

SIMULTANEOUS QUANTIFICATION OF A PLURALITY OF PROTEINS 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. 62/193,819, filed July 17, 2015; U.S. Provisional Application No. 62/261,654, filed December 1, 2015; U.S. Provisional Application No. 62/277,283, filed January 11, 2016; and U.S. Provisional Application No. 62/323,018, 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 methods allow for simultaneous detection of, at most, six to ten protein 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 expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell.

SUMMARY OF THE INVENTION

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

[0003a] In one aspect, the present invention provides a method comprising: (1) contacting at least one protein target in or from at least one cell in a sample with at least one probe comprising a target-binding domain and a signal oligonucleotide; (2) providing a force to a location of the sample sufficient to release the signal oligonucleotide; and (3) collecting and identifying the released signal oligonucleotide, thereby detecting the at least one protein target in or from a specific location of the sample that was provided the force.

[0004] Also disclosed herein is a method including steps of (1) contacting at least one protein 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 protein 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-binding domain comprises a protein-binding molecule, *e.g.*, an antibody, a peptide, an aptamer, and a peptoid. In examples, two or more

protein targets 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.

[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 protein 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 protein 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 protein 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 protein 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 than 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 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 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.