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DESCRIPTION

BACKGROUND

[0001] One of every four men will die of cancer. Further statistics from the American Cancer Society predict that one of every five women will suffer the same fate. Treatments are available for many cancers. However, success for most relies on early detection.

[0002] Cancer is now said to be a disease of the genome. Many oncologists and cancer researchers hope that advances in genomic analysis tools will provide early detection and a path to treatment. However, these tools are more prominent in research labs having not yet matured to the level of being readily available to the vast majority of oncologists. Improvements are needed.

[0003] It has been said that at the time of diagnosis, all cancer patients are mosaics. They are mosaics because they have at least two distinct genomes: the genome they were born with, and the genome that they unwillingly acquired via cancer. Furthermore, as tumors grow, distinct populations of cancer cells become apparent. Leading to even more complex mosaics within the tumor. This cancer cell heterogeneity often results in subpopulations of cells that respond differently to cancer therapies. The end result is often an initial positive response of one subpopulation of cells, resulting in the observation of the patient's tumor shrinking, only to be followed by regrowth of tumor tissue, and in some cases metastasis. Despite early detection of the tumor, an inability to identify the subpopulation of cells that are resistant to the treatment can result in loss of time needed to treat an aggressive cancer. This creates adverse consequences for the patient both emotionally and physically.

[0004] WO 2014/060483 disclosed methods and a product for optimising localised or spatial deletion of gene expression in a tissue sample.

[0005] There is a need for genomic tools that can distinguish subpopulations of cancer cells in tumors. The present disclosure addresses this need and provides other advantages as well.

BRIEF SUMMARY

[0006] The present disclosure provides a method for spatially tagging nucleic acids of a biological specimen. The method can include steps of (a) providing a solid support comprising a plurality of different nucleic acid probes that are randomly located on the solid support, wherein the different nucleic acid probes each includes a barcode sequence that is different from the barcode sequence of other randomly located probes on the solid support; (b) performing a nucleic acid detection reaction on the solid support to locate the barcode sequences on the solid support; (c) contacting a biological specimen with the solid support that

has the randomly located probes; (d) hybridizing the randomly located probes to target nucleic acids from portions of the biological specimen that are proximal to the randomly located probes; and (e) modifying the randomly located probes that are hybridized to the target nucleic acids, thereby producing modified probes that include the barcode sequences and a target specific modification, thereby spatially tagging the nucleic acids of the biological specimen.

[0007] This disclosure further provides a method for spatially tagging nucleic acids of a biological specimen, the method including steps of (a) attaching different nucleic acid probes to a solid support to produce randomly located probes on the solid support, wherein the different nucleic acid probes each includes a barcode sequence, and wherein each of the randomly located probes includes different barcode sequences from other randomly located probes on the solid support; (b) performing a nucleic acid detection reaction on the solid support to determine the barcode sequences of the randomly located probes on the solid support; (c) contacting a biological specimen with the solid support that has the randomly located probes; (d) hybridizing the randomly located probes to target nucleic acids from portions of the biological specimen that are proximal to the randomly located probes; and (e) extending the randomly located probes to produce extended probes that include the barcode sequences and sequences from the target nucleic acids, thereby spatially tagging the nucleic acids of the biological specimen.

[0008] Also provided is a method for spatially tagging nucleic acids of a biological specimen that includes the steps of (a) providing a plurality of nucleic acid primers attached to a solid support, wherein the nucleic acid primers in the plurality include a universal primer sequence that is common to the nucleic acid primers in the plurality; (b) binding a population of nucleic acid probes to the plurality of nucleic acid primers, wherein the nucleic acid probes include a universal primer binding sequence that hybridizes to the universal primer sequence, a target capture sequence and a barcode sequence that differs from barcode sequences of other nucleic acid probes in the population, thereby attaching the different nucleic acid probes at randomly located positions on the solid support; (c) amplifying the different nucleic acid probes by extension of the nucleic acid primers, thereby producing nucleic acid clusters having copies of the barcode sequence and target capture sequence at the randomly located positions on the solid support; (d) performing a sequencing reaction to determine the barcode sequences at the randomly located positions on the solid support; (e) contacting a biological specimen with the nucleic acid clusters on the solid support; (f) hybridizing the target capture sequences of the clusters to target nucleic acids from portions of the biological specimen that are proximal to the clusters; and (g) extending the target capture sequences to produce extended probes that include sequences from the target nucleic acids and the copies of the barcode sequences, thereby tagging the nucleic acids of the biological specimen.

[0009] The invention provides a method for spatially tagging nucleic acids of a biological specimen, the method including steps of (a) providing an array of beads on a solid support, wherein different nucleic acid probes are attached to different beads in the array, wherein the different nucleic acid probes each include a barcode sequence, wherein each bead includes a different barcode sequence from other beads on the solid support, and wherein each of the

different nucleic acid probes includes a target capture sequence; (b) performing a decoder probe hybridization reaction on the solid support to determine the barcode sequences at the randomly located probes on the solid support; (c) contacting a biological specimen with the array of beads; (d) hybridizing the different nucleic acid probes to target nucleic acids from portions of the biological specimen that are proximal to the beads; (e) extending the different nucleic acid probes to produce extended probes that include sequences from the target nucleic acids and the barcode sequences, thereby tagging the nucleic acids of the biological specimen; (f) removing the extended probes from the array of beads, optionally further comprising pooling the extended probes to form a mixture of the extended probes that have been removed from the array of beads; and (g) attaching the extended probes that have been removed from the solid support to a second solid support.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010]

Fig. 1 shows a diagrammatic representation of steps and reagents that can be used to generate barcoded oligo dT probes on an Illumina flow cell, create extended barcoded probes having mRNA sequences and releasing the extended probes from the flow cell.

Fig. 2 shows data indicating the availability of oligo dT capture sequences on probes after bridge amplification of the probes and restriction enzyme digest with BspH1 to remove one of the primer binding sites used for bridge amplification.

Fig. 3 shows sequencing metrics of the flow cell described in Example 1 and shown in Fig. 2.

Fig. 4 the number of unique barcodes determined in 21 tiles of the flow cell described in Example 1 and shown in Fig. 2.

Fig. 5 shows an image of cells captured on a patterned flow cell (Panel A) and cell count data (Panel B).

Fig. 6 shows cells that remain adhered to a flow cell in different conditions.

Fig. 7 shows a diagrammatic representation of steps and reagents used to create probes attached to a gel (Panel A), a diagrammatic representation of steps and reagents used to capture target nucleic acids using the gel-attached probes and fluorescently label the probes (Panel B), and an image created by the fluorescently labeled target nucleic acids following capture by the probes and removal of the tissue from the gel.

Fig. 8 shows a diagrammatic representation of steps and reagents used to capture target nucleic acids using BeadArray™ -attached probes and fluorescently label the probes (Panel A), and an image created by the fluorescently labeled target nucleic acids following capture by the probes and removal of the tissue from the BeadArray™.

DETAILED DESCRIPTION

[0011] The present disclosure provides compositions, apparatus and methods for preserving spatial information when performing multiplex nucleic acid analyses of biological specimens. A variety of tools are available for multiplex nucleic acid analyses including, for example, nucleic acid microarrays and so-called "next generation" sequencing platforms. Such tools allow for parallel detection of very large and complex collections of nucleic acids, including for example, DNA collections that represent all or nearly all of the genetic material of an organism (i.e. the 'genome'), RNA (or cDNA) collections that represent all or nearly all of the complement of expressed genes (i.e. the 'transcriptome') for an organism, and in some cases the collections can include several genomes and/or transcriptomes from several different organisms (e.g. a metabolome or biome from a community or ecosystem). Although these tools provide a vast amount of information about *what* nucleic acid sequences are present in a biological specimen being evaluated, they do not inherently distinguish *where* any particular nucleic acid resided in the biological specimen. Indeed the vast majority of samples applied to multiplex nucleic acid analysis tools are homogenates derived from mixtures of many different cells from a biological specimen. As a result, spatial information is lost and the results obtained from these tools constitute an average transcriptome or average genome for the specimen, important differences between individual cells being lost.

[0012] In particular embodiments, the present disclosure provides new and useful modifications to existing multiplex nucleic acid analysis tools to allow for the preservation of spatial information for biological specimens from which the nucleic acids are obtained. For example, solid supports that are usually used for multiplex sequencing-by-synthesis (SBS) techniques can be modified for use in capturing and spatially tagging nucleic acids from a biological specimen. In an alternative example, arrays of beads, such as those used for genotyping or gene expression analysis, can be used for capturing and spatially tagging nucleic acids from a biological specimen. As set forth in examples below, the solid supports used for an SBS or BeadArray™ platform commercialized by Illumina (San Diego, CA) can be modified for spatial tagging. However, it will be understood that any of a variety of solid supports can be made and used in accordance with the teaching herein. The spatially tagged nucleic acids can be removed from the solid support, pooled together and attached to a second solid support for detection in any of a variety of multiplex nucleic acid analysis systems including, for example, a sequencing platform or microarray platform set forth herein.

[0013] The spatial information provided by a method, composition or apparatus herein can include, for example, the location of one or more cells in a tissue (or other specimen) that has a particular allele at one or more locus (e.g. a genotype), has a particular structural variation in the genome (e.g. fusion, insertion, deletion, rearrangement etc.), has a particular epigenetic signature (e.g. methylation), expresses a particular gene, expresses a particular allele of a gene, expresses a particular splice variant of a gene or the like. In addition to identifying nucleic acids according to their spatial location in a biological specimen, a method, composition

or apparatus of the present disclosure can be used to quantify one or more nucleic acids according to spatial location. For example, the spatial information for one or more cells in a tissue (or other specimen) can include the amount of a particular allele or chromosomal region in a genome (e.g. ploidy); the amount of epigenetic modification of a genetic locus (e.g. methylation); expression level for a particular gene, allele or splice variant; or the like. The amounts can be absolute amounts or relative amounts in accordance with similar measurements obtained in the art for mixed or non-spatially tagged samples.

