SIMULTANEOUS QUANTIFICATION OF GENE EXPRESSION IN A USER-DEFINED REGION OF A CROSS-SECTIONED TISSUE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is claims priority to and the benefit of U.S. Provisional Application No. 62/193,809, filed July 17, 2015; U.S. Provisional Application No. 62/261,657, filed December 1, 2015; U.S. Provisional Application No. 62/277,289, filed January 11, 2016; and U.S. Provisional Application No. 62/323,023, filed April 15, 2016. Each of the above-mentioned applications is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Standard immunohistochemical and *in situ* hybridization methods allow for simultaneous detection of, at most, six to ten protein or nucleic acid targets, with three to four targets being typical. There exists a need for probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell.

SUMMARY

[0003] The present disclosure relates to probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell.

[0004] An example of the present disclosure relates to a method including steps of (1) contacting at least one target in or from at least one cell in a tissue sample with at least one probe comprising a target-binding domain and a signal oligonucleotide; (2) providing a force to a location of the tissue sample sufficient to release the signal oligonucleotide; and (3) collecting and identifying the released signal oligonucleotide, thereby detecting the at least one target in or from a specific location of the tissue sample that was provided the force. The specific location is a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell. The

target may be a nucleic acid, (*e.g.,* mRNA and miRNA) and/or a protein. The target-binding domain may be a single-stranded nucleic acid, a partially double-stranded nucleic acid, or a protein-binding molecule, *e.g.*, an antibody, a peptide, an aptamer, and a peptoid. In examples, two or more targets (*i.e.*, proteins, nucleic acids, and a combination thereof) are detected. In examples, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more targets, and any number therebetween, are detected; for example, 800 or more different targets can be detected. In examples, detecting includes quantifying the abundance of each target.

[0004a] In one aspect, the present disclosure refers to a method of producing a spatially-resolved profile of nucleic acid abundance in a tissue sample comprising: a) contacting the tissue sample with a solution comprising at least 15 species of probes, the probes comprising a target-binding domain, a signal oligonucleotide, and a photo-cleavable motif located between the target-binding domain and the signal oligonucleotide, wherein each species of probe comprises a unique nucleic acid target-binding domain that hybridizes to one of the at least 15 target nucleic acids and a unique signal oligonucleotide specific for the target nucleic acid; b) illuminating a first location of the tissue sample with light sufficient to release signal oligonucleotides at the first location; c) collecting the signal oligonucleotides released in step (b) from the solution proximal to the first location; d) identifying and quantifying via multiplexed immunohistochemistry the signal oligonucleotides collected in step (c), thereby determining the abundance of the at least 15 target nucleic acids in the first location; e) illuminating at least a second location of the tissue sample with light sufficient to release the signal oligonucleotides at the at least second location; f) collecting the signal oligonucleotides released in step (e) from the solution proximal to the at least second location; g) identifying and quantifying via multiplexed immunohistochemistry the signal oligonucleotides collected in step (f), thereby determining the abundance of the at least 15 target nucleic acids in the at least second location; and h) comparing the abundance of the at least 15 target nucleic acids in the first location of the tissue sample to the abundance of the at least 15 target nucleic acids in the at least second location of the tissue sample, thereby generating a spatially-resolved profile of nucleic acid abundance in the tissue sample.

[0005] In examples, the method further includes repeating at least steps (2) and (3) on at least a second specific location of the tissue sample, the second specific location comprising at least a second cell. In examples, detecting includes comparing the abundance of the at least one target in or from the first specific location and in or from the at least second specific location. The at least one cell and at least second cell may be the same cell type or distinct cell types. In some examples, detecting includes quantifying the abundance of the at least one target in or from a first cell type and in or from the at least a second cell type. In examples, first and second cell types are independently selected from a normal cell and an abnormal cell, *e.g.*, a diseased and cancerous cell.

[0006] In examples, the at least one cell is directly immobilized to a surface or is indirectly immobilized to the surface *via* at least one other cell. A tissue sample may be a 2 to 1000 μm thick tissue section, *e.g.*, obtained from a formalin-fixed paraffin embedded (FFPE) sample or from an unfixed sample. The at least one cell may be fixed or unfixed. The at least one cell may be stained or labeled prior to step (2) allowing visualization of a subcellular or cellular structure in the stained or labeled cell. Alternately, for tissue sections, a section adjacent to the section that is contacted with the probes may be stained or labeled prior to step (2), thereby allowing estimation of a subcellular, cellular, or tissue-related structure in the corresponding cell or nearby cell in the section that is contacted with the probes. Such staining or labeling techniques are well known in the art.

[0007] In the above example, at least one probe further includes a linker (*e.g.*, a cleavable linker) located between the target-binding domain and the signal oligonucleotide. The cleavable linker may be photo-cleavable, which is cleaved by light provided by a suitable coherent light source (*e.g.*, a laser and a UV light source) or a suitable incoherent light source (*e.g.*, an arc-lamp and a

light-emitting diode (LED)). The light source may irradiate at least one subcellular structure of the at least one cell and the abundance of the at least one nucleic acid target in or from the at least one subcellular structure of the at least one cell can be detected. Also, the light source may first irradiate at least one subcellular structure in the at least one cell and later irradiate at least one subcellular structure in the at least second cell, allowing a comparison of the abundance of the at least one target in or from the at least one subcellular structure in the at least one cell and the at least one subcellular structure in the at least second cell.

