In some embodiments, the at least one modified oligonucleotide is attached to the at least one analyte binding moiety (*e.g.*, FIGS. 13A-13B, 14A-14B, 15A-15B, 16 and 17). In some embodiments, the at least one modified oligonucleotide is attached to the at least one analyte binding moiety via a conjugation moiety and/or linker. In some embodiments, the at least one modified oligonucleotide comprises at least one primer binding site (*e.g.*, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more).

[0087] The amplification-free probe complexes have many uses, including but not limited to detection and imaging of target analytes on a cell and/or inside a cell (*e.g.*, FIG. 20).

[0088] In some embodiments, individual amplification-free probe complexes comprise at least one modified oligonucleotide (*e.g.*, FIGS. 18A and 18B). In some embodiments, the at least one modified oligonucleotide comprises a nucleic acid, including recombinant and chemically-synthesized forms. In some embodiments, the modified oligonucleotide comprises DNA, RNA or DNA and RNA. In some embodiments, the modified oligonucleotide comprises canonical nucleotides and/or nucleotide analogs. In some embodiments, the modified oligonucleotide comprises polymers of nucleotides, where the nucleotides include natural or non-natural bases and/or sugars. In some embodiments, the modified oligonucleotide comprises naturally-occurring internucleosidic linkages, for

example and without limitation, phosphodiester linkages. In some embodiments, the modified oligonucleotide comprises a 5' phosphate group or lacks a 5' phosphate group. In some embodiments, the modified oligonucleotide comprises non-natural internucleoside linkages, including phosphorothioate, phosphorothiolate, or peptide nucleic acid (PNA) linkages.

[0089] In some embodiments, the modified oligonucleotide comprises a linear oligonucleotide (*e.g.*, FIGS. 13A-13B, 14A-14B, 15A-15B and 16) or a branched oligonucleotide (*e.g.*, FIG. 17).

[0090] In some embodiments, the at least one modified oligonucleotide comprises a conjugation moiety at one end to enable attachment to a cell binding moiety (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises a linker moiety (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises modified nucleotides at one end where the modified nucleotides confer nuclease resistance to the modified oligonucleotide (*e.g.*, FIG. 18A). Exemplary modified nucleotides that confer nuclease resistance include without limitation at least one phosphorothioate linkage and/or at least one 2'-O-methylcytosine bases. For example, the modified oligonucleotide comprises at least one phosphorothioate linkage at the 5' or 3' end which can render the modified oligonucleotide resistant to nuclease degradation. In some embodiments, the modified oligonucleotide comprises 2-5, or more, consecutive phosphorothioate diester bonds at the 5' end. In some embodiments, the oligonucleotide comprises at least one ribonucleotide and/or at least one 2'-O-methyl, 2'-O-methoxyethyl (MOE), 2' fluoro-base nucleotide. In some embodiments, the 3' region of the oligonucleotide can include at least one 2'-O-methyl RNA bases which blocks polymerase-catalyzed extension. In FIG. 18A, the one or more modified nucleotides or modified nucleotide linkages are designated with XXX.

[0091] In some embodiments, the modified oligonucleotide comprises at least one sequencing primer binding site, wherein individual sequencing primer binding sites are positioned in close proximity to a canonical nucleo-base and an abasic site (FIG. 18A). Individual sequencing primer binding sites can hybridize to a sequencing primer to form a nucleic acid duplex which can be used to conduct a polymerase-catalyzed sequencing reaction by binding a detectably labeled multivalent molecule to the 3' end of the sequencing primer at a position that is opposite the canonical nucleo-base. The abasic site inhibits a second sequencing reaction. The amplification-free probe complexes are designed to restrict a sequencing reaction to a single binding event that employs a detectably labeled multivalent molecule. In some embodiments, an amplification-free probe complex comprises: (i) at least

one modified oligonucleotide comprising at least one sequencing primer binding site, at least one canonical nucleobase, and at least one abasic site; and (ii) at least one analyte binding moiety which can bind a target analyte. In some embodiments, the at least one modified oligonucleotide comprises a canonical nucleobase located at a position that is upstream of the beginning of individual sequencing primer binding sites. In some embodiments, the canonical nucleobase located at a position that is upstream of the beginning of individual sequencing primer binding sites. In some embodiments, the at least one modified oligonucleotide comprises an abasic site that is located upstream of the canonical nucleobase. In some embodiments, the abasic site can be located immediately upstream of the canonical nucleobase.

[0092] In some embodiments, the at least one modified oligonucleotide comprises one or more sequencing primer binding sites (*e.g.*, FIG. 18A). In some embodiments, the sequencing primer binding site comprises a universal sequencing primer binding site. In some embodiments, the at least one modified oligonucleotide comprises two or more tandem copies of a sequencing primer binding site (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises a concatemer molecule having two or more tandem copies of a sequencing primer binding site (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises a canonical nucleobase located at a position that is upstream of the beginning of individual sequencing primer binding sites (*e.g.*, FIG. 18A). In some embodiments, the canonical nucleobase located at a position that is immediately upstream of the beginning of individual sequencing primer binding sites (*e.g.*, FIG. 18A). For example, the at least one modified oligonucleotide comprises a canonical nucleobase that is located between 1 and 10 (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10) nucleotides 5' of the 5' end of an individual sequencing primer binding site. Alternatively, the at least one modified oligonucleotide comprises a canonical nucleobase that is located 5' of and immediately adjacent to the 5' end of an individual sequencing primer binding site, *i.e.*, 5' and immediately adjacent to the 3' end of a bound sequencing primer on the complementary strand. In some embodiments, the at least one modified oligonucleotide comprises an abasic site that is located upstream (*i.e.*, 5') of the canonical nucleobase (*e.g.*, FIG. 18A). In some embodiments, the abasic site is located immediately 5' and adjacent to the canonical nucleobase. In some embodiments, the abasic site is located immediately upstream of the canonical nucleobase (*e.g.*, FIG. 18A).

[0093] In some embodiments, the modified oligonucleotide of any of the amplification-free probe complexes comprises one or more sequencing primer binding sites. In some

embodiments, individual sequencing primer binding sites comprise a universal sequencing primer binding site. In some embodiments, the at least one modified oligonucleotide comprises two or more tandem copies of a sequencing primer binding site. In some embodiments, the two or more copies of sequencing primer binding sites can have the same sequence or different sequences.

[0094] In some embodiments, the amplification-free probe complex comprises at least one modified oligonucleotide comprising at least one sequencing primer binding site, at least one target barcode sequence, and a conjugation moiety (*e.g.*, a barcoded amplification-free probe complex) (*e.g.*, FIGS. 19A-19B). In some embodiments, the barcoded amplification-free probe complex comprises a linear or branched structure similar to the amplification-free probe complexes shown in any of FIGS. 13A-13B, 14A-14B, 15A-15B, 16 and 17. In some embodiments, the conjugation moiety can be attached to an analyte binding moiety. In some embodiments, the target barcode sequence of the modified oligonucleotide comprises a short random sequence comprising 3-20 nucleotides in length (*e.g.*, NNN or NNNN), or any range therebetween. The short random sequence is designed, for example, to provide nucleotide diversity and color balance generated by the detectable signals when the barcode is sequenced.

[0095] In some embodiments, in the short random sequence each base “N” at a given position is independently selected from A, G, C, T or U. In some embodiments, the random sequence lacks consecutive repeat sequences having 2 or 3 of the same nucleobase, for example, AA, TT, CC, GG, UU, AAA, TTT, CCC, GGG or UUU.

[0096] In some embodiments, in a population of modified oligonucleotides, the short random sequence comprises a high diversity sequence which includes approximately equal proportions of all four nucleotides (*e.g.*, A, G, C, T and/or U) that will be represented in each cycle of a sequencing run.

[0097] In some embodiments, the short random sequence (*e.g.*, NNN) includes, but is not limited to, AGC, AGT, GAC, GAT, CAT, CAG, TAG, TAC. The skilled artisan will recognize that many more random sequences can be prepared (*e.g.*, 64 possible combinations) where each base “N” at a given position in the random sequence is independently selected from A, G, C, T or U.

[0098] In some embodiments, the modified oligonucleotide is attached to an analyte binding moiety. The analyte binding moiety can bind, without limitation, a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the analyte binding moiety comprises a lipid moiety, a wheat germ agglutinin, an antibody or a biotin moiety. In some

embodiments, the analyte binding moiety comprises a polynucleotide comprising a sequence that is complementary to a target nucleic acid. In some embodiments, the antibody comprises a secondary antibody or a primary antibody. In some embodiments, the modified oligonucleotide can be attached to a primary antibody. In some embodiments, the modified oligonucleotide can be attached to a secondary antibody, and the secondary antibody can be attached/associated to a primary antibody. In some embodiments, the primary antibody binds the target analyte. In some embodiments, the primary and secondary antibodies can be cross-linked.

[0099] In some embodiments, the analyte binding moiety can bind a lipid membrane (*e.g.*, a phospholipid membrane) comprising a lipid moiety. In some embodiments, the lipid moiety comprises a cholesterol moiety, a lipid moiety, a phospholipid moiety, cholic acid, a thioether, *e.g.*, hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, *e.g.*, dodecandiol or undecyl residues, a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glyc-ero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

[00100] In some embodiments, the analyte binding moiety comprises an antibody which can bind a target analyte. In some embodiments, the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the antibody comprises a primary antibody and/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 antibody can comprise 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 cellular sample or on the membrane of the cellular sample, 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 cellular sample 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, actin cytoskeleton, spindle, flagellum, peroxisome and lysosome.

[00101] An antibody, or 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.

[00102] The term “antibody” as used herein includes naturally occurring antibodies as well as non-naturally occurring antibodies. Such non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains. These and other methods of making, for example, chimeric, humanized, CDR-grafted, single chain, and bifunctional antibodies are well known. In addition, modified or derivatized antibodies, or antigen binding fragments of antibodies are considered as within the scope of the instant disclosure. Fab, F(ab')₂, Fd and Fv fragments of an antibody that retain specific binding activity are included within the definition of an antibody fragment.

[00103] In some embodiments, the antibodies can be raised in a host species including for example rabbit, mouse, rat, rabbit, goat, sheep, guinea pig, chicken, hamster, donkey, camel, llama or horse. In some embodiments, the antibodies comprise animal-free antibodies that are produced using recombinant DNA technology. In some embodiments, the antibody is humanized.

[00104] 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, single chain variable fragment (scFv), domain antibodies, VHH, and Fd. In some embodiments, an Fv fragment comprises a variable light chain region (VL) and variable heavy chain region (VH). In some embodiments, a 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, a 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.

[00105] 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.

[00106] Antibodies and antigen-binding fragments of antibodies may be prepared using any suitable standard technique such as, for example, manipulation and expression of the DNA encoding the antibody or antigen binding fragment. Such DNAs are known and/or readily available, *e.g.*, from commercial sources, DNA libraries (including, *e.g.*, phage antibody libraries), or can be synthesized. DNA is sequenced and manipulated chemically or by using molecular biology techniques to, for example, place one or more variable and/or constant domains into a suitable configuration or introduce codons, cysteine residues can be created, amino acids can be modified, added or deleted, and the like.

[00107] The sequences and structures of suitable antibodies and antibody fragments will be known to persons of ordinary skill in the art, and are described for example, in the KEGG Drug database (www.genome.jp/kegg/drug/) and the Protein Data Bank (www.rcsb.org/) as well as other sources known in the art.

[00108] In some embodiments, antibodies can be conjugated to at least one modified oligonucleotide using any well-known linking chemistry. For example, amplification-free probe complexes can be prepared by cross-linking amino groups on the antibody and modified 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.

[00109] In some embodiments, homo-bifunctional or hetero-bifunctional cross-linkers can be introduced as bridges to link together the antibody and modified oligonucleotide.

[00110] In some embodiments, an antibody can be attached to a modified 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.

[00111] In some embodiments, an antibody can be attached to at least one modified 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.

[00112] In some embodiments, an antibody can be attached to at least one modified oligonucleotide using copper-free click chemistry, for example using dibenzocyclooctyne (DBCO). A modified oligonucleotide modified with DBCO can be reacted with an azide-modified antibody to generate an antibody attached to a modified oligonucleotide.

[00113] In some embodiments, an antibody can be attached to at least one modified oligonucleotide using an amine-to-sulfhydryl cross linker succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). A thiol-modified modified oligonucleotide can be reacted with an antibody having an amine-to-thiol linker to generate an antibody attached to a modified oligonucleotide.

[00114] In some embodiments, an antibody can be attached to at least one modified oligonucleotide using a chemically labile linker (*e.g.*, pH sensitive linker) such as for example hydrazone linkers.

[00115] In some embodiments, the modified oligonucleotide can be attached to analyte binding moiety via a linker. In some embodiments, the linker comprises a polymer comprising polyether, polyamine or polyamide. In some embodiments, the linker comprises triethylene glycol (*e.g.*, TEG linker) or polyethylene glycol (*e.g.* PEG linker).

[00116] In some embodiments, linker comprises a polymer spacer arm. In some embodiments, the spacer arm comprises a C3, C6, C9, C12 or C18 spacer arm. In some embodiments, the linker comprises a polymer comprising polyether, polyamine or polyamide. In some embodiments, the linker comprises triethylene glycol (*e.g.*, TEG linker) or polyethylene glycol (*e.g.* PEG linker).

[00117] The present disclosure provides compositions comprising at least one amplification-free probe complex bound to its cognate target analyte. In some embodiments, the at least one amplification-free probe complex is bound to its cognate target analyte on or inside a cellular sample (*e.g.*, FIG. 20).

[00118] The present disclosure provides compositions comprising at least one amplification-free probe complex and at least one sequencing primer. In some embodiments, the at least one amplification-free probe complex is hybridized to at least one sequencing

primer, thereby forming at least one nucleic acid duplex. In some embodiments, the sequencing primer comprises a 3' extendible end. In some embodiments, the sequencing primer comprises a 3' non-extendable (blocked) end which can be converted into a 3' extendible end.

[00119] The present disclosure provides compositions comprising at least one amplification-free probe complex, at least one sequencing primer and at least one sequencing polymerase. In some embodiments, the at least one amplification-free probe complex is hybridized to at least one sequencing primer. In some embodiments, the sequencing primer comprises a 3' extendible end. In some embodiments, the sequencing primer comprises a 3' non-extendable (blocked) end which can be converted into a 3' extendible end. In some embodiments, the at least one amplification-free probe complex is hybridized to at least one sequencing primer thereby forming at least one nucleic acid duplex. In some embodiments, the sequencing polymerase is bound to the at least one nucleic acid duplex.

[00120] The present disclosure provides compositions comprising at least one amplification-free probe complex, at least one sequencing primer, at least one sequencing polymerase and at least one multivalent molecule. In some embodiments, the at least one amplification-free probe complex is hybridized to at least one sequencing primer. In some embodiments, the sequencing primer comprises a 3' extendible end. In some embodiments, the sequencing primer comprises a 3' non-extendable (blocked) end which can be converted into a 3' extendible end. In some embodiments, the at least one amplification-free probe complex is hybridized to at least one sequencing primer thereby forming at least one nucleic acid duplex. In some embodiments, the sequencing polymerase is bound to the at least one nucleic acid duplex. In some embodiments, the multivalent molecule comprises at least one detectable label (*e.g.*, a fluorophore). In some embodiments, a complementary nucleotide moiety of a detectably labeled multivalent molecule binds the sequencing polymerase and binds near the 3' end of the sequencing primer, at a position opposite the canonical nucleobase in the modified oligonucleotide, thereby forming a detectably labeled complex. In some embodiments, the nucleotide moiety of the multivalent molecule is complementary to a nucleotide in the modified oligonucleotide that is 5' and adjacent to the sequencing primer binding site.