[0014] A method set forth herein can be used for localized detection of a nucleic acid in a biological specimen. In some embodiments, a method can be used for identifying or characterizing all of the transcriptome or genome of a biological specimen. Alternatively, a method can be used to identify or characterize only a part of a specimen's transcriptome or genome. A subset of transcripts or genes evaluated in a method herein can be related to a particular disease or condition.

[0015] A method set forth herein can be used for localized or spatial detection of nucleic acids, whether DNA or RNA, in a biological specimen. Thus one or more RNA or DNA molecules can be located with respect to its native position or location within a cell or tissue or other biological specimen. For example, one or more nucleic acids can be localized to a cell or group of adjacent cells, or type of cell, or to particular regions of areas within a tissue sample. The native location or position of individual RNA or DNA molecules can be determined using a method, apparatus or composition of the present disclosure.

[0016] Terms used herein will be understood to take on their ordinary meaning in the relevant art unless specified otherwise. Several terms used herein and their meanings are set forth below.

[0017] As used herein, the term "amplicon," when used in reference to a nucleic acid, means the product of copying the nucleic acid, wherein the product has a nucleotide sequence that is the same as or complementary to at least a portion of the nucleotide sequence of the nucleic acid. An amplicon can be produced by any of a variety of amplification methods that use the nucleic acid, or an amplicon thereof, as a template including, for example, polymerase extension, polymerase chain reaction (PCR), rolling circle amplification (RCA), multiple displacement amplification (MDA), ligation extension, or ligation chain reaction. An amplicon can be a nucleic acid molecule having a single copy of a particular nucleotide sequence (e.g. a PCR product) or multiple copies of the nucleotide sequence (e.g. a concatameric product of RCA). A first amplicon of a target nucleic acid is typically a complementary copy. Subsequent amplicons are copies that are created, after generation of the first amplicon, from the target nucleic acid or from the first amplicon. A subsequent amplicon can have a sequence that is substantially complementary to the target nucleic acid or substantially identical to the target nucleic acid.

[0018] As used herein, the term "array" refers to a population of features or sites that can be differentiated from each other according to relative location. Different molecules that are at

different sites of an array can be differentiated from each other according to the locations of the sites in the array. An individual site of an array can include one or more molecules of a particular type. For example, a site can include a single target nucleic acid molecule having a particular sequence or a site can include several nucleic acid molecules having the same sequence (and/or complementary sequence, thereof). The sites of an array can be different features located on the same substrate. Exemplary features include without limitation, wells in a substrate, beads (or other particles) in or on a substrate, projections from a substrate, ridges on a substrate or channels in a substrate. The sites of an array can be separate substrates each bearing a different molecule. Different molecules attached to separate substrates can be identified according to the locations of the substrates on a surface to which the substrates are associated or according to the locations of the substrates in a liquid or gel. Exemplary arrays in which separate substrates are located on a surface include, without limitation, those having beads in wells.

[0019] As used herein, the term "attached" refers to the state of two things being joined, fastened, adhered, connected or bound to each other. For example, an analyte, such as a nucleic acid, can be attached to a material, such as a gel or solid support, by a covalent or non-covalent bond. A covalent bond is characterized by the sharing of pairs of electrons between atoms. A non-covalent bond is a chemical bond that does not involve the sharing of pairs of electrons and can include, for example, hydrogen bonds, ionic bonds, van der Waals forces, hydrophilic interactions and hydrophobic interactions.

[0020] As used herein, the term "barcode sequence" is intended to mean a series of nucleotides in a nucleic acid that can be used to identify the nucleic acid, a characteristic of the nucleic acid, or a manipulation that has been carried out on the nucleic acid. The barcode sequence can be a naturally occurring sequence or a sequence that does not occur naturally in the organism from which the barcoded nucleic acid was obtained. A barcode sequence can be unique to a single nucleic acid species in a population or a barcode sequence can be shared by several different nucleic acid species in a population. For example, each nucleic acid probe in a population can include different barcode sequences from all other nucleic acid probes in the population. Alternatively, each nucleic acid probe in a population can include different barcode sequences from some or most other nucleic acid probes in a population. For example, each probe in a population can have a barcode that is present for several different probes in the population even though the probes with the common barcode differ from each other at other sequence regions along their length. In particular embodiments, one or more barcode sequences that are used with a biological specimen are not present in the genome, transcriptome or other nucleic acids of the biological specimen. For example, barcode sequences can have less than 80%, 70%, 60%, 50% or 40% sequence identity to the nucleic acid sequences in a particular biological specimen.

[0021] As used herein, the term "biological specimen" is intended to mean one or more cell, tissue, organism or portion thereof. A biological specimen can be obtained from any of a variety of organisms. Exemplary organisms include, but are not limited to, a mammal such as a rodent, mouse, rat, rabbit, guinea pig, ungulate, horse, sheep, pig, goat, cow, cat, dog, primate

(i.e. human or non-human primate); a plant such as *Arabidopsis thaliana,* corn, sorghum, oat, wheat, rice, canola, or soybean; an algae such as *Chlamydomonas reinhardtii;* a nematode such as *Caenorhabditis elegans;* an insect such as *Drosophila melanogaster,* mosquito, fruit fly, honey bee or spider; a fish such as zebrafish; a reptile; an amphibian such as a frog or *Xenopus laevis;* a *Dictyostelium discoideum;* a fungi such as *Pneumocystis carinii, Takifugu rubripes,* yeast, *Saccharamoyces cerevisiae* or *Schizosaccharomyces pombe;* or a Plasmodium falciparum. Target nucleic acids can also be derived from a prokaryote such as a bacterium, *Escherichia coli, Staphylococci* or *Mycoplasma pneumoniae;* an archae; a virus such as Hepatitis C virus or human immunodeficiency virus; or a viroid. Specimens can be derived from a homogeneous culture or population of the above organisms or alternatively from a collection of several different organisms, for example, in a community or ecosystem.

[0022] As used herein, the term "cleavage site" is intended to mean a location in a nucleic acid molecule that is susceptible to bond breakage. The location can be specific to a particular chemical, enzymatic or physical process that results in bond breakage. For example, the location can be a nucleotide that is abasic or a nucleotide that has a base that is susceptible to being removed to create an abasic site. Examples of nucleotides that are susceptible to being removed include uracil and 8-oxo-guanine as set forth in further detail herein below. The location can also be at or near a recognition sequence for a restriction endonuclease such as a nicking enzyme.

[0023] As used herein, the term "cluster," when used in reference to nucleic acids, refers to a population of the nucleic acids that is attached to a solid support to form a feature or site. The nucleic acids are generally members of a single species, thereby forming a monoclonal cluster. A "monoclonal population" of nucleic acids is a population that is homogeneous with respect to a particular nucleotide sequence. Clusters need not be monoclonal. Rather, for some applications, a cluster can be predominantly populated with amplicons from a first nucleic acid and can also have a low level of contaminating amplicons from a second nucleic acid. For example, when an array of clusters is to be used in a detection application, an acceptable level of contamination would be a level that does not impact signal to noise or resolution of the detection technique in an unacceptable way. Accordingly, apparent clonality will generally be relevant to a particular use or application of an array made by the methods set forth herein. Exemplary levels of contamination that can be acceptable at an individual cluster include, but are not limited to, at most 0.1%, 0.5%, 1%, 5%, 10%, 5 25%, or 35% contaminating amplicons. The nucleic acids in a cluster are generally covalently attached to a solid support, for example, via their 5' ends, but in some cases other attachment means are possible. The nucleic acids in a cluster can be single stranded or double stranded. In some but not all embodiments, clusters are made by a solid-phase amplification method known as bridge amplification. Exemplary configurations for clusters and methods for their production are set forth, for example, in U.S. Pat. No. 5,641,658; U.S. Patent Publ. No. 2002/0055100; U.S. Pat. No. 7,115,400; U.S. Patent Publ. No. 2004/0096853; U.S. Patent Publ. No. 2004/0002090; U.S. Patent Publ. No. 2007/0128624; and U.S. Patent Publ. No. 2008/0009420.

[0024] As used herein, the term "different", when used in reference to nucleic acids, means

that the nucleic acids have nucleotide sequences that are not the same as each other. Two or more nucleic acids can have nucleotide sequences that are different along their entire length. Alternatively, two or more nucleic acids can have nucleotide sequences that are different along a substantial portion of their length. For example, two or more nucleic acids can have target nucleotide sequence portions that are different for the two or more molecules while also having a universal sequence portion that is the same on the two or more molecules. Two beads can be different from each other by virtue of being attached to different nucleic acids.

[0025] As used herein, the term "each," when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection. Exceptions can occur if explicit disclosure or context clearly dictates otherwise.

[0026] As used herein, the term "extend," when used in reference to a nucleic acid, is intended to mean addition of at least one nucleotide or oligonucleotide to the nucleic acid. In particular embodiments one or more nucleotides can be added to the 3' end of a nucleic acid, for example, via polymerase catalysis (e.g. DNA polymerase, RNA polymerase or reverse transcriptase). Chemical or enzymatic methods can be used to add one or more nucleotide to the 3' or 5' end of a nucleic acid. One or more oligonucleotides can be added to the 3' or 5' end of a nucleic acid, for example, via chemical or enzymatic (e.g. ligase catalysis) methods. A nucleic acid can be extended in a template directed manner, whereby the product of extension is complementary to a template nucleic acid that is hybridized to the nucleic acid that is extended.