[0008] In examples, the signal oligonucleotide is a single-stranded nucleic acid or a partially double-stranded nucleic acid.

[0009] In examples, the sample may be cultured cells or dissociated cells (fixed or unfixed) that have been immobilized onto a slide. The sample may comprise cells (including both primary cells and cultured cell lines) and/or tissues (including cultured or explanted). The sample may comprise a cultured cell, a primary cell, or a dissociated cell from an explant.

[0010] In examples, the illumination of a region of interest smaller that a field of view (for example a single cell or a subcellular structure within a cell) comprises use of a laser scanning device (e.g., confocal) or a digital mirror device (DMD) to direct the light.

[0011] In examples, a probe is prepared by a cysteine bioconjugation method that is stable, site-specific to, preferably, the antibody's hinge-region heavy-chain. In examples, a probe can comprise a plurality (i.e., more than one, e.g., 2, 3, 4, 5, or more) labeled oligonucleotides per antibody.

[0012] Detecting comprises a polymerase reaction, a reverse transcriptase reaction, hybridization to an oligonucleotide microarray, mass spectrometry, hybridization to a fluorescent molecular beacon, a sequencing reaction, or nCounter® Molecular Barcodes. In preferred examples, nCounter® systems and methods from NanoString Technologies® are used.

[0013] In examples, the signal oligonucleotide is collected from a tissue *via* liquid laminar, turbulent, or transitional flow. The flow may be *via* a channel, *e.g.*, having 25 to 500 μm depth between the tissue and a fluidic device or impermeable barrier placed over the tissue.

[0014] In examples, the signal oligonucleotide is collected from a solution proximal to, e.g., at least immediately above, the at least one cell. The proximal solution may be collected by aspirating, e.g., via a pipette, a capillary tube, a microarray pin, a flow cell comprising holes, or another suitable aspirating system known in the art or any combination thereof. The capillary

tube may comprise an optical device capable of transmitting a light force, e.g., UV light, to the at least one cell. The pipette or a microarray pin may be attached to an array comprising a plurality of pipettes or microarray pins. The proximal solution may comprise an anionic polymer, e.g., dextran sulfate, and/or salmon sperm DNA and/or the collected signal oligonucleotide may be added to a solution comprising an anionic polymer, e.g., dextran sulfate, and/or salmon sperm DNA. Other non-specific blocking agents known in the art in addition to or instead of salmon sperm DNA may be used.

[0015] In examples, the method provides simultaneous spatially-resolved DNA, RNA, and/or protein detection of a tissue sample.

[0016] In examples, digital readout comprises a linear dynamic range of greater than or equal to 5 logs.

[0017] In examples, probes are provided to a sample at concentrations typically less than that used for immunohistochemistry (IHC) or for in situ hybridization (ISH). Alternately, the concentration may be significantly less than that used for IHC or ISH. For example, the probe concentration may be 2 fold less, 5 fold less, 10 fold less, 20 fold less, 25 fold less, 30 fold less, 50 fold less, 60 fold less, 70 fold less, 80 fold less, 90 fold less, 100 fold less, 200 fold less, 300 fold less, 400 fold less, 500 fold less, 600 fold less, 700 fold less, 800 fold less, 900 fold less, 1000 fold less, 2000 fold less, or less and any number in between. In examples, probes are provided at a concentration of 100 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 9 nM, 8 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, 0.1 nM, 0.09 nM, 0.08 nM, 0.07 nM, 0.06 nM, 0.05 nM, 0.04 nM, 0.03 nM, 0.02 nM, 0.01 nM, and less and any concentration in between.

[0018] In examples, a tissue sample is attached to a slide and is first imaged using fluorescence (e.g., fluorescently-labeled antibodies and fluorescent stains (e.g., DAPI)) and then expression of proteins and/or nucleic acids is digitally counted from the sample.

[0019] In examples, a negative purification, e.g., comprising an affinity purification method comprising contacting intact probe molecules with an immobilized oligonucleotide that is complementary to a portion of the intact probe or an immobilized antibody or protein-binding motif that recognizes and binds to a portion of the intact probe, is used to remove intact probe molecules from the released signal oligonucleotides. In examples, the intact probe's target binding domain comprises a universal purification tag or sequence that is partially

complementary to the immobilized oligonucleotide or is capable of being recognized or bound by the immobilized antibody or protein-binding motif. Any such tag or sequence well-known in the art may be used in these examples.

[0020] Any example described herein can be combined with any other example as disclosed herein. While the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the disclosure, which is defined by the scope of the appended claims. Other examples, advantages, and modifications are within the scope of the following claims.

[0021] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0023] **Figure 1**: Shows two exemplary probes. Nucleic acid backbones (either single-stranded DNA or single-stranded RNA) are shown as a straight, black line. The probes each include a target-binding domain, shown in red. The top probe includes labeled RNA segments hybridized to the nucleic acid backbone whereas the bottom probe includes labeled DNA oligonucleotides hybridized to the nucleic acid backbone. A cleavable motif (*e.g.*, a cleavable linker, not shown) may be located between the backbone and a target-binding domain or within the backbone. The cleavable motif allows release of a signal oligonucleotide from a bound target nucleic acid or protein; then, the signal oligonucleotide is collected and detected.

[0024] **Figure 2**: Shows a first type of a probe that can bind directly to a target nucleic acid (top). In the below image, the probe has bound to the target nucleic acid, shown as a blue curvilinear line. In this figure and in later figures, a reporter probe includes six positions