[00121] The present disclosure provides compositions comprising at least one amplification-free probe complex bound to its cognate target analyte, *e.g.* a target analyte of a cellular sample (*e.g.*, FIG. 20), at least one sequencing primer, at least one sequencing polymerase and at least one multivalent molecule. In some embodiments, the at least one

amplification-free probe complex is hybridized to at least one sequencing primer. In some embodiments, the sequencing primer comprises a 3' extendible end. In some embodiments, the sequencing primer comprises a 3' non-extendable (blocked) end which can be converted into a 3' extendible end. In some embodiments, the at least one amplification-free probe complex is hybridized to at least one sequencing primer thereby forming at least one nucleic acid duplex. In some embodiments, the sequencing polymerase is bound to the at least one nucleic acid duplex. In some embodiments, the multivalent molecule comprises at least one detectable label (*e.g.*, a fluorophore). In some embodiments, a complementary nucleotide moiety of a detectably labeled multivalent molecule binds the sequencing polymerase and binds near the 3' end of the sequencing primer, at a position opposite the canonical nucleobase in the modified oligonucleotide thereby forming a detectably labeled complex. In some embodiments, the nucleotide moiety of the multivalent molecule is complementary to a nucleotide in the modified oligonucleotide that is 5' and adjacent to the sequencing primer binding site.

Barcode Sequences for Cell Painting

[00122] The disclosure provides compositions comprising a plurality of amplification-free probe complexes, including at least a first and second sub-population of amplification-free probe complexes. In some embodiments, individual amplification-free probe complexes of the first and second sub-population comprise at least one modified oligonucleotide comprising at least one barcode sequence. In some embodiments, the first sub-population of amplification-free probe complexes comprise barcode sequences having the same sequence. In some embodiments, the second sub-population of amplification-free probe complexes comprise barcode sequences having the same sequence. In some embodiments, the barcode sequences of the first and second sub-populations have different sequences. The barcode sequences can be designed to enable simultaneously detecting and identifying two or more target analytes by conducting a single sequencing cycle and employing multi-color imaging. In some embodiments, the barcode sequences of the amplification-free probe complexes can be used for sequencing-based cell painting.

[00123] In some embodiments, the compositions comprise: a plurality of modified oligonucleotides comprising: at least a first and second sub-population of modified oligonucleotides (*e.g.*, a set comprising at least a first and second sub-population of modified oligonucleotides). In some embodiments, individual modified oligonucleotides in the first sub-population comprise a first barcode sequence which corresponds to a first target analyte,

wherein individual modified oligonucleotides in the second sub-population comprise a second barcode sequence which corresponds to a second target analyte. In some embodiments, the first barcode is at least 2 nucleotides in length. In some embodiments, the second barcode is at least 2 nucleotides in length. In some embodiments, the sequences of the first and second barcodes comprise different sequences. In some embodiments, one nucleobase in a first position in the first barcode sequence comprises a nucleobase that generates a first color signal in a first sequencing cycle, and one nucleobase in a corresponding first position in the second barcode sequence comprises a nucleobase that generates a second color signal in the same first sequencing cycle. In some embodiments, one nucleobase in a second position in the first barcode sequence comprises a nucleobase that generates the second color signal in a second sequencing cycle, and one nucleobase in a corresponding second position in the second barcode sequence comprises a nucleobase that generates the first color signal in the same second sequencing cycle. In some embodiments, the nucleobases in the first and/or second positions of the first and/or second barcodes are identified based on the first and/or second color signals. In some embodiments, the first color signal in the first corresponding position of the first 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 second barcode sequences in the second sequencing cycle identifies the second target analyte (*e.g.*, see the table at FIG. 21). In some embodiments, a sequencing cycle comprises forming a detectably labeled complex, which generates a color signal. In some embodiments, forming the detectably labeled complex comprises hybridizing a sequencing primer to the modified oligonucleotide, thereby forming a nucleic acid duplex, and binding the nucleic acid duplex with a sequencing polymerase and a complementary nucleotide moiety of a multivalent molecule opposite a nucleotide in the modified oligonucleotide thereby forming a detectably labeled complex.

[00124] The present disclosure provides compositions comprising: a plurality of barcoded oligonucleotides comprising at least a first, second and third sub-population of modified oligonucleotides (*e.g.*, a set comprising at least a first, second and third sub-population of modified oligonucleotides). In some embodiments, individual modified oligonucleotides in the first sub-population comprise a first barcode sequence which corresponds to a first target analyte. In some embodiments, the first barcode is at least 3 nucleotides in length. In some embodiments, individual modified oligonucleotides in the second sub-population comprise a second barcode sequence which corresponds to a second target analyte. In some embodiments, the second barcode is at least 3 nucleotides in length. In some embodiments,

individual modified oligonucleotides in the third sub-population comprise a third barcode sequence which corresponds to a third target analyte. In some embodiments, the third barcode is at least 3 nucleotides in length. In some embodiments, the first, second and third barcode sequences comprise different sequences. In some embodiments, one nucleo-base in a first position in the first barcode sequence comprises a nucleo-base that generates a first color signal in a first sequencing cycle. In some embodiments, one nucleo-base in a corresponding first position in the second and third barcode sequences comprises a nucleo-base that generates a second color signal in the same first sequencing cycle. In some embodiments, one nucleo-base in a second position in the first and third barcode sequences comprises a nucleo-base generates the second color signal in a second sequencing cycle. In some embodiments, one nucleo-base in a corresponding second position in the second barcode sequence comprises a nucleo-base that generates the first color signal in the same second sequencing cycle. In some embodiments, one nucleo-base in a third position in the first and second barcode sequences comprises a nucleo-base that generates the second color signal in a third sequencing cycle. In some embodiments, one nucleo-base in a corresponding third position in the third barcode sequence comprise a nucleo-base that generates the first color signal in the same third sequencing cycle. In some embodiments, the first color signal in the first corresponding position of the first barcode sequence in the first sequencing cycle identifies the first target analyte. In some embodiments, the first color signal in the second corresponding position of the second barcode sequence in the second sequencing cycle identifies the second target analyte. In some embodiments, the first color signal in the third corresponding position of the third barcode sequence in the third sequencing cycle identifies the third target analyte (*e.g.*, see the table at FIG. 21). In some embodiments, the second color signal in the first sequencing cycle identifies the second and third target analytes. In some embodiments, the second color signal in the second sequence cycle identifies the first and third target analytes. In some embodiments, the second color signal in the third sequencing cycle identifies the first and second target analytes. In some embodiments, a sequencing cycle comprises forming a detectably labeled complex (which generates a color signal). In some embodiments, forming a detectably labeled complex comprises hybridizing a sequencing primer to the modified oligonucleotide thereby forming a nucleic acid duplex, and binding the nucleic acid duplex with a sequencing polymerase and a complementary nucleotide moiety of a multivalent molecule opposite a nucleotide in the modified oligonucleotide thereby forming a detectably labeled complex.

Methods of Detecting Using Amplification-Free Probe Complexes

[00125] The present disclosure provides methods for detecting target analytes using at least one amplification-free probe complex. In some embodiments, the target analyte can be located inside a cellular sample or on the membrane of a cellular sample. In some embodiments, the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide that is located anywhere in the cellular sample including without limits the cytoplasm and nucleus. In some embodiments, the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide 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. In some embodiments, the cellular sample, comprises, for example and without limitation, a single cell, multiple cells, a tissue, an organ, a tumor, or portions thereof.

[00126] In some embodiments, methods for detecting target analytes comprise **step (a)**: providing a plurality of amplification-free probe complexes described herein. In some embodiments, individual amplification-free probe complexes comprise (i) at least one modified oligonucleotide; and (ii) at least one analyte binding moiety which can bind a cellular target analyte, wherein the at least one modified oligonucleotide is attached to the at least one analyte binding moiety (*e.g.*, FIGS. 13A-13B, 14A-14B, 15A-15B, 16 and 17). In some embodiments, the at least one modified oligonucleotide comprises at least one primer binding site (*e.g.*, at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more).

[00127] In some embodiments, in step (a) of the methods for detecting target analytes, the at least one modified oligonucleotide comprises a nucleic acid including recombinant and chemically-synthesized forms. In some embodiments, the modified oligonucleotide comprises DNA, RNA or DNA and RNA. In some embodiments, the modified oligonucleotide comprises canonical nucleotides and/or nucleotide analogs. In some embodiments, the modified oligonucleotide comprises polymers of nucleotides, where the nucleotides include natural or non-natural bases and/or sugars. In some embodiments, the modified oligonucleotide comprises naturally-occurring internucleosidic linkages, for example and without limitation, phosphodiester linkages. In some embodiments, the modified oligonucleotide comprises a 5' phosphate group or lacks a 5' phosphate group. In some embodiments, the modified oligonucleotide comprises non-natural internucleoside linkages, including phosphorothioate, phosphorothiolate, or peptide nucleic acid (PNA) linkages.

[00128] In some embodiments, in step (a) of the methods for detecting target analytes, the modified oligonucleotide comprises a linear oligonucleotide (*e.g.*, Figures 13A-13B, 14A-14B, 15A-15B and 16) or a branched oligonucleotide (*e.g.*, FIG. 17).

[00129] In some embodiments, in step (a) of the methods for detecting target analytes, the at least one modified oligonucleotide comprises a conjugation moiety at one end to enable attachment to a cell binding moiety (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises a linker moiety (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises modified nucleotides at one end where the modified nucleotides confer nuclease resistance to the modified oligonucleotide (*e.g.*, FIG. 18A). Exemplary modified nucleotides that confer nuclease resistance include without limitation at least one phosphorothioate linkage and/or at least one 2'-O-methylcytosine bases.

[00130] In some embodiments, the at least one modified oligonucleotide comprises one or more sequencing primer binding sites (*e.g.*, FIG. 18A). In some embodiments, the one or more sequencing primer binding sites comprise universal sequencing primer binding sites. In some embodiments, the at least one modified oligonucleotide comprises two or more tandem copies of a sequencing primer binding site (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises a concatemer molecule having two or more tandem copies (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more copies) of a sequencing primer binding site (*e.g.*, FIG. 18A). In some embodiments, the at least one modified oligonucleotide comprises a canonical nucleo-base located at a position that is upstream of the beginning of individual sequencing primer binding sites (*e.g.*, FIG. 18A). For example, the at least one modified oligonucleotide comprises canonical nucleo-bases located at positions 5' to the 5' ends of individual sequencing primer binding sites. In some embodiments, the canonical nucleo-base located at a position that is immediately upstream of the beginning of individual sequencing primer binding sites (*e.g.*, FIG. 18A). For example, the at least one modified oligonucleotide comprises canonical nucleobases located at positions within 1-10 nucleotides, optionally adjacent to, the 5' ends of individual sequencing primer binding sites. In some embodiments, the at least one modified oligonucleotide comprises an abasic site that is located upstream of the canonical nucleo-base (*e.g.*, FIG. 18A). For example, the abasic sites are 5' and within 1-10 nucleotides of the canonical nucleo-bases. In some embodiments, the abasic site is located immediately upstream of the canonical nucleo-base (*e.g.*, FIG. 18A). For example, the abasic sites are 5' and adjacent to the canonical nucleo-bases.

[00131] In some embodiments, in step (a) of the methods for detecting target analytes, the modified oligonucleotide is attached to an analyte binding moiety. In some embodiments, the analyte binding moiety can bind a lipid, polypeptide, nucleic acid or polysaccharide. In some embodiments, the analyte binding moiety comprises a lipid moiety, a wheat germ agglutinin, an antibody or a biotin moiety. In some embodiments, the antibody comprises a secondary antibody or a primary antibody. In some embodiments, the analyte binding moiety comprises a polynucleotide comprising a sequence that is complementary to a target analyte comprising a nucleic acid.

[00132] In some embodiments, in step (a) of the methods for detecting target analytes, the analyte binding moiety comprises a lipid moiety comprising a cholesterol moiety, a lipid moiety, a phospholipid moiety, cholic acid, a thioether, *e.g.*, hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, *e.g.*, dodecandiol or undecyl residues, a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glyc-ero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

[00133] In some embodiments, in step (a) of the methods for detecting target analytes, the analyte binding moiety comprises an antibody. In some embodiments, the antibody comprises a primary and/or secondary antibody. In some embodiments, the modified oligonucleotide can be attached to a primary antibody. In some embodiments, the modified oligonucleotide can be attached to a secondary antibody, and the secondary antibody can be attached/associated to a primary antibody. In some embodiments, the primary antibody binds the target analyte. In some embodiments, the primary and secondary antibodies can be cross-linked.

[00134] 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 can comprise 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 cellular sample and/or on the membrane of the cellular sample. In some embodiments, 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 cellular sample 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, actin cytoskeleton, spindle, flagellum, peroxisome and lysosome.

[00135] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody comprises an immunoglobulin. In some embodiments, an immunoglobulin comprises 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.

[00136] In some embodiments, the antibodies can be raised in a host species including for example rabbit, mouse, rat, rabbit, goat, sheep, guinea pig, chicken, hamster, donkey, camel, llama or horse. In some embodiments, the antibodies comprise animal-free antibodies that are produced using recombinant DNA technology.

[00137] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody comprises an antibody fragment comprising a portion of an intact immunoglobulin that can bind an antigen. Examples of antibody fragments include but are not limited to Fv, scFv, 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, a 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, a 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, a F(ab')₂ fragment comprises a bivalent antibody fragment having two Fab fragments linked via a disulfide bridge at the hinge region.

[00138] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody comprises a single-chain variable fragment (scFv) comprising 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.

[00139] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody can be conjugated to at least one modified oligonucleotide using any well-known linking chemistry. For example, amplification-free probe complexes can be prepared by cross-linking amino groups on the antibody and modified 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. In some embodiments, homo-bifunctional or hetero-bifunctional cross-linkers can be introduced as bridges to link together the antibody and modified oligonucleotide.

[00140] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody can be attached to a modified 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.

[00141] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody can be attached to at least one modified 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.

[00142] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody can be attached to at least one modified oligonucleotide using copper-free click chemistry, for example using dibenzocyclooctyne (DBCO). A modified oligonucleotide modified with DBCO can be reacted with an azide-modified antibody to generate an antibody attached to a modified oligonucleotide.

[00143] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody can be attached to at least one modified oligonucleotide using an amine-to-sulfhydryl cross linker succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate

(SMCC). A thiol-modified modified oligonucleotide can be reacted with an antibody having an amine-to-thiol linker to generate an antibody attached to a modified oligonucleotide.

[00144] In some embodiments, in step (a) of the methods for detecting target analytes, the antibody can be attached to at least one modified oligonucleotide using a chemically labile linker (*e.g.*, pH sensitive linker) such as for example hydrazone linkers.