[0027] As used herein, the term "feature" means a location in an array for a particular species of molecule. A feature can contain only a single molecule or it can contain a population of several molecules of the same species. Features of an array are typically discrete. The discrete features can be contiguous or they can have spaces between each other. The size of the features and/or spacing between the features can vary such that arrays can be high density, medium density or lower density. High density arrays are characterized as having sites separated by less than about 15 µm. Medium density arrays have sites separated by about 15 to 30 µm while low density arrays have sites separated by greater than 30 µm. An array useful herein can have, for example, sites that are separated by less than 100 µm 50 µm 10 µm 5 µm 1 µm or 0.5 µm. An apparatus or method of the present disclosure can be used to detect an array at a resolution sufficient to distinguish sites at the above densities or density ranges.

[0028] As used herein, the term "fluidic mixture" is intended to mean two or more different items that are simultaneously present in a solution. Typically, the two or more items are freely diffusible in the solution. The two or more items can be different types of items (e.g. a nucleic acid and a protein which are different types of molecules) or they can be different species of the same type of items (e.g. two nucleic acid molecules having different sequences). Exemplary items that can be in a fluidic mixture include, but are not limited to, molecules, cells or beads.

[0029] As used herein, the term "flow cell" is intended to mean a vessel having a chamber where a reaction can be carried out, an inlet for delivering reagents to the chamber and an outlet for removing reagents from the chamber. In some embodiments the chamber is configured for detection of the reaction that occurs in the chamber. For example, the chamber can include one or more transparent surfaces allowing optical detection of biological specimens, optically labeled molecules, or the like in the chamber. Exemplary flow cells include, but are not limited to those used in a nucleic acid sequencing apparatus such as flow cells for the Genome Analyzer®, MiSeq®, NextSeq® or HiSeq® platforms commercialized by Illumina, Inc. (San Diego, CA); or for the SOLiD™ or Ion Torrent™ sequencing platform commercialized by Life Technologies (Carlsbad, CA). Exemplary flow cells and methods for their manufacture and use are also described, for example, in WO 2014/142841 A1; U.S. Pat. App. Pub. No. 2010/0111768 A1 and U.S. Pat. No. 8,951,781.

[0030] As used herein, the term "gel" is intended to mean a semi-rigid material that is permeable to liquids and gases. Typically, gel material can swell when liquid is taken up and can contract when liquid is removed by drying. Exemplary gels include, but are not limited to those having a colloidal structure, such as agarose; polymer mesh structure, such as gelatin; or cross-linked polymer structure, such as polyacrylamide, SFA (see, for example, US Pat. App. Pub. No. 2011/0059865 A1) or PAZAM (see, for example, US Pat. App. Publ. No. 2014/0079923 A1). Particularly useful gel material will conform to the shape of a well or other concave feature where it resides.

[0031] As used herein, the terms "nucleic acid" and "nucleotide" are intended to be consistent with their use in the art and to include naturally occurring species or functional analogs thereof. Particularly useful functional analogs of nucleic acids are capable of hybridizing to a nucleic acid in a sequence specific fashion or capable of being used as a template for replication of a particular nucleotide sequence. Naturally occurring nucleic acids generally have a backbone containing phosphodiester bonds. An analog structure can have an alternate backbone linkage including any of a variety of those known in the art. Naturally occurring nucleic acids generally have a deoxyribose sugar (e.g. found in deoxyribonucleic acid (DNA)) or a ribose sugar (e.g. found in ribonucleic acid (RNA)). A nucleic acid can contain nucleotides having any of a variety of analogs of these sugar moieties that are known in the art. A nucleic acid can include native or non-native nucleotides. In this regard, a native deoxyribonucleic acid can have one or more bases selected from the group consisting of adenine, thymine, cytosine or guanine and a ribonucleic acid can have one or more bases selected from the group consisting of uracil, adenine, cytosine or guanine. Useful non-native bases that can be included in a nucleic acid or nucleotide are known in the art. The terms "probe" or "target," when used in reference to a nucleic acid or sequence of a nucleic acid, are intended as semantic identifiers for the nucleic acid or sequence in the context of a method or composition set forth herein and does not necessarily limit the structure or function of the nucleic acid or sequence beyond what is otherwise explicitly indicated. The terms "probe" and "target" can be similarly applied to other analytes such as proteins, small molecules, cells or the like.

[0032] As used herein, the term "pitch," when used in reference to features of an array, is

intended to refer to the center-to-center spacing for adjacent features. A pattern of features can be characterized in terms of average pitch. The pattern can be ordered such that the coefficient of variation around the average pitch is small or the pattern can be random in which case the coefficient of variation can be relatively large. In either case, the average pitch can be, for example, at least about 10 nm, 0.1 µm, 0.5 µm, 1 µm, 5 µm, 10 µm, 100 µm or more. Alternatively or additionally, the average pitch can be, for example, at most about 100 µm, 10 µm, 5 µm, 1 µm, 0.5 µm 0.1 µm or less. Of course, the average pitch for a particular pattern of features can be between one of the lower values and one of the upper values selected from the ranges above.

[0033] As used herein, the term "poly T or poly A," when used in reference to a nucleic acid sequence, is intended to mean a series of two or more thiamine (T) or adenine (A) bases, respectively. A poly T or poly A can include at least about 2, 5, 8, 10, 12, 15, 18, 20 or more of the T or A bases, respectively. Alternatively or additionally, a poly T or poly A can include at most about, 30, 20, 18, 15, 12, 10, 8, 5 or 2 of the T or A bases, respectively.

[0034] As used herein, the term "random" can be used to refer to the spatial arrangement or composition of locations on a surface. For example, there are at least two types of order for an array described herein, the first relating to the spacing and relative location of features (also called "sites") and the second relating to identity or predetermined knowledge of the particular species of molecule that is present at a particular feature. Accordingly, features of an array can be randomly spaced such that nearest neighbor features have variable spacing between each other. Alternatively, the spacing between features can be ordered, for example, forming a regular pattern such as a rectilinear grid or hexagonal grid. In another respect, features of an array can be random with respect to the identity or predetermined knowledge of the species of analyte (e.g. nucleic acid of a particular sequence) that occupies each feature independent of whether spacing produces a random pattern or ordered pattern. An array set forth herein can be ordered in one respect and random in another. For example, in some embodiments set forth herein a surface is contacted with a population of nucleic acids under conditions where the nucleic acids attach at sites that are ordered with respect to their relative locations but 'randomly located' with respect to knowledge of the sequence for the nucleic acid species present at any particular site. Reference to "randomly distributing" nucleic acids at locations on a surface is intended to refer to the absence of knowledge or absence of predetermination regarding which nucleic acid will be captured at which location (regardless of whether the locations are arranged in an ordered pattern or not).

[0035] As used herein, the term "solid support" refers to a rigid substrate that is insoluble in aqueous liquid. The substrate can be non-porous or porous. The substrate can optionally be capable of taking up a liquid (e.g. due to porosity) but will typically be sufficiently rigid that the substrate does not swell substantially when taking up the liquid and does not contract substantially when the liquid is removed by drying. A nonporous solid support is generally impermeable to liquids or gases. Exemplary solid supports include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes,

Teflon™, cyclic olefins, polyimides etc.), nylon, ceramics, resins, Zeonor, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, optical fiber bundles, and polymers. Particularly useful solid supports for some embodiments are located within a flow cell apparatus. Exemplary flow cells are set forth in further detail herein.

[0036] As used herein, the term "spatial tag" is intended to mean a nucleic acid having a sequence that is indicative of a location. Typically, the nucleic acid is a synthetic molecule having a sequence that is not found in one or more biological specimen that will be used with the nucleic acid. However, in some embodiments the nucleic acid molecule can be naturally derived or the sequence of the nucleic acid can be naturally occurring, for example, in a biological specimen that is used with the nucleic acid. The location indicated by a spatial tag can be a location in or on a biological specimen, in or on a solid support or a combination thereof. A barcode sequence can function as a spatial tag.

[0037] As used herein, the term "tissue" is intended to mean an aggregation of cells, and, optionally, intercellular matter. Typically the cells in a tissue are not free floating in solution and instead are attached to each other to form a multicellular structure. Exemplary tissue types include muscle, nerve, epidermal and connective tissues.

[0038] As used herein, the term "universal sequence" refers to a series of nucleotides that is common to two or more nucleic acid molecules even if the molecules also have regions of sequence that differ from each other. A universal sequence that is present in different members of a collection of molecules can allow capture of multiple different nucleic acids using a population of universal capture nucleic acids that are complementary to the universal sequence. Similarly, a universal sequence present in different members of a collection of molecules can allow the replication or amplification of multiple different nucleic acids using a population of universal primers that are complementary to the universal sequence. Thus, a universal capture nucleic acid or a universal primer includes a sequence that can hybridize specifically to a universal sequence. Target nucleic acid molecules may be modified to attach universal adapters, for example, at one or both ends of the different target sequences.

[0039] The embodiments set forth below and recited in the claims can be understood in view of the above definitions.

[0040] The present disclosure provides a method for spatially tagging nucleic acids of a biological specimen. The method can include the steps of (a) attaching different nucleic acid probes to a solid support to produce randomly located probes on the solid support, wherein the different nucleic acid probes each includes a barcode sequence, and wherein each of the randomly located probes includes different barcode sequences from other randomly located probes on the solid support; (b) performing a nucleic acid detection reaction on the solid support to determine the barcode sequences of the randomly located probes on the solid support; (c) contacting a biological specimen with the solid support that has the randomly located probes; (d) hybridizing the randomly located probes to target nucleic acids from portions of the biological specimen that are proximal to the randomly located probes; and (e)

extending the randomly located probes to produce extended probes that include the barcode sequences and sequences from the target nucleic acids, thereby spatially tagging the nucleic acids of the biological specimen.