[00145] In some embodiments, in step (a) of the methods for detecting target analytes, the modified oligonucleotide can be attached to analyte binding moiety via a linker. In some embodiments, the linker comprises a polymer comprising polyether, polyamine or polyamide. In some embodiments, the linker comprises triethylene glycol (*e.g.*, TEG linker) or polyethylene glycol (*e.g.* PEG linker).

[00146] In some embodiments, in step (a) of the methods for detecting target analytes, the linker comprises a polymer spacer arm. In some embodiments, the spacer arm comprises a C3, C6, C9, C12 or C18 spacer arm. In some embodiments, the linker comprises a polymer comprising polyether, polyamine or polyamide. In some embodiments, the linker comprises triethylene glycol (*e.g.*, TEG linker) or polyethylene glycol (*e.g.* PEG linker).

[00147] In some embodiments, the methods for detecting target analytes comprise **step (b)**: providing a cellular sample. In some embodiments the cellular sample is deposited (*e.g.*, seeded) on a support. In some embodiments, the cellular sample comprises a plurality of analytes including at least one target analyte.

[00148] In some embodiments, in step (b) of the methods for detecting target analytes, the cellular sample comprises a plurality of target analytes comprising polypeptides, lipids, nucleic acids and/or polysaccharides. In some embodiments, the target analytes can be located on the cellular sample (*e.g.*, an outer cell membrane). In some embodiments, the target analytes can be located inside the cellular sample (*e.g.*, a nuclear membrane, a nucleolar membrane, a mitochondrial membrane, chloroplast membrane, a Golgi membrane, an endoplasmic reticulum membrane, a peroxisome membrane or a lysosome membrane). In some embodiments, the target analytes can be located inside the cellular sample and on the cellular sample.

[00149] In some embodiments, in step (b) of the methods for detecting target analytes, the cellular sample comprises a whole cell, a plurality of whole cells, an intact tissue or sectioned cellular sample. In some embodiments, the cellular sample comprises a fresh cellular sample, a freshly-frozen cellular sample, a sectioned cellular sample, or an FFPE cellular sample. In some embodiments, the cellular sample can be fixed and/or permeabilized. In some

embodiments, the cellular sample comprises an expanded cellular sample that has been cultured in a simple or complex cell culture media.

[00150] In some embodiments, in step (b) of the methods for detecting target analytes, the cellular sample comprises an intracellular matrix and/or a cross-linked matrix comprising a hydrogel, swellable hydrogel, or cross-linked matrix. In some embodiments, the cellular sample comprises an intracellular matrix wherein the cellular sample is infused with a swellable polyelectrolyte hydrogel (*e.g.*, *see* U.S. patent No. 10,309,879 and Chen 2015 Science 347:543, the contents of these documents are herein incorporated by reference in their entireties). In some embodiments, a fixed and permeabilized cellular sample can be infused with sodium acrylate, acrylamide and a cross-linker, *e.g.*, N-N'-methylenebisacrylamide. In some embodiments, the cellular sample can be infused with an ammonium persulfate (APS) initiator and tetramethylethylenediamine (TEMED) accelerator to achieve polymerization inside the cellular sample. In some embodiments, the cellular sample can be infused with a protease, *e.g.*, proteinase K, for proteolysis and incubated in a digestion buffer. In some embodiments, the gel inside the cellular sample can be swelled by addition of water. In some embodiments, the cellular sample lacks an intracellular matrix.

[00151] In some embodiments, in step (b) of the methods for detecting target analytes, the cellular sample can be deposited (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.

[00152] In some embodiments, in step (b) of the methods for detecting target analytes, the cellular sample can be deposited (seeded) onto a support having a coating that promotes proliferation, migration, differentiation and/or adhesion of cultured cell or living *ex vivo* cells or tissue samples. Embodiments of the coating are described herein. In some embodiments, the cellular sample can be deposited on a support that lacks immobilized capture primers which can bind target polynucleotides from the cellular sample. In some embodiments, the cellular sample can be deposited (*e.g.*, seeded) onto an uncoated support. In some embodiments, in step (b) of the methods for detecting target analytes, the cellular sample can be deposited (*e.g.*, seeded) on a support that lacks immobilized capture primers which can bind target polynucleotide analytes from the cellular sample. Alternatively, the support

includes a plurality of immobilized capture primers which can bind target polynucleotide analytes from the cellular sample.

[00153] In some embodiments, the methods for detecting target analytes comprise **step (c)**: contacting the cellular sample with the plurality of amplification-free probe complexes, wherein the contacting is conducting under a condition suitable for binding the analyte binding moieties of the amplification-free probe complexes to their cognate target analytes.

[00154] In some embodiments, a plurality of amplification-free probe complexes can bind a cellular sample (*e.g.*, FIG. 20). In some embodiments, the plurality of amplification-free probe complexes can bind an outer cell membrane (*e.g.*, FIG. 20). In some embodiments, the plurality of amplification-free probe complexes can enter a cell and can bind a cell membrane of a cellular organelle located inside the cellular sample, including for example a nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, actin cytoskeleton, spindle, flagellum, peroxisome or lysosome.

[00155] In some embodiments, the methods for detecting target analytes comprise **step (d)**: contacting the plurality of modified oligonucleotides of the plurality of amplification-free probe complexes of step (c) with a plurality of sequencing primers under a condition suitable for individual sequencing primers to bind their cognate (*i.e.* complementary) sequencing primer binding sites on individual modified oligonucleotides to form a plurality of nucleic acid duplexes, wherein individual nucleic acid duplexes comprise a sequencing primer binding site hybridized to a sequencing primer (*e.g.*, FIGS. 13B, 14B, 15B, 16, 17 and 18A). In some embodiments, the sequencing primer hybridizes to the modified oligonucleotide at a position that is immediately downstream (3') of a canonical nucleobase on the modified oligonucleotide. For example, the sequencing primers hybridizes to a sequencing primer binding site that is located between 1-10 nucleotides 3' of, or alternatively, 3' of and adjacent to, the canonical nucleobase. In some embodiments, the sequencing primer hybridizes to the modified oligonucleotide at a position that is two base positions downstream of (*i.e.* 3' to) an abasic site on the modified oligonucleotide. In some embodiments, the sequencing primer comprises a 3' extendible end. In some embodiments, the sequencing primer comprises a 3' non-extendable (blocked) end which can be converted into a 3' extendible end.

[00156] In some embodiments, the methods for detecting target analytes comprise **step (e)**: contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases and a plurality of multivalent molecules. In some embodiments, individual multivalent molecules comprise a core attached to a plurality of nucleotide-arms, wherein individual

nucleotide-arms are attached to a nucleotide moiety (*e.g.*, FIGS. 1-4A and 4B). In some embodiments, individual multivalent molecules are labeled with a detectably reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, the core of the multivalent molecule is labeled with a fluorophore. In some embodiments, the fluorophore attached to a given core of the multivalent molecule corresponds to the nucleotide base (*e.g.*, adenine, guanine, cytosine, thymine or uracil) of the nucleotide arm, allowing for identification of the complementary nucleobase in the modified oligonucleotide. In some embodiments, at least one of the nucleotide arms of the multivalent molecule comprises a linker and/or nucleotide base that is attached to a fluorophore. In some embodiments, the fluorophore which is attached to a given nucleotide base corresponds to the nucleotide base (*e.g.*, adenine, guanine, cytosine, thymine or uracil) of the nucleotide arm, allowing for identification of the complementary nucleobase in the modified oligonucleotide.

[00157] In some embodiments, individual sequencing polymerase(s) is/are capable of binding a nucleic acid duplex (*e.g.*, FIG. 18B). In some embodiments, individual sequencing polymerase(s) is/are capable of binding a nucleic acid duplex and binding a complementary nucleotide moiety of a multivalent molecule opposite a nucleotide (*e.g.*, comprising the canonical nucleobase) in a given modified oligonucleotide (*e.g.*, FIG. 18B), thereby forming a plurality of detectably labeled complexes. In some embodiments, the detectably labeled complexes generate a color signal as described herein. In some embodiments, individual detectably labeled complexes comprise a modified oligonucleotide hybridized to a sequencing primer, thereby forming a nucleic acid duplex, a sequencing polymerase bound to the nucleic acid duplex, and a nucleotide moiety of a multivalent molecule bound to the 3' end of the sequencing primer and opposite the canonical nucleobase in the modified oligonucleotide.

[00158] In some embodiments, the detectably labeled complex can form in less than about 30 minutes, or less than about 20 minutes, or less than about 10 minutes, or less than about 5 minutes.

[00159] In some embodiments, the contacting of step (e) is conducted under a condition suitable for inhibiting polymerase-catalyzed incorporation of a nucleotide moiety into the 3' end of the sequencing primer. In some embodiments, the contacting of step (e) comprises contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases, a plurality of multivalent molecules and a plurality of non-catalytic cations. In some embodiments, the non-catalytic cations comprise strontium, barium and/or calcium. In some embodiments, in step (e), the complementary nucleotide moiety of a multivalent molecule

binds opposite a nucleotide in a given modified oligonucleotide, and the complementary nucleotide moiety of a multivalent molecule does not incorporate into the terminal 3' end of the sequencing primer. In some embodiments, the binding of a complementary nucleotide moiety of a multivalent molecule in step (e) does not result in primer extension. In some embodiments, the binding of a complementary nucleotide moiety of a multivalent molecule in step (e) does not result in nucleic acid amplification.

[00160] In some embodiments, the modified oligonucleotide is designed to include at least one abasic site which can inhibit a second binding event by another detectably labeled multivalent molecule so that in the contacting of step (e), one nucleotide moiety of one detectably labeled multivalent molecule binds one nucleic acid duplex and one sequencing polymerase to emit a signal that is associated with one detectably labeled complex. In some embodiments, the signal can be imaged.

[00161] In some embodiments, the detectably labeled complexes are stable and do not dissociate until subjected to a condition that causes dissociation. In some embodiments, the detectably labeled complexes have 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 detectably labeled complexes has a persistence time of greater than about 0.1-0.25 seconds, about 0.25-0.5 seconds, about 0.5-0.75 seconds, about 0.75-1 second, about 1-2 seconds, about 2-3 seconds, 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, at or above 72 °C, or at or above 80 °C, or within a range defined by any of the foregoing. In some embodiments, the detectably labeled complexes remain stable until subjected to a condition that causes dissociation of interactions between any of the sequencing polymerase, modified oligonucleotide, sequencing primer and/or the nucleotide moiety of the multivalent molecule. For example, a dissociating condition comprises contacting the detectably labeled complexes with any one or any combination of a detergent, EDTA and/or water.

[00162] In some embodiments, in the contacting of step (e), individual multivalent molecules comprise multiple nucleotide-arms wherein each nucleotide-arm carries at least one nucleotide moiety. In some embodiments, the nucleotide moieties of a single multivalent molecule can form two or more detectably labeled complexes comprising an avidity complex.

[00163] In some embodiments, a first nucleotide moiety of a multivalent molecule can form a first detectably labeled complex on a first amplification-free probe complex, and a second nucleotide moiety of the same multivalent molecule can form a second detectably

labeled complex on a second amplification-free probe complex, wherein the first and second amplification-free probe complexes are bound to the same cellular sample. In some embodiments, the first and second detectably labeled complexes form an avidity complex.

[00164] In some embodiments, a first nucleotide moiety of a multivalent molecule can form a first detectably labeled complex on a first region of a first amplification-free probe complex, and a second nucleotide moiety of the same multivalent molecule can form a second detectably labeled complex on a second region of the same amplification-free probe complex, wherein the first amplification-free probe complex is bound to a cellular sample. In some embodiments, the first and second detectably labeled complexes form an avidity complex.

[00165] In some embodiments, the methods for detecting target analytes comprise **step (f)**: imaging the cellular sample which is bound to a detectably labeled complex of step (e) (*e.g.*, FIG. 20).

[00166] In some embodiments, the imaging of step (f) comprises employing an optical imaging system comprising a field-of-view (FOV) greater than 1.0 mm². In some embodiments, the detectably labeled complex of step (e) can form in less than about 30 minutes, less than about 20 minutes, less than about 10 minutes, or less than about 5 minutes. In some embodiments, the field of view (FOV) can exceed 1 mm² and the cycle time for scanning large area (> 10 mm²) can be less than 5 minutes.

[00167] In some embodiments, in the method for detecting target analytes of step (f), the modified oligonucleotides of the amplification-free probe complexes are hybridized with the plurality of sequencing primers in the presence of a hybridization solution. In some embodiments, the hybridization solution comprises a saline-sodium citrate (SSC) solution. In some embodiments, the hybridization solution comprises a 2X, 3X, 4X, 5X, 7X, 8X, 9X or 10X SSC solution. In some embodiments, the hybridization solution comprises a 2X, 3X or 5X SSC solution. In some embodiments, the hybridization solution comprises acetonitrile. In some embodiments, the hybridization solution comprises dimethyl sulfoxide (DMSO). In some embodiments, the hybridization solution comprises Denhardt's solution which includes Ficoll (*e.g.*, Ficoll 400), polyvinylpyrrolidone and bovine serum albumin (BSA). In some embodiments, the hybridization solution comprises betaine. In some embodiments, the hybridization solution comprises trehalose. In some embodiments, the hybridization solution comprises guanidinium isothiocyanate (GITC). In some embodiments, the hybridization solution comprises any one or any combination of two or more of a pH buffering agent, a chelator, a detergent, a denaturing agent, a crowding agent and/or a blocking agent. In some

embodiments, the hybridization solution comprises a sodium salt (*e.g.*, NaCl). In some embodiments, the pH buffering agent comprises HEPES (*e.g.*, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or MES (*e.g.*, 2-(*N*-morpholino)ethanesulfonic acid). In some embodiments, the chelator comprises EDTA (ethylenediaminetetraacetic acid) and/or EGTA (ethylene glycol tetraacetic acid). In some embodiments, the detergent comprises Tween-20, Triton-X 100 and/or SDS. In some embodiments, the denaturing agent comprises formamide, 2-pyrrolidone, urea and/or ethylene carbonate. In some embodiments, the crowding agent comprises dextran sulfate and/or polyethylene glycol (PEG), for example and without limitation, 1KPEG, 2KPEG, 4KPEG, 5KPEG and/or PEG200. In some embodiments, the blocking agent comprises BSA.

Barcode Sequences for Methods for Cell Painting

[00168] The disclosure provides amplification-free probe complexes comprising barcode sequences, and methods for sequencing the barcode sequences. The disclosure provides a plurality of amplification-free probe complexes comprising barcode sequences, the plurality comprising sub-populations of modified oligonucleotides. In some embodiments, the sub-populations comprise modified oligonucleotides in which the modified oligonucleotides of the different sub-population comprise different barcode sequences. For example, all amplification-free probe complexes of one sub-population comprise the same barcode sequence, which differs from the barcode sequences of the other sub-populations. The barcode sequences can be employed for simultaneously detecting and identifying two or more target analytes by conducting a single sequencing cycle and employing multi-color imaging. In some embodiments, the barcode sequences of the amplification-free probe complexes can be used for sequencing-based cell painting methods.