[0041] Any of a variety of solid supports can be used in a method, composition or apparatus of the present disclosure. Particularly useful solid supports are those used for nucleic acid arrays. Examples include glass, modified glass, functionalized glass, inorganic glasses, microspheres (e.g. inert and/or magnetic particles), plastics, polysaccharides, nylon, nitrocellulose, ceramics, resins, silica, silica-based materials, carbon, metals, an optical fiber or optical fiber bundles, polymers and multiwell (e.g. microtiter) plates. Exemplary plastics include acrylics, polystyrene, copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes and Teflon™. Exemplary silica-based materials include silicon and various forms of modified silicon.

[0042] In particular embodiments, a solid support can be within or part of a vessel such as a well, tube, channel, cuvette, Petri plate, bottle or the like. A particularly useful vessel is a flow-cell, for example, as described in WO 2014/142841 A1; U.S. Pat. App. Pub. No. 2010/0111768 A1 and U.S. Pat. No. 8,951,781 or Bentley et al., Nature 456:53-59 (2008), each of which is incorporated herein by reference. Exemplary flow-cells are those that are commercially available from Illumina, Inc. (San Diego, CA) for use with a sequencing platform such as a Genome Analyzer®, MiSeq®, NextSeq® or HiSeq® platform. Another particularly useful vessel is a well in a multiwell plate or microtiter plate.

[0043] Optionally, a solid support can include a gel coating. Attachment of nucleic acids to a solid support via a gel is exemplified by flow cells available commercially from Illumina Inc. (San Diego, CA) or described in US Pat. App. Pub. Nos. 2011/0059865 A1, 2014/0079923 A1, or 2015/0005447 A1; or PCT Publ. No. WO 2008/093098. Exemplary gels that can be used in the methods and apparatus set forth herein include, but are not limited to, those having a colloidal structure, such as agarose; polymer mesh structure, such as gelatin; or cross-linked polymer structure, such as polyacrylamide, SFA (see, for example, US Pat. App. Pub. No. 2011/0059865 A1) or PAZAM (see, for example, US Pat. App. Publ. Nos. 2014/0079923 A1, or 2015/0005447 A1).

[0044] In some embodiments, a solid support can be configured as an array of features to which nucleic acids can be attached. The features can be present in any of a variety of desired formats. For example, the features can be wells, pits, channels, ridges, raised regions, pegs, posts or the like. In some embodiments, the features can contain beads. However, in particular embodiments the features need not contain a bead or particle. Exemplary features include wells that are present in substrates used for commercial sequencing platforms sold by 454 LifeSciences (a subsidiary of Roche, Basel Switzerland) or Ion Torrent (a subsidiary of Life Technologies, Carlsbad California). Other substrates having wells include, for example, etched fiber optics and other substrates described in US Pat Nos. 6,266,459; 6,355,431; 6,770,441; 6,859,570; 6,210,891; 6,258,568; 6,274,320; US Pat app. Publ. Nos. 2009/0026082 A1; 2009/0127589 A1; 2010/0137143 A1; 2010/0282617 A1 or PCT Publication No. WO 00/63437.

In some embodiments, wells of a substrate can include gel material (with or without beads) as set forth in US Pat. App. Publ. No. 2014/0243224 A1.

[0045] The features on a solid support can be metal features on a non-metallic surface such as glass, plastic or other materials exemplified above. A metal layer can be deposited on a surface using methods known in the art such as wet plasma etching, dry plasma etching, atomic layer deposition, ion beam etching, chemical vapor deposition, vacuum sputtering or the like. Any of a variety of commercial instruments can be used as appropriate including, for example, the FlexAL®, OpAL®, Ionfab 300plus®, or Optofab 3000® systems (Oxford Instruments, UK). A metal layer can also be deposited by e-beam evaporation or sputtering as set forth in Thornton, Ann. Rev. Mater. Sci. 7:239-60 (1977). Metal layer deposition techniques, such as those exemplified above, can be combined with photolithography techniques to create metal regions or patches on a surface. Exemplary methods for combining metal layer deposition techniques and photolithography techniques are provided in US Pat. No. 8,895,249 or US Pat App. Pub. No. 2014/0243224 A1.

[0046] Features can appear on a solid support as a grid of spots or patches. The features can be located in a repeating pattern or in an irregular, non-repeating pattern. Particularly useful repeating patterns are hexagonal patterns, rectilinear patterns, grid patterns, patterns having reflective symmetry, patterns having rotational symmetry, or the like. Asymmetric patterns can also be useful. The pitch can be the same between different pairs of nearest neighbor features or the pitch can vary between different pairs of nearest neighbor features.

[0047] High density arrays are characterized as having average pitch of less than about 15 μm. Medium density arrays have average pitch of about 15 to 30 μm while low density arrays have average pitch greater than 30 μm. An array useful in the invention can have average pitch that is less than 100 μm 50 μm 10 μm 5 μm, 1 μm or 0.5 μm. The average pitch values and ranges set forth above or elsewhere herein are intended to be applicable to ordered arrays or random arrays.

[0048] In particular embodiments, features on a solid support can each have an area that is larger than about 100 nm^2, 250 nm^2, 500 nm^2, 1 μm^2, 2.5 μm^2, 5 μm^2, 10 μm^2, 100 μm^2, or 500 μm^2. Alternatively or additionally, features can each have an area that is smaller than about 1 mm^2, 500 μm^2, 100 μm^2, 25 μm^2, 10 μm^2, 5 μm^2, 1 μm^2, 500 nm^2, or 100 nm^2. The above ranges can describe the apparent area of a bead or other particle on a solid support when viewed or imaged from above.

[0049] In particular embodiments, a solid support can include a collection of beads or other particles. The particles can be suspended in a solution or they can be located on the surface of a substrate. Examples of arrays having beads located on a surface include those wherein beads are located in wells such as a BeadChip array (Illumina Inc., San Diego CA), substrates used in sequencing platforms from 454 LifeSciences (a subsidiary of Roche, Basel Switzerland) or substrates used in sequencing platforms from Ion Torrent (a subsidiary of Life

Technologies, Carlsbad California). Other solid supports having beads located on a surface are described in US Pat. Nos. 6,266,459; 6,355,431; 6,770,441; 6,859,570; 6,210,891; 6,258,568; or 6,274,320; US Pat. App. Publ. Nos. 2009/0026082 A1; 2009/0127589 A1; 2010/0137143 A1; or 2010/0282617 A1 or PCT Publication No. WO 00/63437. Several of the above references describe methods for attaching nucleic acid probes to beads prior to loading the beads in or on a solid support. As such, the collection of beads can include different beads each having a unique probe attached. It will however, be understood that the beads can be made to include universal primers, and the beads can then be loaded onto an array, thereby forming universal arrays for use in a method set forth herein. As set forth previously herein, the solid supports typically used for bead arrays can be used without beads. For example, nucleic acids, such as probes or primers can be attached directly to the wells or to gel material in wells. Thus, the above references are illustrative of materials, compositions or apparatus that can be modified for use in the methods and compositions set forth herein.

[0050] Accordingly, a solid support used in a method set forth herein can include an array of beads, wherein different nucleic acid probes are attached to different beads in the array. In this embodiment, each bead can be attached to a different nucleic acid probe and the beads can be randomly distributed on the solid support in order to effectively attach the different nucleic acid probes to the solid support. Optionally, the solid support can include wells having dimensions that accommodate no more than a single bead. In such a configuration, the beads may be attached to the wells due to forces resulting from the fit of the beads in the wells. It is also possible to use attachment chemistries or adhesives to hold the beads in the wells.

[0051] Nucleic acid probes that are attached to beads can include barcode sequences. A population of the beads can be configured such that each bead is attached to only one type of barcode and many different beads each with a different barcode are present in the population. In this embodiment, randomly distributing the beads to a solid support will result in randomly locating the nucleic acid probes (and their respective barcode sequences) on the solid support. In some cases there can be multiple beads with the same barcode sequence such that there is redundancy in the population. Randomly distributing a redundant population of beads on a solid support that has a capacity that is greater than the number of unique barcodes in the bead population will result in redundancy of barcodes on the solid support. Alternatively, the number of different barcodes in a population of beads can exceed the capacity of the solid support in order to produce an array that is not redundant with respect to the population of barcodes on the solid support. The capacity of the solid support will be determined in some embodiments by the number of features (e.g. single-bead occupancy wells) that attach or otherwise accommodate a bead.

[0052] A solid support can include, or can be made by the methods set forth herein to attach, a plurality of different nucleic acid probes. For example, a solid support can include at least 10, 100, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 or more different probes. Alternatively or additionally, a solid support can include at most 1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 100, or fewer different probes. It will be understood that each of

the different probes can be present in several copies, for example, when the probes have been amplified to form a cluster. Thus, the above ranges can describe the number of different nucleic acid clusters on a solid support. It will also be understood that the above ranges can describe the number of different barcodes, target capture sequences, or other sequence elements set forth herein as being unique to particular nucleic acid probes. Alternatively or additionally, the ranges can describe the number of extended probes or modified probes created on a solid support using a method set forth herein.