[00169] In some embodiments, in the methods for sequencing barcodes, the sequencing comprises **step (a)**: sequencing a plurality of modified oligonucleotides by conducting at least two sequencing cycles comprising, in each sequencing cycle, sequencing the same corresponding nucleo-base position of all of the barcodes in the plurality of sub-populations of modified oligonucleotides. In some embodiments, a first barcode of a modified oligonucleotide in the first sub-population generates a first color signal in one particular sequencing cycle, and the barcodes of modified oligonucleotides in the other sub-populations generate a second color signal in the same particular sequencing cycle. In some embodiments, the first and second color signals are distinguishable in the same sequencing cycle. In some embodiments, the first color signal of any of the barcodes in any one

sequencing cycle identifies the corresponding nucleotide identity in the barcode, and thereby the target analyte which corresponds to the barcode sequence of the amplification-free probe complex. In some embodiments, the plurality of modified oligonucleotides are on and/or inside a cellular sample.

[00170] In some embodiments, the sequencing comprises **step (b)**: imaging the first and second color signals in the one particular sequencing cycle, and identifying the target analyte that corresponds to a barcode sequence from the amplification-free probe complex that generates the first color signal. In some embodiments, the first and second color signals are generated on and/or inside the cellular sample.

[00171] In some embodiments, in steps (a) and (b), at least two different target analytes can be simultaneously identified by imaging the first and second color signals generated in a single sequencing cycle. For example, the plurality of the amplification-free probe complexes comprises a first sub-population comprising a first barcode sequence and a first analyte binding moiety that binds a first analyte, and a second sub-population comprising a second barcode sequence and a second analyte binding moiety that binds a second analyte. In this example, without limitation, the first barcode sequence and the second barcode sequence are not the same, and the first analyte binding moiety and second binding moiety are not the same. Imaging the first and second color signals allows for imaging the positions of the sub-populations of amplification-free probe complexes comprising the first and second barcode sequences, which correspond to the first and second analyte binding moieties bound to the first and second analytes, in a single sequencing cycle.

[00172] In some embodiments, in steps (a) and (b), at least two different cellular structures can be simultaneously identified by imaging the first and second color signals generated in a single sequencing cycle. In some embodiments, the cellular structures include without limits any cellular membrane, nucleus, nucleolus, mitochondria, chloroplast, Golgi apparatus, ribosome, endoplasmic reticulum, microtubules, actin cytoskeleton, spindle, flagellum, peroxisome and lysosome.

[00173] In some embodiments, in steps (a) and (b), the plurality of sub-populations of modified oligonucleotides are located on and/or inside a cellular sample.

[00174] In some embodiments, in steps (a) and (b), all of the modified oligonucleotides can be sequenced essentially simultaneously to detect and identify target analytes using a plurality of sequencing primers comprising the same sequence.

[00175] In some embodiments, in steps (a) and (b), all of the modified oligonucleotides can be sequenced essentially simultaneously to detect and identify target analytes using a plurality of sequencing primers comprising different sequences.

[00176] In some embodiments, in steps (a) and (b), the modified oligonucleotide can be sequenced in separate batches to detect and identify target analytes using a plurality of sequencing primers having different sequences.

[00177] In some embodiments, in steps (a) and (b), only a partial length of the barcode needs to be sequenced in order to identify the different target analytes. In some embodiments, the full length of the barcodes can be sequenced to detect and identify the different target analytes. In some embodiments, the barcode can be 2-20 nucleotides in length.

[00178] The present disclosure provides methods for sequencing barcodes comprising **step (a)**: sequencing a plurality of modified oligonucleotides comprising a plurality of sub-populations of modified oligonucleotides by conducting at least two sequencing cycles. In some embodiments, in each sequencing cycle, the sequencing comprises sequencing the same corresponding nucleo-base position of all of the barcodes in the plurality of sub-populations of modified oligonucleotides. In some embodiments, a first barcode of a modified oligonucleotide in the first sub-population generates a first color signal in one particular sequencing cycle, and the barcodes of modified oligonucleotides in the other sub-populations generate a second color signal in the same particular sequencing cycle. In some embodiments, the first and second color signals are distinguishable in the same sequencing cycle. In some embodiments, the first color signal of any of the barcodes in any one sequencing cycle identifies the target analyte which corresponds to its cognate barcode. In some embodiments, the plurality of subpopulations of modified oligonucleotides are on and/or inside a cellular sample.

[00179] In some embodiments, the methods for sequencing barcodes comprises **step (b)**: imaging the first and second color signals in the one particular sequencing cycle, and identifying the target analyte that corresponds to the barcode sequence that generates the first color signal. In some embodiments, the first and second color signals are generated on and/or inside the cellular sample.

[00180] 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.

[00181] In some embodiments, the plurality of sub-populations of modified oligonucleotides are located on and/or inside a cellular sample.

[00182] In some embodiments, all of the barcodes can be sequenced essentially simultaneously to detect and identify target analytes using a plurality of sequencing primers comprising the same sequence.

[00183] In some embodiments, all of the barcodes can be sequenced essentially simultaneously to detect and identify target analytes using a plurality of sequencing primers comprising different sequences.

[00184] In some embodiments, the 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.

[00185] In some embodiments, only a partial length of the barcode needs to be sequenced in order to identify the different target analytes. In some embodiments, the full length of the barcodes can be sequenced to detect and identify the different target analytes. In some embodiments, the barcode can be 2-20 nucleotides in length.

[00186] In some embodiments, the target analyte is located inside the cellular sample and/or on the membrane of the cellular sample. In some embodiments, 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 cellular sample 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, actin cytoskeleton, spindle, flagellum, peroxisome and lysosome.

[00187] In some embodiments, simultaneously sequencing two or more sub-populations of modified oligonucleotides 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.

[00188] The present disclosure provides methods for sequencing barcodes comprising **step (a)**: providing a plurality of modified oligonucleotides including at least a first sub-population of modified oligonucleotides comprising a plurality of a first barcode which corresponds to a first target analyte and a second sub-population of modified oligonucleotides comprising a plurality of a second barcode which corresponds to a second target analyte. In some embodiments, the first barcode is 2-20 nucleobases in length. In some embodiments,

the second barcode is 2-20 nucleo-bases in length. In some embodiments, the plurality of modified oligonucleotides is located on and/or inside a cellular sample.

[00189] In some embodiments, the methods for sequencing barcodes comprise **step (b)**: conducting a first sequencing cycle, wherein the sequencing comprises sequencing essentially simultaneously the first nucleo-base position of the first and second barcodes in the first and second sub-populations of modified oligonucleotides. In some embodiments, the sequencing is conducted using a plurality of sequencing primers having the same sequence. In some embodiments, the first nucleo-base position of the first barcode generates a first color signal and the first nucleo-base position of the second barcode generates a second color signal. In some embodiments, the first and second color signals are distinguishable from each other in the first sequencing cycle. In some embodiments, the first color signal identifies the first target analyte. In some embodiments, the second color signal identifies the second target analyte. In some embodiments, the first sequencing cycle is conducted on and/or inside the cellular sample.

[00190] In some embodiments, the first sequencing cycle of step (b) can be conducted using any sequencing methods described herein or known in the art, including, without limitation, two-stage sequencing using multivalent molecules, sequencing-by-binding, or sequencing using chain terminator nucleotides.

[00191] In some embodiments, the methods for sequencing barcodes comprise **step (c)**: conducting a second sequencing cycle. In some embodiments, the sequencing comprises sequencing essentially simultaneously the second nucleo-base position of the first and second barcodes in the first and second sub-populations of modified oligonucleotides. In some embodiments, the second nucleo-base position of the first barcode generates the second color signal. In some embodiments, the second nucleo-base position of the second barcode generates the first color signal. In some embodiments, the first and second color signals are distinguishable from each other in the second sequencing cycle. In some embodiments, the first color signal identifies the second target analyte. In some embodiments, the second color signal identifies the first target analyte. In some embodiments, the second sequencing cycle is conducted on and/or inside the cellular sample.

[00192] In some embodiments, the second sequencing cycle of step (c) can be conducted using any sequencing methods described herein or known in the art including, without limitation, two-stage sequencing using multivalent molecules, sequencing-by-binding, or sequencing using chain terminator nucleotides.

[00193] In some embodiments, the methods for sequencing barcodes further comprises **step (d)**: imaging the first and second color signals in the first sequencing cycle and identifying the first target analyte. In some embodiments, the first and second color signals are generated on and/or in the cellular sample.

[00194] In some embodiments, the methods for sequencing barcodes further comprises **step (e)**: imaging the first and second color signals in the second sequencing cycle and identifying the second target analyte.

[00195] In some embodiments, the methods for sequencing barcodes further comprises: imaging the first and second color signals 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.

[00196] In some embodiments, the methods for sequencing barcodes further comprises: imaging the first and second color signals 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.

[00197] In some embodiments, the first and second color signals are generated on and/or in the cellular sample.

[00198] 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.

[00199] In some embodiments, the first and second target analytes can be identified by conducting no more than two sequencing cycles.

[00200] In some embodiments, the first and second barcodes can be identified by sequencing the full length of the first and second barcodes.

[00201] In some embodiments, the methods comprise sequencing the first nucleo-base position of the first barcodes of step (b), and sequencing the second nucleo-base position of the first barcodes of step (c). In some embodiments, the first and second nucleo-base positions are consecutive nucleo-base positions in the first barcodes. In some embodiments, the first and second nucleo-base positions are non-consecutive nucleo-base positions in the first barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[00202] In some embodiments, the methods comprise sequencing the first nucleo-base position of the second barcodes of step (b), and sequencing the second nucleo-base position of the second barcodes of step (c). In some embodiments, the first and second nucleo-base

positions are consecutive nucleo-base positions in the second barcodes. In some embodiments, the first and second nucleo-base positions are non-consecutive nucleo-base positions in the second barcodes. For example, the first and second non-consecutive nucleo-base positions can have a gap of 1-10 nucleo-base positions.

[00203] In some embodiments, the first and second target analytes are located inside the cellular sample and/or on the membrane of the cellular sample. In some embodiments, the first and/or second target analyte comprises a polypeptide, lipid, nucleic acid or polysaccharide. In some embodiments, the first and/or second target analytes comprise a polypeptide, enzyme or lipid located anywhere in the cellular sample including, without limits, the cytoplasm and nucleus. In some embodiments, the first and/or 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, actin cytoskeleton, spindle, flagellum, peroxisome and lysosome.

[00204] In some embodiments, simultaneously sequencing at least two sub-populations of modified oligonucleotides 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 sequencing-based cell painting.

[00205] The present disclosure provides methods for sequencing barcodes comprising **step (a)**: providing a plurality of modified oligonucleotides including at least a first sub-population of modified oligonucleotides comprising a plurality of a first barcode which corresponds to a first target analyte, a second sub-population of modified oligonucleotides comprising a plurality of a second barcode which corresponds to a second target analyte, and a third sub-population of modified oligonucleotides comprising a plurality of a third barcode which corresponds to a third target. In some embodiments, the plurality of modified oligonucleotides are located inside a cellular sample. In some embodiments, the first barcode is 4-20 nucleo-bases in length. In some embodiments, the second barcode is 4-20 nucleo-bases in length. In some embodiments, the third barcode is 4-20 nucleo-bases in length. In some embodiments, the first, second and/or third barcode is 3-20 nucleo-bases in length. In some embodiments, the first, second and/or third barcode is 2-20 nucleo-bases in length.

[00206] In some embodiments, the methods for sequencing barcodes comprise **step (b)**: conducting a first sequencing cycle, wherein the sequencing cycle comprises sequencing essentially simultaneously the first nucleo-base position of the first, second and third

barcodes in the first, second and third sub-populations of modified oligonucleotides. In some embodiments, the first sequencing cycle comprises using a plurality of sequencing primers having the same sequence. In some embodiments, the first nucleo-base position of the first barcode generates a first color signal and the first nucleo-base position of the second and third barcode generates a second color signal. In some embodiments, the first and second color signals are distinguishable from each other in the first sequencing cycle. In some embodiments the first color signal identifies the first target analyte. In some embodiments, the first sequencing cycle occurs on and/or inside a cellular sample.

[00207] In some embodiments, the first sequencing cycle of step (b) can be conducted using any sequencing methods described herein or known in the art including, without limitation, including two-stage sequencing using multivalent molecules, sequencing-by-binding, or sequencing using chain terminator nucleotides.

[00208] In some embodiments, the methods for sequencing barcodes comprise **step (c)**: conducting a second sequencing cycle, wherein the sequencing comprises sequencing essentially simultaneously the second nucleo-base position of the first, second and third barcodes in the first, second and third sub-populations of modified oligonucleotides. In some embodiments, the second nucleo-base position of the first barcode generates the second color signal. In some embodiments, the second nucleo-base position of the second barcode generates the first color signal. In some embodiments, the second nucleo-base position of the third barcode generates the second color signal. In some embodiments, the first and second color signals are distinguishable from each other in the second sequencing cycle. In some embodiments, the first color signal identifies the second target analyte. In some embodiments, the second sequencing cycle occurs on and/or inside the cellular sample.

[00209] In some embodiments, the second sequencing cycle of step (c) can be conducted using any sequencing methods described herein or known in the art including, without limitation, two-stage sequencing using multivalent molecules, sequencing-by-binding, or sequencing using chain terminator nucleotides.

[00210] In some embodiments, methods for sequencing barcodes comprise **step (d)**: conducting a third sequencing cycle, wherein the sequencing comprises sequencing essentially simultaneously the third nucleo-base position of the first, second and third barcodes in the first, second and third sub-populations of modified oligonucleotides. In some embodiments, the third nucleo-base position of the first barcode generates the second color signal. In some embodiments, the third nucleo-base position of the second barcode generates the second color signal. In some embodiments, the third nucleo-base position of the third

barcode generates the first color signal. In some embodiments, the first and second color signals are distinguishable from each other in the third sequencing cycle. In some embodiments, the first color signal identifies the third target analyte. In some embodiments, the third sequencing cycle occurs on and/or inside the cellular sample.

[00211] In some embodiments, the third sequencing cycle of step (d) can be conducted using any sequencing methods described herein or known in the art including, without limitation, two-stage sequencing using multivalent molecules, sequencing-by-binding, or sequencing using chain terminator nucleotides.

[00212] In some embodiments, the methods for sequencing barcodes further comprise: imaging the first and second color signals in the first sequencing cycle, and identifying the first target analyte.

[00213] In some embodiments, the methods for sequencing barcodes further comprise: imaging the first and second color signals in the second sequencing cycle and identifying the second target analyte.

[00214] In some embodiments, the methods for sequencing barcodes further comprise: imaging the first and second color signals in the third sequencing cycle and identifying the third target analyte.

[00215] In some embodiments, the methods for sequencing barcodes further comprise: imaging the first and second color signals 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.

[00216] In some embodiments, the methods for sequencing barcodes further comprise: imaging the first and second color signals 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.

[00217] In some embodiments, the methods for sequencing barcodes further comprise: imaging the first and second color signals generated inside the cellular sample 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.

[00218] In some embodiments, the first and second color signals generated at any of the first, second and/or third sequencing cycles are generated on and/or inside the cellular sample.

[00219] 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.

[00220] In some embodiments, the first, second and third target analytes can be identified by conducting no more than three sequencing cycles.

[00221] In some embodiments, the first, second and third barcodes can be identified by sequencing the full length of the first, second and third barcodes.

[00222] In some embodiments, the methods comprise sequencing the first nucleobase position of the first barcodes of step (b), and sequencing the second nucleobase position of the first barcodes of step (c). In some embodiments the first and second nucleobase positions are consecutive nucleobase positions in the first barcodes. In some embodiments, the first and second nucleobase positions are non-consecutive nucleobase positions in the first barcodes. For example, the first and second non-consecutive nucleobase positions can have a gap of 1-10 nucleobase positions.

[00223] In some embodiments, the methods comprise sequencing the first nucleobase position of the second barcodes of step (b), and sequencing the second nucleobase position of the second barcodes of step (c). In some embodiments, the first and second nucleobase positions are consecutive nucleobase positions in the second barcodes. In some embodiments, the first and second nucleobase positions are non-consecutive nucleobase positions in the second barcodes. For example, the first and second non-consecutive nucleobase positions can have a gap of 1-10 nucleobase positions.

[00224] In some embodiments, the methods comprise sequencing the first nucleobase position of the third barcodes of step (b), and sequencing the second nucleobase position of the third barcodes of step (c). In some embodiments, the first and second nucleobase positions are consecutive nucleobase positions in the third barcodes. In some embodiments, the first and second nucleobase positions are non-consecutive nucleobase positions in the third barcodes. For example, the first and second non-consecutive nucleobase positions can have a gap of 1-10 nucleobase positions.

[00225] In some embodiments, the first, second and third target analytes are located inside the cellular sample and/or on the membrane of the cellular sample. In some embodiments, 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 cellular sample 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, actin cytoskeleton, spindle, flagellum, peroxisome and lysosome.

[00226] In some embodiments, simultaneously sequencing two or more sub-populations of modified oligonucleotides 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.

[00227] In some embodiments, the first sequencing cycle of step (b), 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.

Two-Stage Methods for Nucleic Acid Sequencing

[00228] The present disclosure provides a two-stage method for sequencing any of the modified oligonucleotides of the amplification-free probe complexes described herein (*e.g.*, FIGS. 13A-13B, 14A-14B, 15A-15B, 16, 17, 18A-18B and 19A-19B). In some embodiments, the two-stage sequencing method can be conducted on and/or inside a cellular sample. In some embodiments, the two-stage sequencing method can be employed to sequence the canonical nucleo-base of any of the modified oligonucleotides described herein (*e.g.*, FIGS. 18A-18B). In some embodiments, the two-stage sequencing method can be employed to sequence the target barcode sequence (or a portion thereof) of any of the modified oligonucleotides described herein (*e.g.*, FIGS. 19A-19B).

[00229] In some embodiments, the first stage generally comprises binding multivalent molecules to polymerase complexes to form multivalent-binding polymerase complexes, and detecting the multivalent-binding polymerase complexes. In some embodiments, the second stage comprises nucleotide incorporation and extension of the sequencing primer. In some embodiments, one sequencing cycle comprises completion of a first and second stage.

[00230] In some embodiments, the first stage comprises **step (a)**: contacting (i) a first plurality of sequencing polymerases, (ii) a plurality of modified oligonucleotides located inside a cellular sample and (iii) a plurality of sequencing primers, where the contacting is conducted under a condition suitable to form a first plurality of sequencing polymerase complexes, each complex comprising a first sequencing polymerase bound to a nucleic acid

duplex, wherein the nucleic acid duplex comprises a portion of a modified oligonucleotide hybridized to a sequencing primer. In some embodiments, the sequencing primers comprise 3' extendible ends or 3' non-extendible ends. In some embodiments, the first sequencing polymerase comprises a recombinant mutant sequencing polymerase.

[00231] In some embodiments, the sequencing method comprises **step (b)**: contacting the first plurality of polymerase complexes with a plurality of multivalent molecules to form a plurality of multivalent-binding polymerase complexes (*e.g.*, binding complexes) inside the cellular sample. In some embodiments, individual multivalent molecules in the plurality of multivalent molecules comprise a core attached to multiple nucleotide arms and individual nucleotide arms are attached to a nucleotide moiety (*e.g.*, FIGS. 1-3 and 4A-4B). In some embodiments, the contacting of step (b) is conducted under a condition suitable for binding complementary nucleotide moieties of the multivalent molecules to at least two of the polymerase complexes in the first plurality thereby forming a plurality of multivalent-binding polymerase complexes. In some embodiments, the condition is suitable for inhibiting polymerase-catalyzed incorporation of the complementary nucleotide moieties into the primers of the plurality of multivalent-binding polymerase complexes. In some embodiments, the contacting of step (b) is conducted in the presence of at least one non-catalytic cation which inhibits polymerase-catalyzed nucleotide incorporation. In some embodiments, the at least one non-catalytic cation comprises strontium, barium and/or calcium.

[00232] In some embodiments, in the method of step (b), at least one of the multivalent molecules in the plurality of multivalent molecules is labeled with a detectable reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore.

[00233] In some embodiments, in the method of step (b), individual nucleotide arms of a multivalent molecule 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 (*e.g.*, FIG. 4B). In some embodiments, the labeled multivalent molecules comprise a fluorophore attached to the core, spacer, linker and/or nucleotide moiety of the multivalent molecules.

[00234] In some embodiments, in the method of step (b), the plurality of multivalent molecules comprises at least one multivalent molecule having multiple nucleotide arms (*e.g.*, FIGS. 1-3 and 4A) each attached with a nucleotide analog (*e.g.*, nucleotide analog moiety), where the nucleotide analog includes a chain terminating moiety (*e.g.*, blocking moiety) at the sugar 2' and/or 3' position. In some embodiments, the plurality of multivalent molecules

comprises at least one multivalent molecule comprising multiple nucleotide arms each attached with a nucleotide moiety that lacks a chain terminating moiety.

[00235] In some embodiments, the sequencing method comprises **step (c)**: detecting the plurality of multivalent-binding polymerase complexes. In some embodiments, the detecting includes detecting the multivalent molecules that are bound to the polymerase complexes in the first plurality, where the complementary nucleotide moieties of the multivalent molecules are bound to the primers but incorporation of the complementary nucleotide moieties is inhibited. In some embodiments, the multivalent molecules are labeled with a detectable reporter moiety to permit detection. In some embodiments, the detectable reporter moiety comprises a fluorophore which generates a color signal. In some embodiments, the detecting of step (c) comprises imaging the plurality of multivalent-binding polymerase complexes.

[00236] In some embodiments, the methods for sequencing further comprise **step (d)**: identifying the nucleobase of the complementary nucleotide moieties that are bound to the first plurality of polymerase complexes, thereby determining the sequence of the modified oligonucleotides. In some embodiments, the multivalent molecules are labeled with a detectable reporter moiety that corresponds to the particular nucleotide moieties attached to the nucleotide arms to permit identification of the complementary nucleotide moieties (*e.g.*, nucleotide base adenine, guanine, cytosine, thymine or uracil) that are bound to the first plurality of polymerase complexes.

[00237] In some embodiments, sequencing a modified oligonucleotide comprising a canonical nucleobase and an abasic site (*e.g.*, FIG. 18A-18B) comprises conducting the first stage of the two-stage sequencing method (*e.g.*, steps (a) – (d)), and optionally conducting the second stage (*e.g.*, steps (e) – (k), described below).

[00238] In some embodiments, sequencing a modified oligonucleotide comprising a target barcode sequence (*e.g.*, FIGS. 19A-19B) comprises conducting the first stage of the two-stage sequencing method (*e.g.*, steps (a) – (d)), and conducting the second stage (*e.g.*, steps (e) – (k)).

[00239] In some embodiments, the second stage of the two-stage sequencing method generally comprises nucleotide incorporation. In some embodiments, the sequencing method comprises **step (e)**: dissociating the plurality of multivalent-binding polymerase complexes and removing the first plurality of sequencing polymerases and their bound multivalent molecules, and retaining the plurality of nucleic acid duplexes inside the cellular sample.

[00240] In some embodiments, the sequencing method comprises **step (f)**: contacting the plurality of the nucleic acid duplexes retained at step (e) inside the cellular sample with a

second plurality of sequencing polymerases, wherein the contacting is conducted under a condition suitable for binding the second plurality of sequencing polymerases to the plurality of the nucleic acid duplexes, thereby forming a second plurality of polymerase complexes, each complex comprising a second sequencing polymerase bound to a nucleic acid duplex. In some embodiments, the second sequencing polymerase comprises a recombinant mutant sequencing polymerase.

[00241] In some embodiments, the plurality of first sequencing polymerases of step (a) have an amino acid sequence that is 100% identical to the amino acid sequence of the plurality of the second sequencing polymerases of step (f). In some embodiments, the plurality of first sequencing polymerases of step (a) have an amino acid sequence that differs from the amino acid sequence of the plurality of the second sequencing polymerases of step (f).

[00242] In some embodiments, the methods for sequencing further comprise **step (g):** contacting the second plurality of polymerase complexes with a plurality of nucleotides, wherein the contacting is conducted under a condition suitable for binding complementary nucleotides from the plurality of nucleotides to at least two of the second polymerase complexes thereby forming a plurality of nucleotide-polymerase complexes. In some embodiments, the contacting of step (g) is conducted under a condition that is suitable for promoting polymerase-catalyzed incorporation of the bound complementary nucleotides into the primers of the nucleotide-polymerase complexes. In some embodiments, the incorporating the nucleotide into the 3' end of the primer in step (g) comprises a primer extension reaction. In some embodiments, the contacting of step (g) is conducted in the presence of at least one catalytic cation which promotes nucleotide incorporation. In some embodiments, the at least one catalytic cation comprises magnesium and/or manganese. In some embodiments, the plurality of nucleotides comprise native nucleotides (*e.g.*, non-analog nucleotides) or nucleotide analogs. In some embodiments, the plurality of nucleotides comprise a 2' and/or 3' chain terminating moiety which is removable or is not removable. In some embodiments, at least one of the nucleotides in the plurality is not labeled with a detectable reporter moiety. In some embodiments, the plurality of nucleotides are non-labeled nucleotides. In some embodiments, the plurality of nucleotides comprises a plurality of nucleotides labeled with detectable reporter moiety. The detectable reporter moiety can comprise 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 or is not removable from the base. 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.

[00243] In some embodiments, when the plurality of nucleotides in steps (g) comprise labeled nucleotides, the methods for sequencing further comprise **step (h)**: detecting the complementary nucleotides which are incorporated into the primers of the nucleotide-polymerase complexes. In some embodiments, the plurality of nucleotides are labeled with a detectable reporter moiety to permit detection. In some embodiments, when the plurality of nucleotides in steps (g) comprises non-labeled nucleotides, the detecting of step (h) is omitted.

[00244] In some embodiments, when the plurality of nucleotides in steps (g) comprises labeled nucleotides, the methods for sequencing further comprise **step (i)**: identifying the bases of the complementary nucleotides which are incorporated into the primers of the nucleotide-polymerase complexes. In some embodiments, the identification of the incorporated complementary nucleotides in step (i) can be used to confirm the identity of the complementary nucleotides of the multivalent molecules that are bound to the first plurality of polymerase complexes in step (d). In some embodiments, the identifying of step (i) can be used to determine the sequence of the modified oligonucleotide. In some embodiments, when the plurality of nucleotides in steps (g) comprises non-labeled nucleotides, the identifying of step (i) is omitted.

[00245] In some embodiments, when the plurality of nucleotides in step (g) comprise 2' and/or 3' chain terminating nucleotides, the methods for sequencing further comprise **step (j)**: removing the chain terminating moiety from the incorporated nucleotides.

[00246] In some embodiments, the methods for sequencing further comprise **step (k)**: repeating steps (a) – (j) at least once. In some embodiments, the sequence of the modified oligonucleotides can be determined by detecting and identifying the multivalent molecules that bind the sequencing polymerases but do not incorporate into the 3' end of the primer at steps (c) and (d). In some embodiments, the sequence of the modified oligonucleotides can be determined (or confirmed) by detecting and identifying the nucleotide that incorporates into the 3' end of the primer at steps (h) and (i).

Forming Avidity Complexes

[00247] In some embodiments, in any of the two-stage sequencing methods, the binding of the first plurality of polymerase complexes with the plurality of multivalent molecules forms

at least one avidity complex, the method comprising the steps: **(1)** binding a first sequencing primer, a first sequencing polymerase, and a first multivalent molecule to a first portion of a modified oligonucleotide, thereby forming a first binding complex, wherein a first nucleotide moiety of the first multivalent molecule binds to the first sequencing polymerase; and **(2)** binding a second sequencing primer, a second sequencing polymerase, and the first multivalent molecule to a second portion of the same modified oligonucleotide 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, modified oligonucleotide comprises tandem repeat sequences of at least one universal site for binding a sequencing primer, a canonical nucleobase and an abasic site. In some embodiments, modified oligonucleotide comprises tandem repeat sequences of at least one universal site for binding a sequencing primer and a target barcode sequence. The first and second sequencing primers can bind to a sequencing primer binding site along the modified oligonucleotide. Exemplary multivalent molecules are shown in FIGS. 1-3 and 4A-4B.

Forming Avidity Complexes with Detecting and Identifying

[00248] In some embodiments, in any of the two-stage sequencing methods, the method includes binding the first plurality of polymerase complexes with the plurality of multivalent molecules to form at least one avidity complex, and the method comprises the steps: **(1)** contacting the plurality of sequencing polymerases and the plurality of sequencing primers with different portions of a modified oligonucleotide to form at least first and second polymerase complexes on the same modified oligonucleotide; **(2)** contacting a plurality of multivalent molecules to the at least first and second polymerase complexes on the same modified oligonucleotide, under conditions suitable to bind a single multivalent molecule from the plurality to the first and second polymerase complexes, wherein at least a first nucleotide moiety of the single multivalent molecule is bound to the first polymerase complex which includes a first sequencing primer hybridized to a first portion of the modified oligonucleotide 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 polymerase complex which includes a second sequencing primer hybridized to a second portion of the modified oligonucleotide 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 modified oligonucleotide forms an avidity complex; **(3)** detecting the first and second binding complexes on the same modified oligonucleotide, and **(4)** identifying the first nucleotide moiety in the first binding complex thereby determining the sequence of the first portion of the modified oligonucleotide, and identifying the second nucleotide moiety in the second binding complex thereby determining the sequence of the second portion of the modified oligonucleotide.

[00249] In some embodiments, the modified oligonucleotide comprises tandem repeat sequences of at least one universal site for binding a sequencing primer, a canonical nucleobase and an abasic site. In some embodiments, the modified oligonucleotide comprises tandem repeat sequences of at least one universal site for binding a sequencing primer and a target barcode sequence. The plurality of sequencing primers can bind to a sequencing primer binding site along the modified oligonucleotide. Exemplary multivalent molecules are shown in FIGS. 1-3 and 4A-4B.

Sequencing-by-Binding Methods

[00250] In some embodiments, any of the modified oligonucleotides of the amplification-free probe complexes described herein (*e.g.*, FIGS. 13A-13B, 14A-14B, 15A-15B, 16, 17, 18A-18B and 19A-19B) can be sequenced by conducting sequencing-by-binding (SBB) reactions. In some embodiments, the SBB reactions occur on and/or inside a cellular sample. 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 modified oligonucleotide (*e.g.*, a modified oligonucleotide 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 modified oligonucleotide being contacted, under the ternary complex stabilizing conditions, with nucleotide cognates for first, second and third base types in the modified oligonucleotide; **(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 modified oligonucleotide, 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 modified oligonucleotide after step (b), thereby producing an extended primer; and **(e)** repeating steps (a) through (d) at least once on the primed modified oligonucleotide 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).