[0053] Features, may be present on a solid support prior to contacting the solid support with nucleic acid probes. For example, in embodiments where probes are attached to a support via hybridization to primers, the primers can be attached at the features, whereas interstitial areas outside of the features substantially lack any of the primers. Nucleic acid probes can be captured at preformed features on a solid support, and optionally amplified on the solid support, using methods set forth in US Pat. No. 8,895,249, US Pat. No. 8,778,849, or US Pat App. Pub. No. 2014/0243224 A1. Alternatively, a solid support may have a lawn of primers or may otherwise lack features. In this case, a feature can be formed by virtue of attachment of a nucleic acid probe on the solid support. Optionally, the captured nucleic acid probe can be amplified on the solid support such that the resulting cluster becomes a feature. Although attachment is exemplified above as capture between a primer and a complementary portion of a probe, it will be understood that capture moieties other than primers can be present at pre-formed features or as a lawn. Other exemplary capture moieties include, but are not limited to, chemical moieties capable of reacting with a nucleic acid probe to create a covalent bond or receptors capable of biding non-covalently to a ligand on a nucleic acid probe.

[0054] A step of attaching nucleic acid probes to a solid support can be carried out by providing a fluid that contains a mixture of different nucleic acid probes and contacting this fluidic mixture with the solid support. The contact can result in the fluidic mixture being in contact with a surface to which many different nucleic acid probes from the fluidic mixture will attach. Thus, the probes have random access to the surface (whether the surface has pre-formed features configured to attach the probes or a uniform surface configured for attachment). Accordingly, the probes can be randomly located on the solid support.

[0055] The total number and variety of different probes that end up attached to a surface can be selected for a particular application or use. For example, in embodiments where a fluidic mixture of different nucleic acid probes is contacted with a solid support for purposes of attaching the probes to the support, the number of different probe species can exceed the occupancy of the solid support for probes. Thus, the number and variety of different probes that attach to the solid support can be equivalent to the probe occupancy of the solid support. Alternatively, the number and variety of different probe species on the solid support can be less than the occupancy (i.e. there will be redundancy of probe species such that the solid support may contain multiple features having the same probe species). Such redundancy can be achieved, for example, by contacting the solid support with a fluidic mixture that contains a number and variety of probe species that is substantially lower than the probe occupancy of the solid support.

[0056] Attachment of the nucleic acid probes can be mediated by hybridization of the nucleic acid probes to complementary primers that are attached to the solid support, chemical bond formation between a reactive moiety on the nucleic acid probe and the solid support (examples are set forth in US Pat. No. 8,895,249, US Pat. No. 8,778,849, or US Pat App. Pub. No. 2014/0243224 A1), affinity interactions of a moiety on the nucleic acid probe with a solid support-bound moiety (e.g. between known receptor-ligand pairs such as streptavidinbiotin, antibody-epitope, lectin-carbohydrate and the like), physical interactions of the nucleic acid probes with the solid support (e.g. hydrogen bonding, ionic forces, van der Waals forces and the like), or other interactions known in the art to attach nucleic acids to surfaces.

[0057] In some embodiments, attachment of a nucleic acid probe is non-specific with regard to any sequence differences between the nucleic acid probe and other nucleic acid probes that are or will be attached to the solid support. For example, different probes can have a universal sequence that complements surface-attached primers or the different probes can have a common moiety that mediates attachment to the surface. Alternatively, each of the different probes (or a subpopulation of different probes) can have a unique sequence that complements a unique primer on the solid support or they can have a unique moiety that interacts with one or more different reactive moiety on the solid support. In such cases, the unique primers or unique moieties can, optionally, be attached at predefined locations in order to selectively capture particular probes, or particular types of probes, at the respective predefined locations.

[0058] One or more features on a solid support can each include a single molecule of a particular probe. The features can be configured, in some embodiments, to accommodate no more than a single nucleic acid probe molecule. However, whether or not the feature can accommodate more than one nucleic acid probe molecule, the feature may nonetheless include no more than a single nucleic acid probe molecule. Alternatively, an individual feature can include a plurality of nucleic acid probe molecules, for example, an ensemble of nucleic acid probe molecules having the same sequence as each other. In particular embodiments, the ensemble can be produced by amplification from a single nucleic acid probe template to produce amplicons, for example, as a cluster attached to the surface.

[0059] A method set forth herein can use any of a variety of amplification techniques. Exemplary techniques that can be used include, but are not limited to, polymerase chain reaction (PCR), rolling circle amplification (RCA), multiple displacement amplification (MDA), or random prime amplification (RPA). In some embodiments the amplification can be carried out in solution, for example, when features of an array are capable of containing amplicons in a volume having a desired capacity. Preferably, an amplification technique used in a method of the present disclosure will be carried out on solid phase. For example, one or more primer species (e.g. universal primers for one or more universal primer binding site present in a nucleic acid probe) can be attached to a solid support. In PCR embodiments, one or both of the primers used for amplification can be attached to a solid support (e.g. via a gel). Formats that utilize two species of primers attached to a solid support are often referred to as bridge amplification because double stranded amplicons form a bridge-like structure between the two

surface attached primers that flank the template sequence that has been copied. Exemplary reagents and conditions that can be used for bridge amplification are described, for example, in U.S. Pat. Nos. 5,641,658, 7,115,400, or 8,895,249; or U.S. Pat. Publ. Nos. 2002/0055100 A1, 2004/0096853 A1, 2004/0002090 A1, 2007/0128624 A1 or 2008/0009420 A1. Solid-phase PCR amplification can also be carried out with one of the amplification primers attached to a solid support and the second primer in solution. An exemplary format that uses a combination of a surface attached primer and soluble primer is the format used in emulsion PCR as described, for example, in Dressman et al., Proc. Natl. Acad. Sci. USA 100:8817-8822 (2003), WO 05/010145, or U.S. Pat. App. Publ. Nos. 2005/0130173 A1 or 2005/0064460 A1. Emulsion PCR is illustrative of the format and it will be understood that for purposes of the methods set forth herein the use of an emulsion is optional and indeed for several embodiments an emulsion is not used.

[0060] RCA techniques can be modified for use in a method of the present disclosure. Exemplary components that can be used in an RCA reaction and principles by which RCA produces amplicons are described, for example, in Lizardi et al., Nat. Genet. 19:225-232 (1998) and US Pat. App. Publ. No. 2007/0099208 A1. Primers used for RCA can be in solution or attached to a solid support. The primers can be one or more of the universal primers described herein.

[0061] MDA techniques can be modified for use in a method of the present disclosure. Some basic principles and useful conditions for MDA are described, for example, in Dean et al., Proc Natl. Acad. Sci. USA 99:5261-66 (2002); Lage et al., Genome Research 13:294-307 (2003); Walker et al., Molecular Methods for Virus Detection, Academic Press, Inc., 1995; Walker et al., Nucl. Acids Res. 20:1691-96 (1992); US 5,455,166; US 5,130,238; and US 6,214,587. Primers used for MDA can be in solution or attached to a solid support at an amplification site. Again, the primers can be one or more of the universal primers described herein.

[0062] In particular embodiments a combination of the above-exemplified amplification techniques can be used. For example, RCA and MDA can be used in a combination wherein RCA is used to generate a concatameric amplicon in solution (e.g. using solution-phase primers). The amplicon can then be used as a template for MDA using primers that are attached to a solid support (e.g. universal primers). In this example, amplicons produced after the combined RCA and MDA steps will be attached to the solid support.

[0063] Nucleic acid probes that are used in a method set forth herein or present in an apparatus or composition of the present disclosure can include barcode sequences, and for embodiments that include a plurality of different nucleic acid probes, each of the probes can include a different barcode sequence from other probes in the plurality. Barcode sequences can be any of a variety of lengths. Longer sequences can generally accommodate a larger number and variety of barcodes for a population. Generally, all probes in a plurality will have the same length barcode (albeit with different sequences), but it is also possible to use different length barcodes for different probes. A barcode sequence can be at least 2, 4, 6, 8, 10, 12, 15, 20 or more nucleotides in length. Alternatively or additionally, the length of the

barcode sequence can be at most 20, 15, 12, 10, 8, 6, 4 or fewer nucleotides. Examples of barcode sequences that can be used are set forth, for example in, US Pat. App. Publ. No. 2014/0342921 A1 and US Pat. No. 8,460,865.

[0064] A method of the present disclosure can include a step of performing a nucleic acid detection reaction on a solid support to determine barcode sequences of nucleic acid probes that are located on the solid support. In many embodiments the probes are randomly located on the solid support and the nucleic acid detection reaction provides information to locate each of the different probes. Exemplary nucleic acid detection methods include, but are not limited to nucleic acid sequencing of a probe, hybridization of nucleic acids to a probe, ligation of nucleic acids that are hybridized to a probe, extension of nucleic acids that are hybridized to a probe, extension of a first nucleic acid that is hybridized to a probe followed by ligation of the extended nucleic acid to a second nucleic acid that is hybridized to the probe, or other methods known in the art such as those set forth in US Pat. No. 8,288,103 or 8,486,625.

[0065] Sequencing techniques, such as sequencing-by-synthesis (SBS) techniques, are a particularly useful method for determining barcode sequences. SBS can be carried out as follows. To initiate a first SBS cycle, one or more labeled nucleotides, DNA polymerase, SBS primers etc., can be contacted with one or more features on a solid support (e.g. feature(s) where nucleic acid probes are attached to the solid support). Those features where SBS primer extension causes a labeled nucleotide to be incorporated can be detected. Optionally, the nucleotides can include a reversible termination moiety that terminates further primer extension once a nucleotide has been added to the SBS primer. For example, a nucleotide analog having a reversible terminator moiety can be added to a primer such that subsequent extension cannot occur until a deblocking agent is delivered to remove the moiety. Thus, for embodiments that use reversible termination, a deblocking reagent can be delivered to the solid support (before or after detection occurs). Washes can be carried out between the various delivery steps. The cycle can then be repeated n times to extend the primer by n nucleotides, thereby detecting a sequence of length n. Exemplary SBS procedures, fluidic systems and detection platforms that can be readily adapted for use with a composition, apparatus or method of the present disclosure are described, for example, in Bentley et al., Nature 456:53-59 (2008), PCT Publ. Nos. WO 91/06678, WO 04/018497 or WO 07/123744; US Pat. Nos. 7,057,026, 7,329,492, 7,211,414, 7,315,019 or 7,405,281, and US Pat. App. Publ. No. 2008/0108082.

[0066] Other sequencing procedures that use cyclic reactions can be used, such as pyrosequencing. Pyrosequencing detects the release of inorganic pyrophosphate (PPi) as particular nucleotides are incorporated into a nascent nucleic acid strand (Ronaghi, et al., Analytical Biochemistry 242(1), 84-9 (1996); Ronaghi, Genome Res. 11(1), 3-11 (2001); Ronaghi et al. Science 281(5375), 363 (1998); or US Pat. Nos. 6,210,891, 6,258,568 or 6,274,320). In pyrosequencing, released PPi can be detected by being immediately converted to adenosine triphosphate (ATP) by ATP sulfurylase, and the level of ATP generated can be detected via luciferase-produced photons. Thus, the sequencing reaction can be monitored via a luminescence detection system. Excitation radiation sources used for fluorescence based

detection systems are not necessary for pyrosequencing procedures. Useful fluidic systems, detectors and procedures that can be used for application of pyrosequencing to apparatus, compositions or methods of the present disclosure are described, for example, in PCT Pat. App. Publ. No. WO2012/058096, US Pat. App. Publ. No. 2005/0191698 A1, or US Pat. Nos. 7,595,883 or 7,244,559.

[0067] Sequencing-by-ligation reactions are also useful including, for example, those described in Shendure et al. Science 309:1728-1732 (2005); or US Pat. Nos. 5,599,675 or 5,750,341. Some embodiments can include sequencing-by-hybridization procedures as described, for example, in Bains et al., Journal of Theoretical Biology 135(3), 303-7 (1988); Drmanac et al., Nature Biotechnology 16, 54-58 (1998); Fodor et al., Science 251(4995), 767-773 (1995); or PCT Pat. App. Publ. No. WO 1989/10977. In both sequencing-by-ligation and sequencing-by-hybridization procedures, target nucleic acids (or amplicons thereof) that are present at sites of an array are subjected to repeated cycles of oligonucleotide delivery and detection. Compositions, apparatus or methods set forth herein or in references cited herein can be readily adapted for sequencing-by-ligation or sequencing-by-hybridization procedures. Typically, the oligonucleotides are fluorescently labeled and can be detected using fluorescence detectors similar to those described with regard to SBS procedures herein or in references cited herein.

[0068] Some sequencing embodiments can utilize methods involving the real-time monitoring of DNA polymerase activity. For example, nucleotide incorporations can be detected through fluorescence resonance energy transfer (FRET) interactions between a fluorophore-bearing polymerase and y-phosphate-labeled nucleotides, or with zeromode waveguides (ZMWs). Techniques and reagents for FRET-based sequencing are described, for example, in Levene et al. Science 299, 682-686 (2003); Lundquist et al. Opt. Lett. 33, 1026-1028 (2008); Korlach et al. Proc. Natl. Acad. Sci. USA 105, 1176-1181 (2008).

[0069] Some sequencing embodiments include detection of a proton released upon incorporation of a nucleotide into an extension product. For example, sequencing based on detection of released protons can use an electrical detector and associated techniques that are commercially available from Ion Torrent (Guilford, CT, a Life Technologies and Thermo Fisher subsidiary) or sequencing methods and systems described in US Pat app. Publ. Nos. 2009/0026082 A1; 2009/0127589 A1; 2010/0137143 A1; or US 2010/0282617 A1.

[0070] Nucleic acid hybridization techniques are also useful method for determining barcode sequences. In some cases combinatorial hybridization methods can be used such as those used for decoding of multiplex bead arrays (see e.g. US Pat. No. 8,460,865). Such methods utilize labelled nucleic acid decoder probes that are complementary to at least a portion of a barcode sequence. A hybridization reaction can be carried out using decoder probes having known labels such that the location where the labels end up on the solid support identifies the nucleic acid probes according to rules of nucleic acid complementarity. In some cases, pools of many different probes with distinguishable labels are used, thereby allowing a multiplex decoding operation. The number of different barcodes determined in a decoding operation can

exceed the number of labels used for the decoding operation. For example, decoding can be carried out in several stages where each stage constitutes hybridization with a different pool of decoder probes. The same decoder probes can be present in different pools but the label that is present on each decoder probe can differ from pool to pool (i.e. each decoder probe is in a different "state" when in different pools). Various combinations of these states and stages can be used to expand the number of barcodes that can be decoded well beyond the number of distinct labels available for decoding. Such combinatorial methods are set forth in further detail in US Pat. No. 8,460,865 or Gunderson et al., Genome Research 14:870-877 (2004).

[0071] A method of the present disclosure can include a step of contacting a biological specimen with a solid support that has nucleic acid probes attached thereto. In some embodiments the nucleic acid probes are randomly located on the solid support. The identity and location of the nucleic acid probes may have been decoded prior to contacting the biological specimen with the solid support. Alternatively, the identity and location of the nucleic acid probes can be determined after contacting the solid support with the biological specimen.

[0072] In some embodiments the biological specimen is one or more cells. The cell(s) can be individual and free from any tissue or multicellular structure at the time contact is made with the solid support. For example, the cell(s) can be present in a fluid (e.g. when a plurality of different cells are present the fluid can be a fluidic mixture of the different cells) and the fluid can be contacted with the solid support to which the different probes are attached. Any of a variety of cells can be used including, for example, those from a prokaryote, archae or eukaryote. One or more cells used in a method, composition or apparatus of the present disclosure can be a single celled organisms or from a multicellular organism. Exemplary organisms from which one or more cell can be obtained include, but are not limited to a mammal, plant, algae, nematode, insect, fish, reptile, amphibian, fungi or Plasmodium falciparum. Exemplary species are set forth previously herein or known in the art.

[0073] Embodiments of the present disclosure can also use one or more subcellular components as a biological specimen. For example a fluidic mixture can include one or more nuclei, golgi apparatus, mitochondria, chloroplasts, membrane fractions, vesicles, endoplasmic reticulum, or other components known in the art. Other useful types of biological specimens are one or more viruses or a viroids.

[0074] It will be understood that a biological specimen can be a homogeneous culture or population of the above cells, subcellular components, viruses or viroids. Alternatively the biological specimen can be a non-homogenous collection of cells, subcellular components, viruses or viroids, for example, derived from several different organisms in a community or ecosystem. An exemplary community is the collection of bacteria present in the digestive system, lung or other organ of a multicellular organism such as a mammal.

[0075] One or more cells, subcellular components, viruses or viroids that are contacted with a solid support in a method set forth herein can be attached to the solid support. Attachment can be achieved using methods known in the art such as those exemplified herein with respect to

attachment of nucleic acids to a solid support. In some embodiments, attachment is selective for specific types of cells, subcellular components, viruses or viroids. For example, the solid support can include antibodies or other receptors that are selective for epitopes or ligands present on one or a subset of different cells, subcellular components, viruses or viroids present in a fluidic mixture. In other embodiments, the attachment of cells, subcellular components, viruses or viroids can be mediated by non-selective moieties such as chemical moieties that are broadly reactive.

[0076] In particular embodiments, one or more cells, subcellular components, viruses or viroids that have been contacted with a solid support can be lysed to release target nucleic acids. Lysis can be carried out using methods known in the art such as those that employ one or more of chemical treatment, enzymatic treatment, electroporation, heat, hypotonic treatment, sonication or the like. Exemplary lysis techniques are set forth in Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Ed., Cold Spring Harbor Laboratory, New York (2001) and in Ansubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1999).

[0077] In some embodiments the biological specimen is a tissue section. The tissue can be derived from a multicellular organism such as those exemplified above in regard to cells. A tissue section can be contacted with a solid support, for example, by laying the tissue on the surface of the solid support. The tissue can be freshly excised from an organism or it may have been previously preserved for example by freezing, embedding in a material such as paraffin (e.g. formalin fixed paraffin embedded samples), formalin fixation, infiltration, dehydration or the like. Optionally, a tissue section can be attached to a solid support, for example, using techniques and compositions exemplified herein with regard to attaching nucleic acids, cells, viruses, beads or the like to a solid support. As a further option, a tissue can be permeabilized and the cells of the tissue lysed when the tissue is in contact with a solid support. Any of a variety of treatments can be used such as those set forth above in regard to lysing cells. Target nucleic acids that are released from a tissue that is permeabilized can be captured by nucleic acid probes on the surface.

[0078] A tissue can be prepared in any convenient or desired way for its use in a method, composition or apparatus herein. Fresh, frozen, fixed or unfixed tissues can be used. A tissue can be fixed or embedded using methods described herein or known in the art.

[0079] A tissue sample for use herein, can be fixed by deep freezing at temperature suitable to maintain or preserve the integrity of the tissue structure, e.g. less than -20° C. In another example, a tissue can be prepared using formalin-fixation and paraffin embedding (FFPE) methods which are known in the art. Other fixatives and/or embedding materials can be used as desired. A fixed or embedded tissue sample can be sectioned, i.e. thinly sliced, using known methods. For example, a tissue sample can be sectioned using a chilled microtome or cryostat, set at a temperature suitable to maintain both the structural integrity of the tissue sample and the chemical properties of the nucleic acids in the sample.