Methods for Sequencing using Nucleotide Analogs

[00251] In some embodiments, any of the modified oligonucleotides of the amplification-free probe complexes described herein (*e.g.*, FIGS. 13A-13B, 14A-14B, 15A-15B, 16, 17, 18A-18B and 19A-19B) can be sequenced by conducting a sequencing reaction using at least one nucleotide analog comprising a 2' or 3' chain terminating moiety.

[00252] In some embodiments, the chain terminating sequencing method comprises **step (a)**: contacting a sequencing polymerase to (i) a modified oligonucleotide and (ii) a sequencing primer, wherein the contacting is conducted under a condition suitable to bind the sequencing polymerase to the modified oligonucleotide which is hybridized to the sequencing primer, wherein the modified oligonucleotide hybridized to the sequencing primer forms the nucleic acid duplex. In some embodiments, the sequencing polymerase comprises a recombinant mutant sequencing polymerase that can bind and incorporate nucleotide analogs. In some embodiments, the sequencing primer comprises a 3' extendible end. In some embodiments, the sequencing primer comprises a 3' non-extendible end that can be converted to a 3' extendible end.

[00253] In some embodiments, the methods for sequencing comprises **step (b)**: contacting the sequencing polymerase with a plurality of nucleotides under a condition suitable for binding at least one nucleotide to the sequencing polymerase which is bound to the nucleic acid duplex and suitable for polymerase-catalyzed nucleotide incorporation. In some embodiments, the sequencing polymerase is contacted with the plurality of nucleotides in the presence of at least one catalytic cation comprising magnesium and/or manganese. In some embodiments, the plurality of nucleotides comprises at least one nucleotide analog having a chain terminating moiety at the sugar 2' or 3' position. In some embodiments, the chain terminating moiety is removable from the sugar 2' or 3' position to convert the chain terminating moiety to an OH or H group. In some embodiments, the plurality of nucleotides comprises at least one nucleotide that lacks a chain terminating moiety. In some

embodiments, at least one nucleotide is labeled with a detectable reporter moiety (*e.g.*, fluorophore). In some embodiments, the plurality of nucleotides comprises a plurality of labeled nucleotides.

[00254] In some embodiments, the methods for sequencing comprises **step (c)**: incorporating at least one nucleotide into the 3' end of the extendible primer under a condition suitable for incorporating the at least one nucleotide. In some embodiments, the suitable conditions for nucleotide binding the polymerase and for incorporation the nucleotide can be the same or different. In some embodiments, conditions suitable for incorporating the nucleotide comprise inclusion of at least one catalytic cation comprising magnesium and/or manganese. In some embodiments, the at least one nucleotide binds the sequencing polymerase and incorporates into the 3' end of the extendible primer. In some embodiments, the incorporating the nucleotide into the 3' end of the sequencing primer in step (c) comprises a primer extension reaction.

[00255] In some embodiments, the methods for sequencing comprises **step (d)**: detecting and identifying the nucleotides incorporated into the 3' ends of the sequencing primers.

[00256] In some embodiments, the methods for sequencing comprises **step (e)**: removing the chain terminating moiety from the incorporated nucleotides and generating a plurality of sequencing primers having extendible 3' ends.

[00257] In some embodiments, the methods for sequencing comprises **step (f)**: repeating steps (a) – (e) at least once.

[00258] In some embodiments, in step (b), the plurality of nucleotides comprises a plurality of nucleotides labeled with detectable reporter moiety. In some embodiments, detectable reporter moiety comprises a fluorophore. In some embodiments, the fluorophore can be attached to the nucleotide base. In some embodiments, the fluorophore can be 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.

Cellular Samples

[00259] In some embodiments, in any of the compositions or methods described herein, the cellular sample 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 cellular sample comprises a fresh cellular sample, a freshly-frozen cellular sample, a sectioned cellular sample, or an FFPE cellular sample. In some embodiments, the cellular sample comprises one or more living cells or non-living cells. In some embodiments, the cellular sample can be obtained from a virus, fungus, prokaryote or eukaryote. In some embodiments, the cellular sample 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 cellular sample comprises one or more virally-infected cells. In some embodiments, the cellular sample comprises a biofilm, *i.e.* a consortium of microorganisms that adhere together. In some embodiments, the cellular sample can be obtained from any organism including human, simian, ape, canine, feline, bovine, equine, murine, porcine, caprine, lupine, ranine, piscine, plant, insect, fungus, yeast or bacterium. In some embodiments, the cellular sample 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. In some embodiments, the cellular sample comprises cells that can form an adherent monolayer when cultured on a support (*e.g.*, coated or non-coated support). In some embodiments, the cellular sample comprises human cells that can form adherent cells (*e.g.*, adherent monolayer) when cultured on a support, including for example HeLa, HEK293, HUVEC, HCT116, A549, PC3, HepG2, MCF7 and U2OS. In some embodiments, the cellular sample comprises human stem cells. In some embodiments, the cellular sample comprises cells that have been differentiated in vitro into one or more cell types including, without limitation, neurons, glia, myocytes, epithelia cells, T cells, B cells, NK cells and the like.

Culturing Cellular Samples on a Support

[00260] In some embodiments, in any of the compositions or methods described herein, the cellular sample can be cultured on a support (*e.g.*, a flowcell). In some embodiments, the methods comprise culturing the cellular sample on the support under condition suitable for expanding the cellular sample 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 cellular sample to confluence or to non-confluence. In some embodiments, the methods comprise culturing the cells of the cellular sample, and then inducing terminal differentiation of the cells using methods known in the art. In some

embodiments, the methods comprise culturing the cellular sample 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 cellular sample 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 cellular sample can be cultured with suitable aeration, *e.g.*, with oxygen and/or nitrogen.

[00261] 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 cellular sample. 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 cellular sample can be placed in a simple cell medium prior to or during the step of conducting any of the nucleic acid methods described herein.

[00262] 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 cellular sample can be placed in a complex cell medium prior to, or during, the step of conducting any of the nucleic acid methods described herein.

[00263] In some embodiments, the cellular sample 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.

[00264] In some embodiments, the cellular sample can be deposited (*e.g.*, seeded) onto a support which is passivated with a coating that promotes proliferation, migration, differentiation and/or adhesion of cultured cell or living *ex vivo* cells or tissue samples. In some embodiments, the cellular sample can be deposited on a support that lacks immobilized capture primers which can bind target polynucleotides from the cellular sample. In some embodiments, the support can be coated with a solubilized basement membrane matrix. Suitable methods for coating a support with solubilized basement membrane matrix will be known to persons of ordinary skill in the art. In some embodiments, the solubilized basement membrane matrix is secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, for example MATRIGEL®. In some embodiments, the support can be coated with a gel matrix, including for example collagen gel, alginate gel or lactate gel. In some embodiments, the

support can be coated with one or more animal-derived protein including for example aggrecan, brevican, collagen (*e.g.*, collagen type I, II, III or IV), fibronectin, elastin, laminin, laminin/fibronectin, laminin/poly-D-lysine, laminin/poly-D-ornithine, vitronectin, osteopontin, gelatin (*e.g.*, porcine), fibrillin, fibrinogen, plasminogen, plasmin, tenascin, hyaluronic acid proteoglycan, keratan sulfate proteoglycan, heparan sulfate proteoglycan, chondroitin sulfate proteoglycan, syndecan-1 (*e.g.*, proteoglycan), and IGF binding protein. 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 poly-amino acid including and without limitation, a poly-lysine compound (*e.g.*, poly-L-lysine (PLL) or poly-D-lysine (PDL)), an arginine compound, a poly-arginine compound, a poly-ornithine 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-aminoisobutyric 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 short peptides of an extracellular matrix protein, including and without limitation, Arg-Gly-Asp (RGD), RGD-coupled alginate, Ile-Lys-Val-Ala-Val (IKVAV), Lys-Gln-Ala-Gly-Asp-Val (KQAGDV), Val-Ala-Pro-Gly (VAPG), Phe-Gly-Leu (FGL), fibronectin domains, and laminin. 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). In some embodiments, the support is coated with any compound that is presently known in the art to be useful for promoting proliferation, migration, differentiation and/or adhesion of cultured cells or living *ex vivo* cells or tissue samples. In some embodiments, the cellular sample can be deposited (*e.g.*, seeded) onto an uncoated support.

Cell Fixation

[00265] In some embodiments, in any of the compositions or methods described herein, the cellular sample comprises a fixed cellular sample. In some embodiments, the cellular sample 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 cellular sample. In some embodiments, the fixation reagent inhibits loss of nucleic acids from the cellular sample.

[00266] In some embodiments, the fixation reagent can cross-link the RNA to prevent the RNA from escaping the cellular sample. 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).

[00267] 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.

[00268] 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.

[00269] In some embodiments, the cellular sample can be fixed on the support with 4% paraformaldehyde for about 30-60 minutes, or any range therebetween, and washed with PBS.

[00270] In some embodiments, the cellular sample can be stained, de-stained (*e.g.*, with stain removed), or un-stained.

Cell Permeabilization

[00271] In some embodiments, in any of the compositions or methods described herein, the cellular sample comprises a permeabilized cellular sample. In some embodiments, the methods comprise treating the cellular sample with a permeabilization reagent that alters the

cell membrane to permit penetration of reagents, such as multivalent molecules, nucleotides, chain terminator nucleotides, sequencing polymerases, sequencing primers, rolling circle amplification (RCA) reagents, polymerase chain reaction (PCR) reagents, sequencing reagents and the like, into the cells. For example, and without limitation, the permeabilization reagent removes membrane lipids from the cell membrane. In some embodiments, the cellular sample 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.

[00272] 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.

Target Analytes

[00273] In some embodiments, the analyte binding moieties conjugated to the modified oligonucleotides described herein can selectively bind to target analytes located on and/or inside a cell. In some embodiments, the modified oligonucleotide (and conjugated analyte binding moiety) can enter a cell. In some embodiments, the modified oligonucleotide (and conjugated analyte binding moiety) can be located in a cytoplasm of a cell. In some embodiments, the analyte binding moiety of the modified oligonucleotide can bind a cellular organelle or can enter a cellular organelle including for example a nucleus, a nucleolus, a mitochondrion, a Golgi apparatus, an endoplasmic reticulum, flagellum, cilium or a chloroplast.

[00274] In some embodiments, the analyte binding moieties bind a target analyte comprising naturally-occurring molecules, recombinant molecules, modified molecules and/or synthetic molecules. In some embodiments, the target analyte comprises a peptide,

including without limitations, polypeptides, proteins, protein fragments and enzymes. In some embodiments, the target analyte comprises cell surface receptors, including without limitation ion channel receptors (*e.g.*, ligand-gated ion channel receptors), G-protein coupled receptors, enzyme-linked receptors and intracellular cell receptors. In some embodiments, the target analyte comprises a cluster of differentiation (CD) including CDs that act as cell receptors, cell ligands, cell signaling and cell adhesion. In some embodiments, the target analyte comprises an immunoglobulin molecule, including, without limitations, antibodies, antibody fragments and single-chain antibodies. In some embodiments, the target analyte comprises saccharides, including without limitations, monosaccharides, disaccharides, oligosaccharides, polysaccharides, homopolysaccharides and heteropolysaccharides. In some embodiments, the target analyte comprises lipids, including without limitations, triglycerides, phospholipids, steroids, fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides (*e.g.*, which are derived from condensation of ketoacyl subunits), sterol lipids and prenol lipids (*e.g.*, which are derived from condensation of isoprene subunits). In some embodiments, the target analyte comprises nucleic acids, including without limitation, polynucleotides, oligonucleotides, DNA, cDNA and RNA. In some embodiments, the target analyte comprises glycosylated molecules, including without limitation, glycoproteins and glycolipids.

[00275] In some embodiments, the analyte binding moiety can bind to a cell membrane protein. In some embodiments, the analyte binding moiety can bind to PMCA (plasma membrane Ca^{2+} ATPase) wherein the analyte binding moiety comprises calmodulin, RASSF1 (Ras-associated factor 1), calcineurin A, α -1 syntrophin, or caloxin. In some embodiments, the analyte binding moiety can bind to ezrin, wherein the analyte binding moiety comprises DAG1 (dystroglycan), SLC9A3R1 (Na^{+})/ H^{+} exchange regulatory cofactor NHR-RF1), RDX (radixin), ARHGAP18 (rho GTPase-activating protein 18), or SDC4 (syndecan-4). In some embodiments, the analyte binding moiety can bind to ZO1 (zonula occludens-1), wherein the analyte binding moiety comprises DbpA (DNA binding protein A), occludin, afadin, or α -catenin. In some embodiments, the analyte binding moiety can bind to claudin-1, wherein the analyte binding moiety comprises occludin, ZO-1, ZO-2 or ZO-3 (zonula occludens-1, -2 or -3), as well as homologs and orthologs thereof.

[00276] In some embodiments, the analyte binding moiety can bind to G-actin (globular actin) or F-actin (filamentous actin). In some embodiments, the analyte binding moiety comprises phalloidin which can bind F-actin.

[00277] In some embodiments, the analyte binding moiety can bind to proteins located on a nuclear membrane, including without limitation SUN2 (Sad1 and UNC84 domain containing 2), TMPO (thymopoietin), SUN1 (sad1 and UNC84 domain containing 1), LEMD2 (LEM domain nuclear envelope protein 2), LMNB1 (lamin B), TOR1AIP1 (torsin 1A interacting protein 1), LBR (lamin B receptor), and LMNB2 (lamin B2) and type A lamin, as well as homologs and orthologs thereof.

[00278] In some embodiments, the analyte binding moiety can bind to proteins located in a nucleus. In some embodiments, the analyte binding moiety can bind to histones, TAF15 (TAT-box binding protein associated factor 15), SMARCAD1 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily A containing DEAD/H box 1), SRRM2 (serine/arginine repetitive matrix 2), RBM25 (RNA binding motif protein 25), PML (PML nuclear body scaffold), SMN2 (survival of motor neuron 2 centromeric) and MKI67 (marker of proliferation Ki-67), as well as homologs and orthologs thereof.

[00279] In some embodiments, the analyte binding moiety can bind to a protein located in the cytoplasm. In some embodiments, the analyte binding moiety can bind to ATXN2 (ataxin 2), G3BP2 (G3BP stress granule assembly factor 2), AIMP1 (aminoacyl tRNA synthetase complex interacting multifunctional protein 1), SERBP1 (SERPINE1 mRNA binding protein 1), CCDC43 (coiled-coil domain containing 43), ATXN2L (ataxin 2 like), AMPD2 (adenosine monophosphate deaminase 2) and RABGAP1 (RAB GTPase activating protein 1). In some embodiments, the analyte binding moiety can bind to tubulin, *e.g.* the analyte binding moiety comprises colchicine, vinblastine, pironetin, maytansine, taxane, laulimalide, peloruside, cevipabulin, or rhizosin.