[0080] In some embodiments, a tissue sample will be treated to remove embedding material (e.g. to remove paraffin or formalin) from the sample prior to release, capture or modification of nucleic acids. This can be achieved by contacting the sample with an appropriate solvent (e.g. xylene and ethanol washes). Treatment can occur prior to contacting the tissue sample with a solid support set forth herein or the treatment can occur while the tissue sample is on the solid support. Exemplary methods for manipulating tissues for use with solid supports to which nucleic acids are attached are set forth in US Pat. App. Publ. No. 2014/0066318 A1.

[0081] The thickness of a tissue sample or other biological specimen that is contacted with a solid support in a method, composition or apparatus set forth herein can be any suitable thickness desired. In representative embodiments, the thickness will be at least 0.1 µm, 0.25 µm, 0.5 µm, 0.75 µm, 1 µm, 5 µm, 10 µm, 50 µm, 100 µm or thicker. Alternatively or additionally, the thickness of a biological specimen that is contacted with a solid support will be no more than 100 µm, 50 µm, 10 µm, 5 µm, 1 µm, 0.5 µm, 0.25 µm, 0.1 µm or thinner.

[0082] A particularly relevant source for a biological specimen is a human being. The specimen can be derived from an organ, including for example, an organ of the musculoskeletal system such as muscle, bone, tendon or ligament; an organ of the digestive system such as salivary gland, pharynx, esophagus, stomach, small intestine, large intestine, liver, gallbladder or pancreas; an organ of the respiratory system such as larynx, trachea, bronchi, lungs or diaphragm; an organ of the urinary system such as kidney, ureter, bladder or urethra; a reproductive organ such as ovary, fallopian tube, uterus, vagina, placenta, testicle, epididymis, vas deferens, seminal vesicle, prostate, penis or scrotum; an organ of the endocrine system such as pituitary gland, pineal gland, thyroid gland, parathyroid gland, or adrenal gland; an organ of the circulatory system such as heart, artery, vein or capillary; an organ of the lymphatic system such as lymphatic vessel, lymph node, bone marrow, thymus or spleen; an organ of the central nervous system such as brain, brainstem, cerebellum, spinal cord, cranial nerve, or spinal nerve; a sensory organ such as eye, ear, nose, or tongue; or an organ of the integument such as skin, subcutaneous tissue or mammary gland. In some embodiments, a biological specimen is obtained from a bodily fluid or excreta such as blood, lymph, tears, sweat, saliva, semen, vaginal secretion, ear wax, fecal matter or urine.

[0083] A specimen from a human can be considered (or suspected) healthy or diseased when used. In some cases, two specimens can be used: a first being considered diseased and a second being considered as healthy (e.g. for use as a healthy control). Any of a variety of conditions can be evaluated, including but not limited to, an autoimmune disease, cancer, cystic fibrosis, aneuploidy, pathogenic infection, psychological condition, hepatitis, diabetes, sexually transmitted disease, heart disease, stroke, cardiovascular disease, multiple sclerosis or muscular dystrophy. Particularly relevant conditions are genetic conditions or conditions associated with pathogens having identifiable genetic signatures.

[0084] As set forth above, a flow cell provides a convenient apparatus for use in a method set forth herein. For example, a flow cell is a convenient apparatus for housing a solid support that will be treated with multiple fluidic reagents such as the repeated fluidic deliveries used for

some nucleic acid sequencing protocols or some nucleic acid hybridization protocols. In some embodiments, a biological specimen can be delivered to a solid support in a flow cell, for example, when a fluidic mixture of cells, subcellular components, viruses or viroids is delivered to the solid support. In some embodiments it may be preferable to open a flow cell to expose a solid support inside or to remove the solid support from the flow cell in order to allow convenient delivery of a biological specimen to the solid support. For example, opening the flow cell or removing the solid support can allow a user or robotic device to lay a tissue section on the solid support. The opening of a flow cell or removal of a solid support from a flow cell can be temporary. Thus, the flow cell can subsequently be closed or the solid support returned to the flow cell to proceed with one or more subsequent steps of a method set forth herein.

[0085] In some embodiments, a flow cell can have a construction that allows it to be opened or taken apart. For example the flow cell can be in a closed state while performing a sequencing reaction, for example to decode barcodes. Then the flow cell can be taken apart so that tissue can be placed on the flow cell surface. The flow cell can be held together by adhesive such that one or more surface can be removed to open it. For example, a flow cell can have a spacer with adhesive surfaces on the top or bottom (akin to single-sided or double-sided sticky tape) and this spacer can occur between two solid supports. One or both of the solid supports can be configured to attach nucleic acids and support a biological specimen as set forth herein. The spacer can have open regions (e.g. created by laser cutting of the spacer material) that create fluidic channels bound by the two solid supports and the spacer. Thus, one or both of the solid supports can be non-permanently adhered to the spacer to allow one or both of them to be removed to allow access to the surface when placing a tissue or other specimen thereon.

[0086] A nucleic acid probe used in a composition, apparatus or method set forth herein can include a target capture moiety. In particular embodiments, the target capture moiety is a target capture sequence. The target capture sequence is generally complementary to a target sequence such that target capture occurs by formation of a probe-target hybrid complex. A target capture sequence can be any of a variety of lengths including, for example, lengths exemplified above in the context of barcode sequences.

[0087] In multiplex embodiments, a plurality of different nucleic acid probes can include different target capture sequences that hybridize to different target nucleic acid sequences from a biological specimen. Different target capture sequences can be used to selectively bind to one or more desired target nucleic acids from a biological specimen. In some cases, the different nucleic acid probes can include a target capture sequence that is common to all or a subset of the probes on a solid support. For example, the nucleic acid probes on a solid support can have a poly A or poly T sequence. Such probes or amplicons thereof can hybridize to mRNA molecules, cDNA molecules or amplicons thereof that have poly A or poly T tails. Although the mRNA or cDNA species will have different target sequences, capture will be mediated by the common poly A or poly T sequence regions.

[0088] Any of a variety of target nucleic acids can be captured and analyzed in a method set forth herein including, but not limited to, messenger RNA (mRNA), copy DNA (cDNA), genomic

DNA (gDNA), ribosomal RNA (rRNA) or transfer RNA (tRNA). Particular target sequences can be selected from databases and appropriate capture sequences designed using techniques and databases known in the art.

[0089] Other target capture moieties that are useful include, for example, the moieties set forth herein as useful for attaching nucleic acid probes to a solid support.

[0090] A method set forth herein can include a step of hybridizing nucleic acid probes, that are on a solid support, to target nucleic acids that are from portions of the biological specimen that are proximal to the probes. Generally, a target nucleic acid will diffuse from a region of the biological specimen to an area of the solid support that is in proximity with that region of the specimen. Here the target nucleic acid will interact with nucleic acid probes that are proximal to the region of the specimen from which the target nucleic acid was released. A target-probe hybrid complex can form where the target nucleic acid encounters a complementary target capture sequence on a nucleic acid probe. The location of the target-probe hybrid complex will generally correlate with the region of the biological specimen from where the target nucleic acid was derived. In multiplex embodiments, the solid support will include a plurality of nucleic acid probes, the biological specimen will release a plurality of target nucleic acids and a plurality of target-probe hybrids will be formed on the solid support. The sequences of the target nucleic acids and their locations on the support will provide spatial information about the nucleic acid content of the biological specimen. Although the example above is described in the context of target nucleic acids that are released from a biological specimen, it will be understood that the target nucleic acids need not be released. Rather, the target nucleic acids may remain in contact with the biological specimen, for example, when they are attached to an exposed surface of the biological specimen in a way that the target nucleic acids can also bind to appropriate nucleic acid probes on the solid support.

[0091] A method of the present disclosure can include a step of extending solid support-attached probes to which target nucleic acids are hybridized. In embodiments where the probes include barcode sequences, the resulting extended probes will include the barcode sequences and sequences from the target nucleic acids (albeit in complementary form). The extended probes are thus spatially tagged versions of the target nucleic acids from the biological specimen. The sequences of the extended probes identify what nucleic acids are in the biological specimen and where in the biological specimen the target nucleic acids are located. It will be understood that other sequence elements that are present in the nucleic acid probes can also be included in the extended probes. Such elements include, for example, primer binding sites, cleavage sites, other tag sequences (e.g. sample identification tags), capture sequences, recognition sites for nucleic acid binding proteins or nucleic acid enzymes, or the like.

[0092] Extension of probes can be carried out using methods exemplified herein or otherwise known in the art for amplification of nucleic acids or sequencing of nucleic acids. In particular embodiments one or more nucleotides can be added to the 3' end of a nucleic acid, for example, via polymerase catalysis (e.g. DNA polymerase, RNA polymerase or reverse

transcriptase). Chemical or enzymatic methods can be used to add one or more nucleotide to the 3' or 5' end of a nucleic acid. One or more oligonucleotides can be added to the 3' or 5' end of a nucleic acid, for example, via chemical or enzymatic (e.g. ligase catalysis) methods. A nucleic acid can be extended in a template directed manner, whereby the product of extension is complementary to a template nucleic acid that is hybridized to the nucleic acid that is extended. In some embodiments, a DNA primer is extended by a reverse transcriptase using an RNA template, thereby producing a cDNA. Thus, an extended probe made in a method set forth herein can be a reverse transcribed DNA molecule. Exemplary methods for extending nucleic acids are set forth in US Pat. App. Publ. No. US 2005/0037393 A1 or US Pat. No. 8,288,103 or 8,486,625.