[00280] In some embodiments, the analyte binding moiety can bind to a Golgi apparatus. In some embodiments, the analyte binding moiety can bind to Golgin 97, *e.g.* when the analyte binding moiety comprises FIP1/RCP (family interacting proteins) (Rab-coupling protein). In some embodiments, the analyte binding moiety can bind to TGN 46 (trans-Golgi network 46) wherein the analyte binding moiety comprises PKD (protein kinase D), or OSBP (oxysterol-binding protein), as well as homologs and orthologs thereof.

[00281] In some embodiments, the analyte binding moiety can bind to an endoplasmic reticulum. In some embodiments, the analyte binding moiety can bind to HSP90B1 (heat shock protein 90 beta family member 1), CANX (calnexin), KTN1 (kinectin 1), PDIA3 (protein disulfide isomerase family A member 3), RCN1 (reticulocalbin 1), RRBP1 (ribosome binding protein 1), SEC61B (SEC61 translocon subunit beta), and CY51A1

(cytochrome P450 family 51 subfamily A member 1), as well as homologs and orthologs thereof.

[00282] In some embodiments, the analyte binding moiety can bind to mitochondria. In some embodiments, the analyte binding moiety can bind to CS (citrate synthase), LRPPRC (leucine rich pentatricopeptide repeat containing), SLC25A24 (solute carrier family 25 member 24), TIMM44 (translocase of inner mitochondrial membrane 44), GCDH (glutaryl-CoA dehydrase) and TRAP1 (TNF receptor associated protein 1), as well as homologs and orthologs thereof.

[00283] In some embodiments, the analyte binding moiety can bind to a cytokine. In some embodiments, the cytokine comprises a protein expressed on the surface of immune and non-immune cells. In some embodiments, the cytokine comprises a human cytokine. Cytokines can include, without limitation, interleukins, interferons, tumor necrosis factors, transforming growth factors, chemokines, lymphokines, monokines and colony stimulating factors.

[00284] In some embodiments, the analyte binding moiety can bind to an interleukin including and without limitation IL1alpha (hematopoietin-1), IL-1beta (catabolin), IL-1RA (IL-1 receptor antagonist), IL-18 (interferon-gamma inducing factor), IL-2, IL-4, IL-7, IL-9, IL-13, IL15, IL-3, IL-5, GM-CSF, IL-6, IL-11, G-CSF, IL-12, LIF (leukemia inhibitory factor), OSM (oncostatin-M), IL-10, IL-20, IL-14, IL16, IL-17, IL-17A, IL-17F, IL-21, IL-23, IL-22 and IL-35, as well as homologs and orthologs thereof.

[00285] In some embodiments, the analyte binding moiety can bind to an interferon including and without limitation IFN-alpha, IFN-beta and IFN-gamma, as well as homologs and orthologs thereof.

[00286] In some embodiments, the analyte binding moiety can bind to a tumor necrosis factor including and without limitation CD154 (CD40L or TRAP), LT-beta, TNF-alpha (cachectin), TNF-beta (LT-alpha), 4-1BBL, APRIL (TALL-2), CD70, CD153, CD178, GITRL, LIGHT, OX40L, TALL-1, TRAIL (Apo2L), TWEAK (Apo3L) and TRANCE (OPGL), as well as homologs and orthologs thereof.

[00287] In some embodiments, the analyte binding moiety can bind to a transforming growth factor including and without limitation TGF-beta1, TGF-beta2 and TGF-beta3, as well as homologs and orthologs thereof.

[00288] In some embodiments, the analyte binding moiety can bind to a chemokine including and without limitation XCL1, XCL2, CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CXCL1, CXCL2, CXCL3, CXCL4,

CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CX3CL1, as well as homologs and orthologs thereof.

[00289] In some embodiments, the analyte binding moiety can bind to a vascular growth factor including and without limitation VEGF-A, VEGF-B, VEGF-C and VEGF-D, as well as homologs and orthologs thereof.

[00290] In some embodiments, the analyte binding moiety can bind to a platelet derived growth factor including and without limitation PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and PDGF-AB, as well as homologs and orthologs thereof.

[00291] In some embodiments, the analyte binding moiety can bind to a cytokine including and without limitation erythropoietin (EPO), M-CSF (macrophage colony-stimulating factor) and GM-CSF (granulocyte-macrophage colony-stimulating factor), as well as homologs and orthologs thereof.

[00292] In some embodiments, the analyte binding moiety can bind to a peptide hormone. In some embodiments, the peptide hormone comprises a human peptide hormone. In some embodiments, the analyte binding moiety can bind to a peptide hormone including and without limitation adrenocorticotrophic hormone (ACTH), adropin, amylin, angiotensin, anti-Müllerian hormone (AMH), atrial natriuretic peptide (ANP), calcitonin, cholecystikinin (CCK), chorionic somatomammotropin hormone 1 (CSH1), chorionic somatomammotropin hormone 2 (CSH2), corticotropin releasing hormone (CRH), gastrin, ghrelin, glucagon, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), gonadotropin releasing hormone 1 (GNRH1), gonadotropin releasing hormone 2 (GNRH2), growth hormone, growth hormone releasing hormone (GHRH), follicle-stimulating hormone (FSH), insulin, leptin, luteinizing hormone (LH), melanocyte-stimulating hormone (MSH), oxytocin, parathyroid hormone (PTH), parathyroid hormone like hormone (PTH LH), pro-melanin concentrating hormone (PMCH), prolactin, renin, resistin (RETN), somatostatin, somatotropin (GH1), thyroid-stimulating hormone (TSH), thyrotropin-releasing hormone (TRH), vasopressin and vasoactive intestinal peptide (VIP), as well as homologs and orthologs thereof.

[00293] In some embodiments, the analyte binding moiety may comprise an antibody or antibody fragment, including, without limitation, a single-chain antibody, a minibody, a single chain variable fragment (scFv), Fab fragment, F(ab)₂ fragment, F(ab')₂ fragment, a domain antibody, a VHH antibody, one or more isolated complementarity-determining regions (CDRs), an antigen binding domain of a T-cell receptor or synthetic T-cell receptor, and the like.

[00294] In some embodiments, the analyte binding moiety comprises a lectin, including but not limited to concavalin A, wheat germ agglutinin, or other lectins as are known in the art.

[00295] In some embodiments, the analyte binding moiety comprises a hemagglutinin or other phage or viral cell surface or cell binding protein.

[00296] In some embodiments, the analyte binding moiety comprises a peptide hormone or peptide signaling molecule, including but not limited to endocrine or paracrine signaling peptides and bacterial signaling peptides.

[00297] In some embodiments, the analyte binding moiety comprises a surface binding peptide such as an amphipathic peptide or antimicrobial peptide, examples of which include but are not limited to melittin, colicin, antibiotic antimicrobials, brevinin, esculentin, ranacyclin, and the like.

[00298] In some embodiments, the analyte binding moiety comprises a polynucleotide comprising a sequence that is complementary to a target analyte comprising a nucleic acid.

[00299] The target analyte can comprise molecules from any source including molecules borne in air, water, soil or food. In some embodiments, the target analyte can be isolated from any organism including viruses, fungi, prokaryotes or eukaryotes. In some embodiments, the target analyte can be isolated from any organism including human, simian, ape, canine, feline, bovine, equine, murine, porcine, caprine, lupine, ranine, piscine, plant, insect or bacteria. In some embodiments, the target analyte can be isolated from any biological fluid, including blood, urine, serum, lymph, tumor, saliva, anal secretions, vaginal secretions, amniotic samples, perspiration, semen, environmental samples or culture samples. In some embodiments, the target analyte can be isolated 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. In some embodiments, the target analyte comprises naturally-occurring molecules, recombinant molecules, modified molecules and/or synthetic molecules. In some embodiments, the target analyte can be prepared using recombinant nucleic acid technology including but not limited to any combination of vector cloning, transgenic host cell preparation, host cell culturing and/or PCR amplification.

Sequencing Polymerases

[00300] 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 template molecule (*e.g.*, a modified oligonucleotide). 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 template molecule (*e.g.*, a modified oligonucleotide). In some embodiments, the plurality of sequencing polymerases comprise recombinant mutant polymerases.

[00301] 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 altiarthaeales* archaeon; *Candidatus Hadarchaeum Yellowstoneense*; *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*, *Archaeoglobus*, *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. The phi29 DNA polymerase can be wild type phi29 DNA polymerase (*e.g.*, MagniPhi™ from Expedeon™), or variant EquiPhi29™ DNA polymerase (*e.g.*, from Thermo Fisher Scientific®), or chimeric QualiPhi™ DNA polymerase (*e.g.*, from 4basebio™). Additional polymerases are described in U.S. Patent No. 11,859,241, the contents of which are incorporated by reference in their entirety herein.

Nucleotides and Chain-Terminating Nucleotides

[00302] 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.

[00303] 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 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.

[00304] 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 phosphoramidate 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.

[00305] 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) ($\text{Pd}(\text{PPh}_3)_4$) 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 H_2 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 (K_2CO_3) 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.

[00306] 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(hydroxypropyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[00307] 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'-sulfhydryl, 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.

[00308] 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.

[00309] In some embodiments, the nucleotide comprises a cleavable linker on the nucleotide base. 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) ($\text{Pd(PPh}_3)_4$) 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 H_2 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 (K_2CO_3) 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.

[00310] 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(hydroxypropyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[00311] 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

[00312] 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.

[00313] 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. 1). 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. 5. Exemplary multivalent molecules are shown in FIGS. 1-4. An exemplary spacer is shown in FIG. 6 (top) and exemplary linkers are shown in FIG. 6 (bottom) and FIG. 7. Exemplary nucleotides attached to a linker are shown in FIGS. 8-11. An exemplary biotinylated nucleotide arm is shown in FIG. 12.

[00314] 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.

[00315] 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 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.

[00316] 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 phosphoramidate 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.

[00317] 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) ($\text{Pd(PPh}_3)_4$) 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 H_2 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 (K_2CO_3) 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.

[00318] 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-TTP) or Tri(hydroxypropyl)phosphine (THPP). In some embodiments, the cleaving agent comprises 4-dimethylaminopyridine (4-DMAP).

[00319] 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'-sulfhydryl, 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.

[00320] 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.

[00321] 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.

[00322] 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™.

[00323] 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 template molecule (*e.g.*, a modified oligonucleotide) duplexed with a primer, and a nucleotide, or the binding complex comprises (ii) a polymerase, a template molecule (*e.g.*, a modified oligonucleotide) 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. In some embodiments, 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, 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 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 cellular sample 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.

Multivalent Probes Comprising Target-Specific Oligonucleotide Probes

[00324] In some embodiments, in any of the compositions or methods described herein, the sequencing can employ at least one multivalent probe comprising a core attached to a plurality of probe arms wherein individual probe arms comprises a polymer arm linked to a target-specific oligonucleotide probe (*e.g.*, FIGS. 5A-5B). In some embodiments, the multivalent probes can be used to conduct a sequencing-by-hybridization method.

[00325] In some embodiments in the method for sequencing-by-hybridization, the multivalent probe comprises: (1) a core; and (2) a plurality of probe arms which comprise (i) a core attachment moiety, (ii) a spacer comprising a PEG moiety, (iii) a linker, and (iv) a target-specific oligonucleotide probe. In some embodiments, the core is attached to the plurality of probe arms, wherein the spacer is attached to the linker, wherein the linker is attached to the target-specific probe. In some embodiments, the linker is attached to the target-specific probe through the base of one of the nucleotides in the oligonucleotide probe. 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.

[00326] In some embodiments, the target-specific probes can be linked to the end of a probe arm or the target-specific probes can be linked to an internal region of the probe arm. In some embodiments, the structure of a multivalent probe is shown in FIG. 5A. In some embodiments, the structure of a probe arm comprising a target-specific oligonucleotide probe is shown in FIG. 5B.

[00327] In some embodiments, the spacer comprises the structure shown in FIG. 6 (top). In some embodiments, the linker comprises any of the structures shown in FIG. 6 (bottom) and FIG. 7.

[00328] In some embodiments, the core of a multivalent probe 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™.

[00329] In some embodiments, individual multivalent probes comprise a core linked to a plurality of probe arms wherein each probe arm comprises the same target-specific sequence. For example, the core can be linked to 2, 3, 4, 5, 6, 7, 8, 9, 10 or more probe arms wherein each probe arm comprises the same target-specific sequence. In some embodiments, the probe arms comprising the same target-specific sequences can selectively hybridize to the same target sequence at multiple regions on the same template molecule (*e.g.*, a modified oligonucleotide) or can selectively hybridize to the same target sequence on different template molecules (*e.g.*, modified oligonucleotides).

[00330] In some embodiments, individual multivalent probes comprise a core linked to a plurality of probe arms wherein individual probe arms comprise different target-specific sequences. For example, the core can be linked to probe arms having 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different target-specific sequences. In some embodiments, the probe arms comprising different target-specific sequences can selectively hybridize to different target

sequences on the same template molecule or can selectively hybridize to different target sequences on different template molecules.

[00331] In some embodiments, a target-specific probe of a multivalent probe comprises an oligonucleotide that is 10-20 nucleotides in length, 20-30 nucleotides in length, 30-40 nucleotides in length, 40-50 nucleotides in length, 50-60 nucleotides in length, 60-70 nucleotides in length, 70-80 nucleotides in length, 80-90 nucleotides in length, 90-100 nucleotides in length, any range therebetween, or longer.

[00332] In some embodiments, individual multivalent probes can be labeled with a detectably reporter moiety. In some embodiments, the detectable reporter moiety comprises a fluorophore. In some embodiments, the core of the multivalent probe can be labeled with at least one fluorophore, wherein the fluorophore which is attached to a given core of the multivalent probe corresponds to a particular target-specific probe sequence of the probe arms. In some embodiments, the linker of the multivalent probe can be labeled with at least one fluorophore, wherein the fluorophore which is attached to a given linker of the multivalent probe corresponds to a particular target-specific probe sequence of the probe arms. In some embodiments, the spacer of the multivalent probe can be labeled with at least one fluorophore, wherein the fluorophore which is attached to a given spacer of the multivalent probe corresponds to a particular target-specific probe sequence of the probe arms. In some embodiments, the target-specific oligonucleotide of the multivalent probe can be labeled with at least one fluorophore, wherein the fluorophore which is attached to a given target-specific oligonucleotide of the multivalent probe corresponds to a particular target-specific probe sequence of the probe arms.

[00333] In some embodiments, the sequencing comprises: **(1)** contacting a plurality of template molecules (*e.g.*, modified oligonucleotides) with a plurality of first detectably labeled multivalent probes (*e.g.*, first fluorophore-labeled multivalent probes) under a condition suitable for selectively hybridizing a target-specific probe of individual first detectably labeled multivalent probes to a first target sequence region of a template molecule, thereby forming a first plurality of detectably labeled template-probe complexes, wherein the target-specific probes of the first detectably labeled multivalent probes comprise a first probe sequence; **(2)** detecting and imaging the fluorescent signal and color emitted by the first plurality of detectably labeled multivalent probes that are selectively hybridized to the first target sequence region of the template molecules (*e.g.*, first plurality of template-probe complexes); **(3)** removing the first plurality of detectably labeled multivalent probes from the first template-probe complexes and retaining the template molecules; **(4)** contacting the

template molecules retained at step (3) with a plurality of second detectably labeled multivalent probes (*e.g.*, second fluorophore-labeled multivalent probes) under a condition suitable for selectively hybridizing a target-specific probe of individual second detectably labeled multivalent probes to a second target sequence region of the same template molecule, thereby forming a second plurality of detectably labeled template-probe complexes, wherein the target-specific probes of the second detectably labeled multivalent probes comprise a second probe sequence; and **(5)** detecting and imaging the fluorescent signal and color emitted by the second plurality of detectably labeled multivalent probes that are selectively hybridized to the second target sequence of the template molecules (*e.g.*, second plurality of template-probe complexes). In some embodiments, the plurality of template molecules are on and/or inside a cellular sample.

[00334] In some embodiments, steps (4) – (5) can be repeated at least once. In some embodiments, repeating steps (4)-(5) comprises contacting the template molecules with a plurality of third detectably labeled multivalent probes under a condition suitable for selectively hybridizing a target-specific probe of individual third detectably labeled multivalent probes to a third target sequence region of the same template molecule, thereby forming a third plurality of detectably labeled template-probe complexes wherein the target-specific probes of the third detectably labeled multivalent probes comprise a third probe sequence. In some embodiments, any of the first, second and third target sequence regions of the template molecule overlap or do not overlap. In some embodiments, the first, second and third probe sequences are not the same.

[00335] In some embodiments, a sequencing-by-hybridization cycle comprises steps (1) – (2). In some embodiments, a sequencing-by-hybridization cycle comprises steps (1) – (3).

[00336] In some embodiments, images of sequential sequencing-by-hybridization cycles using the multivalent probes can be used to decode the target sequence of the template molecules on and/or inside the cellular sample.

[00337] In some embodiments, in a given sequencing-by-hybridization cycle, the template molecules can be contacted with one type of detectably labeled multivalent probes comprising the same target-specific probe sequence.

[00338] In some embodiments, in a given sequencing-by-hybridization cycle, the template molecules can be contacted with a mixture of different types of detectably labeled multivalent probes where individual multivalent probes carry a different target-specific probe sequence which can selectively hybridize to their cognate target sequence.

[00339] In some embodiments, in a given sequencing-by-hybridization cycle, the mixture of different types of detectably labeled multivalent probes can selectively hybridize to different target sequences on the same template molecule.

[00340] In some embodiments, in a given sequencing-by-hybridization cycle, the mixture of different types of detectably labeled multivalent probes can selectively hybridize to different target sequences on different template molecules.

[00341] In some embodiments, at least two sequencing-by-hybridization cycles can be conducted as described herein employing the plurality of detectably labeled multivalent probes comprising target-specific probes.

[00342] In some embodiments, 2 - 50 sequencing-by-hybridization cycles can be conducted as described herein employing the plurality of detectably labeled multivalent probes comprising target-specific probes.

Flow cells

[00343] In some embodiments, in any of the compositions or methods described herein, the cellular sample can be deposited onto a solid support (*e.g.*, a flowcell). In some embodiments, the cellular sample is deposited onto a flowcell having walls (*e.g.*, top or first wall, and bottom or second wall) and a gap in-between, where the gap can be filled with a fluid, where the flowcell is positioned in a fluorescence optical imaging system. The cellular sample has a thickness that may require using the imaging system to focus separately on the first and second surfaces of the flowcell, when using a traditional imaging system. For improved imaging of the sequencing reaction of the amplification-free probe complexes on and/or inside the cellular sample, the flowcell can be positioned in a high-performance fluorescence imaging system, which comprises two or more tube lenses which are designed to provide optimal imaging performance for the first and second surfaces of the flowcell at two or more fluorescence wavelengths. In some embodiments, the high-performance imaging system further comprises a focusing mechanism configured to refocus the optical system between acquiring images of the first and second surfaces of the flowcell. In some embodiments, the high-performance imaging system is configured to image two or more fields-of-view on at least one of the first flowcell surface or the second flowcell surface.

Automated Mode

[00344] Any combination of the steps of the methods described herein can be performed in an automated mode using a fluid dispensing system known in the art, including cell

deposition (*e.g.*, cell seeding), culturing the cellular sample on the flowcell, cell fixation, cell permeabilization, reverse transcription reactions, contacting the cellular samples with any of the amplification-free probe complexes described herein, and sequencing.

[00345] In some embodiments, the present disclosure provides apparatuses and methods for growing/culturing a cellular sample on a flowcell and conducting nucleic acid workflows of the cultured cellular sample on the flowcell.

[00346] In some embodiments, the cellular sample can be deposited on the flowcell, where the flowcell can be coated with a reagent that promotes cell adhesion to the flowcell. The flowcell, having a cell sample adhered thereon, can be placed onto a sequencing apparatus having a flowcell holder/cradle which is fluidically connected to an automated fluid dispensing system and configured on a fluorescent microscope. In some embodiments, the sequencing apparatus can be configured with at least one fluidic delivery device, at least one fluidics device (*e.g.*, a microfluidics device), at least one imaging device and/or at least one sensor to detect signals from the sequencing reactions.

[00347] In some embodiments, the automated fluid dispensing system can be used to deliver simple and/or complex cell culture media to the cellular sample on the flowcell. In some embodiments, the cellular sample can be cultured/expanded on the flowcell for 2-10 generations (*e.g.*, cell passages) or more. In some embodiments, the cellular sample can be expanded to confluence or non-confluence.

[00348] In some embodiments, the automated fluid dispensing system can be used to deliver fixation reagents to the expanded cellular sample on the flowcell, and the cellular sample can be incubated under conditions suitable for cell fixation.

[00349] In some embodiments, the automated fluid dispensing system can be used to deliver permeabilization reagents to the fixed cellular sample on the flowcell, and the cellular sample can be incubated under conditions suitable for cell permeabilization.

[00350] In some embodiments, the automated fluid dispensing system can be used to deliver reagents for conducting reverse transcription of RNA inside the fixed and permeabilized cellular sample under a condition suitable for generating a plurality of cDNA inside the cellular sample. In some embodiments, the reverse transcriptase reaction can be omitted.

[00351] In some embodiments, the automated fluid dispensing system can be used to deliver reagents for hybridizing the amplification-free probe complexes to the cellular sample.

[00352] In some embodiments, the automated fluid dispensing system can be used to deliver reagents for conducting rolling circle amplification under a condition suitable for generating a plurality of concatemer molecules inside the cellular sample.

[00353] In some embodiments, the automated fluid dispensing system can be used to deliver sequencing reagents for conducting one or more sequencing cycles of the template molecules or modified oligonucleotides described herein under a condition suitable for generating a plurality of sequencing read products inside the cellular sample. In some embodiments, individual cycle times can be achieved in less than 30 minutes. In some embodiments, the field of view (FOV) can exceed 1 mm² and the cycle time for scanning large area (> 10 mm²) can be less than 5 minutes.

[00354] In some embodiments, the automated fluid dispensing system can be used to deliver reagents for removing the plurality of sequencing read products from the template molecules and retaining the template molecules on or inside the cellular sample.

[00355] In some embodiments, the automated fluid dispensing system can be used to deliver sequencing reagents for conducting one or more sequencing cycles of the template molecules under a condition suitable for generating another plurality of sequencing read products inside the cellular sample (*e.g.*, separate batches of sequencing or re-iterative sequencing). In some embodiments, individual cycle times can be achieved in less than 30 minutes. In some embodiments, the field of view (FOV) can exceed 1 mm² and the cycle time for scanning large area (> 10 mm²) can be less than 5 minutes.

INCORPORATION BY REFERENCE

[00356] Throughout this application various publications, patents, and/or patent applications are referenced. The disclosures of the publications, patents and/or patent applications are hereby incorporated by reference in their entireties into this application in order to more fully describe the state of the art to which this disclosure pertains.

EQUIVALENTS

[00357] The details of one or more embodiments of the disclosure are set forth in the accompanying description above. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, the preferred methods and materials are now described. Other features, objects, and advantages of the disclosure will be apparent from the description and from the claims. In the specification and the appended claims, the singular forms include plural referents unless the

context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. All patents and publications cited in this specification are incorporated by reference.

CLAIMS**WHAT IS CLAIMED IS:**

1. An amplification-free probe complex comprising:
 - (i) at least one modified oligonucleotide; and
 - (ii) at least one analyte binding moiety which can bind a target analyte, wherein the at least one modified oligonucleotide is attached to the at least one analyte binding moiety,
wherein the at least one modified oligonucleotide comprises two or more tandem copies of a sequencing primer binding site,
wherein the at least one modified oligonucleotide comprises canonical nucleobases located at a position that is immediately 5' of the 5' ends of individual sequencing primer binding sites, and
wherein the at least one modified oligonucleotide comprises an abasic site that is located immediately 5' of the canonical nucleobase.
2. The amplification-free probe complex of claim 1, wherein the canonical nucleobases are located between 1 and 10 nucleotides 5' of the 5' ends of the individual sequencing primer binding sites.
3. The amplification-free probe complex of claim 1, wherein the canonical nucleobases are 5' and adjacent to the 5' ends of the individual sequencing primer binding sites.
4. An amplification-free probe complex comprising:
 - (i) at least one modified oligonucleotide; and
 - (ii) at least one analyte binding moiety which can bind a target analyte, wherein the at least one modified oligonucleotide is attached to the at least one analyte binding moiety,
wherein the at least one modified oligonucleotide comprises at least one sequencing primer binding site and a barcode sequence.
5. The amplification-free probe complex of claim 4, wherein the at least one modified oligonucleotide comprises two or more tandem copies of the primer binding site and the barcode sequence.

6. The amplification-free probe complex of any one of claims 1-5, wherein the target analyte comprises a lipid, polypeptide, nucleic acid or polysaccharide.
7. The amplification-free probe complex of any one of claims 1-5, wherein the analyte binding moiety comprises a lipid moiety, a wheat germ agglutinin, an antibody or a biotin moiety.
8. The amplification-free probe complex of any one of claims 1-7, wherein the target analyte is inside a cellular sample and/or on the surface of a cellular sample.
9. The amplification-free probe complex of claim 8, wherein the cellular sample comprises a whole single cell, a plurality of whole cells, an intact tissue, sectioned cell or a sectioned tissue sample.
10. The amplification-free probe complex of claim 8, wherein the cellular sample comprises a fresh cellular sample, a freshly-frozen cellular sample, or a formalin-fixed and paraffin-embedded (FFPE) cellular sample.
11. The amplification-free probe complex of claim 8, wherein the cellular sample comprises a fixed and permeabilized cellular sample.
12. The amplification-free probe complex of any one of claims 1-11, wherein the analyte binding moiety comprises a secondary antibody or a primary antibody.
13. A method for detecting a target analyte, comprising:
 - a) providing a plurality of the amplification-free probe complexes of any one of claims 1-3 or 6-12;
 - b) providing a sample comprising a plurality of analytes, including at least one target analyte, wherein the sample is deposited on a support;
 - c) contacting the sample with the plurality of amplification-free probe complexes, wherein the contacting is conducted under a condition suitable for binding the analyte binding moieties to the target analytes;
 - d) contacting the plurality of modified oligonucleotides of the plurality of amplification-free probe complexes of step (c) with a plurality of sequencing

primers under a condition suitable for individual sequencing primers to bind the sequencing primer binding sites on the modified oligonucleotides to form a plurality of nucleic acid duplexes, wherein individual nucleic acid duplexes comprise a sequencing primer binding site on a modified oligonucleotide hybridized to a sequencing primer;

- e) contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases and a plurality of detectably labeled multivalent molecules, wherein individual nucleic acid duplexes bind a sequencing polymerase and a nucleotide moiety of a multivalent molecule, thereby forming a plurality of detectably labeled complexes, wherein a nucleotide moiety of a detectably labeled multivalent molecule is bound to the 3' end of the sequencing primer and opposite the canonical nucleobase in the modified oligonucleotide, wherein the contacting of step (e) is conducted under a condition suitable for inhibiting polymerase-catalyzed incorporation of the nucleotide moiety into the 3' end of the sequencing primer, and wherein the nucleotide moiety of the multivalent molecule binds opposite and is complementary to a nucleotide in the modified oligonucleotide, thereby producing a plurality of detectably labeled complexes; and
- f) imaging the sample which is bound to the plurality of detectably labeled complexes thereby detecting a target analyte on a cellular sample or inside a cellular sample.

14. A method for detecting a target analyte, comprising:

- a) providing a plurality of the amplification-free probe complexes of any one of claims 4-12;
- b) providing a sample comprising a plurality of analytes, including at least one target analyte, wherein the sample is deposited on a support;
- c) contacting the sample with the plurality of amplification-free probe complexes, wherein the contacting is conducted under a condition suitable for binding the analyte binding moieties to the target analyte;
- d) contacting the plurality of modified oligonucleotides of the plurality of amplification-free probe complexes of step (c) with a plurality of sequencing primers under a condition suitable for individual sequencing primers to bind the sequencing primer binding sites on the modified oligonucleotides to form a

plurality of nucleic acid duplexes, wherein individual nucleic acid duplexes comprise a sequencing primer binding site on the modified oligonucleotide hybridized to a sequencing primer;

- e) contacting the plurality nucleic acid duplexes with a plurality of sequencing polymerases and a plurality of detectably labeled multivalent molecules, wherein individual nucleic acid duplexes bind a sequencing polymerase and a nucleotide moiety of a multivalent molecule, thereby forming a plurality of detectably labeled complexes, wherein the nucleotide moiety of a detectably labeled multivalent molecule is bound to the 3' end of the sequencing primer and opposite the barcode sequence in the modified oligonucleotide, and wherein the nucleotide moiety of the multivalent molecule binds opposite and is complementary to a nucleotide in the modified oligonucleotide, thereby producing a plurality of detectably labeled complexes; and
 - f) imaging the sample, thereby detecting the target analyte.
15. The method of claim 14, wherein the contacting of step (e) is conducted under a condition suitable for inhibiting polymerase-catalyzed incorporation of the nucleotide moiety into the 3' end of the sequencing primer.
16. The method of any one of claims 13-15, wherein individual multivalent molecules comprise a core attached to a plurality of nucleotide-arms, wherein each nucleotide-arm comprises a nucleotide moiety.
17. The method of any one of claims 13-15, wherein the complementary nucleotide moiety of the multivalent molecule does not incorporate into the terminal 3' end of the sequencing primer.
18. The method of any one of claims 13-17, wherein the nucleotide moiety of the multivalent molecule is complementary to a nucleotide in the modified oligonucleotide that is 5' and adjacent to the sequencing primer binding site.
19. The method of any one of claims 13-18, wherein the sample is a cellular sample.

20. The method of claim 19, wherein the target analyte is on the cellular sample and/or inside the cellular sample.
21. The method of claim 19 or 20, wherein the cellular sample comprises a whole single cell, a plurality of whole cells, an intact tissue, sectioned cell or a sectioned tissue sample.
22. The method of claim 19 or 20, wherein the cellular sample comprises a fresh cellular sample, a freshly-frozen cellular sample, or a formalin-fixed and paraffin-embedded (FFPE) cellular sample.
23. The method of claim 19 or 20, wherein the cellular sample comprises a fixed and permeabilized cellular sample.