[0093] All or part of a target nucleic acid that is hybridized to a nucleic acid probe can be copied by extension. For example, an extended probe can include at least, 1, 2, 5,10, 25, 50, 100, 200, 500, 1000 or more nucleotides that are copied from a target nucleic acid. The length of the extension product can be controlled, for example, using reversibly terminated nucleotides in the extension reaction and running a limited number of extension cycles. The cycles can be run as exemplified for SBS techniques and the use of labeled nucleotides is not necessary. Accordingly, an extended probe produced in a method set forth herein can include no more than 1000, 500, 200, 100, 50, 25, 10, 5, 2 or 1 nucleotides that are copied from a target nucleic acid. Of course extended probes can be any length within or outside of the ranges set forth above.

[0094] Although the methods of the present disclosure are exemplified by an embodiment where probes that are hybridized to target nucleic acids are extended to copy at least a portion of the target nucleic acid, it will be understood that the probes can be modified in alternative ways. The probes that are hybridized to target nucleic acids can be subjected to a reaction that creates a target specific modification of the probe. A target specific modification will result only when the probe interacts with a target nucleic acid, for example, via complementary based hybridization. In many embodiments, the target specific modification will be specific to the sequence of the particular target nucleic acid that interacts with the probe. Examples of useful target specific modifications, include but are not limited to, insertion or addition of a sequence by ligation or transposition (see, for example, US Pat. App. Publ. No. 2010/0120098 A1), chemical modifications such as psoralen crosslinking or addition of a detectable tag moiety, modifications by nucleic acid enzymes, ligation of a hairpin linker, or other modifications set forth in the nucleic acid assays of US Pat. App. Publ. No. US 2005/0037393 A1 or US Pat. No. 8,288,103 or 8,486,625.

[0095] It will be understood that probes used in a method, composition or apparatus set forth herein need not be nucleic acids. Other molecules can be used such as proteins, carbohydrates, small molecules, particles or the like. Probes can be a combination of a nucleic acid component (e.g. having a barcode, primer binding site, cleavage site and/or other sequence element set forth herein) and another moiety (e.g. a moiety that captures or modifies a target nucleic acid).

[0096] A method set forth herein can further include a step of acquiring an image of a biological specimen that is in contact with a solid support. The solid support can be in any of a variety of states set forth herein. For example, the solid support can include attached nucleic acid probes or clusters derived from attached nucleic acid probes. Alternatively, the solid support may not include nucleic acid probes, instead being in a state that precedes attachment of nucleic acid probes or in a state that follows removal of nucleic acid probes from the solid support. Accordingly, an image can be obtained at any of a variety of points in a method set forth herein.

[0097] An image can be obtained using detection devices known in the art. Examples include microscopes configured for light, bright field, dark field, phase contrast, fluorescence, reflection, interference, or confocal imaging. A biological specimen can be stained prior to imaging to provide contrast between different regions or cells. In some embodiments, more than one stain can be used to image different aspects of the specimen (e.g. different regions of a tissue, different cells, specific subcellular components or the like). In other embodiments, a biological specimen can be imaged without staining.

[0098] In particular embodiments, a fluorescence microscope (e.g. a confocal fluorescent microscope) can be used to detect a biological specimen that is fluorescent, for example, by virtue of a fluorescent label. Fluorescent specimens can also be imaged using a nucleic acid sequencing device having optics for fluorescent detection such as a Genome Analyzer®, MiSeq®, NextSeq® or HiSeq® platform device commercialized by Illumina, Inc. (San Diego, CA); or a SOLiD™ sequencing platform commercialized by Life Technologies (Carlsbad, CA). Other imaging optics that can be used include those that are found in the detection devices described in Bentley et al., Nature 456:53-59 (2008), PCT Publ. Nos. WO 91/06678, WO 04/018497 or WO 07/123744; US Pat. Nos. 7,057,026, 7,329,492, 7,211,414, 7,315,019 or 7,405,281, and US Pat. App. Publ. No. 2008/0108082.

[0099] An image of a biological specimen can be obtained at a desired resolution, for example, to distinguish tissues, cells or subcellular components. Accordingly, the resolution can be sufficient to distinguish components of a biological specimen that are separated by at least 0.5 µm, 1 µm, 5 µm, 10 µm, 50 µm, 100 µm, 500 µm, 1 mm or more. Alternatively or additionally, the resolution can be set to distinguish components of a biological specimen that are separated by at least 1 mm, 500 µm, 100 µm, 50 µm, 10 µm, 5 µm, 1 µm, 0.5 µm or less.

[0100] A method set forth herein can include a step of correlating locations in an image of a biological specimen with barcode sequences of nucleic acid probes that are attached to a surface to which the biological specimen is, was or will be contacted. Accordingly, characteristics of the biological specimen that are identifiable in the image can be correlated with the nucleic acids that are found to be present in their proximity. Any of a variety of morphological characteristics can be used in such a correlation, including for example, cell shape, cell size, tissue shape, staining patterns, presence of particular proteins (e.g. as detected by immunohistochemical stains) or other characteristics that are routinely evaluated in pathology or research applications. Accordingly, the biological state of a tissue or its

components as determined by visual observation can be correlated with molecular biological characteristics as determined by spatially resolved nucleic acid analysis.

[0101] A solid support upon which a biological specimen is imaged can include fiducial markers to facilitate determination of the orientation of the specimen or the image thereof in relation to probes that are attached to the solid support. Exemplary fiducials include, but are not limited to beads (with or without fluorescent moieties or moieties such as nucleic acids to which labeled probes can be bound), fluorescent molecules attached at known or determinable features, or structures that combine morphological shapes with fluorescent moieties. Exemplary fiducials are set forth in US Pat. App. Publ. No. 2002/0150909 A1 or US Pat. No. 10,540,783. One or more fiducials are preferably visible while obtaining an image of a biological specimen. Preferably, the solid support includes at least 2, 3, 4, 5, 10, 25, 50, 100 or more fiducial markers. The fiducials can be provided in a pattern, for example, along an outer edge of a solid support or perimeter of a location where a biological specimen resides. In a preferred embodiment, one or more fiducials are detected using the same imaging conditions used to visualize a biological specimen. However if desired separate images can be obtained (e.g. one image of the biological specimen and another image of the fiducials) and the images can be aligned to each other.

[0102] Optionally, a biological specimen, can be removed from a solid support after an image has been obtained and after target nucleic acids have been captured by nucleic acid probes on the solid support. Thus, a method of the present disclosure can include a step of washing a solid support to remove cells, tissue or other materials from a biological specimen. Removal of the specimen can be performed using any suitable technique and will be dependent on the tissue sample. In some cases, the solid support can be washed with water. The water can contain various additives, such as surfactants (e.g. detergents), enzymes (e.g. proteases and collagenases), cleavage reagents, or the like, to facilitate removal of the specimen. In some embodiments, the solid support is treated with a solution comprising a proteinase enzyme. Alternatively or additionally, the solution can include cellulase, hemicelluase or chitinase enzymes (e.g. if desiring to remove a tissue sample from a plant or fungal source). In some cases, the temperature of a wash solution will be at least 30°C, 35°C, 50°C, 60°C or 90°C. Conditions can be selected for removal of a biological specimen while not denaturing hybrid complexes formed between target nucleic acids and solid support-attached nucleic acid probes.

[0103] A method of the present disclosure can further include a step of removing one or more extended probes from a solid support. In particular embodiments, the probes will have included a cleavage site such that the product of extending the probes will also include the cleavage site. Alternatively, a cleavage site can be introduced into a probe during a modification step. For example a cleavage site can be introduced into an extended probe during the extension step.

[0104] Exemplary cleavage sites include, but are not limited to, moieties that are susceptible to a chemical, enzymatic or physical process that results in bond breakage. For example, the

location can be a nucleotide sequence that is recognized by an endonuclease. Suitable endonucleases and their recognition sequences are well known in the art and in many cases are even commercially available (e.g. from New England Biolabs, Beverley MA; ThermoFisher, Waltham, MA or Sigma Aldrich, St. Louis MO). A particularly useful endonuclease will break a bond in a nucleic acid strand at a site that is 3'-remote to its binding site in the nucleic acid, examples of which include Type II or Type IIs restriction endonucleases. In some embodiments an endonuclease will cut only one strand in a duplex nucleic acid (e.g. a nicking enzyme). Examples of endonucleases that cleave only one strand include Nt.BstNBI and Nt.AlwI.

[0105] In some embodiments, a cleavage site is an abasic site or a nucleotide that has a base that is susceptible to being removed to create an abasic site. Examples of nucleotides that are susceptible to being removed to form an abasic site include uracil and 8-oxo-guanine. Abasic sites can be created by hydrolysis of nucleotide residues using chemical or enzymatic reagents. Once formed, abasic sites may be cleaved (e.g. by treatment with an endonuclease or other single-stranded cleaving enzyme, exposure to heat or alkali), providing a means for site-specific cleavage of a nucleic acid. An abasic site may be created at a uracil nucleotide on one strand of a nucleic acid. The enzyme uracil DNA glycosylase (UDG) may be used to remove the uracil base, generating an abasic site on the strand. The nucleic acid strand that has the abasic site may then be cleaved at the abasic site by treatment with endonuclease (e.g. EndoIV endonuclease, AP lyase, FPG glycosylase/AP lyase, EndoVIII glycosylase/AP lyase), heat or alkali. In a particular embodiment, the USER™ reagent available from New England Biolabs is used for the creation of a single nucleotide gap at a uracil base in a nucleic acid.

[0106] Abasic sites may also be generated at non-natural/modified deoxyribonucleotides other than uracil and cleaved in an analogous manner by treatment with endonuclease, heat or alkali. For example, 8-oxo-guanine can be converted to an abasic site by exposure to FPG glycosylase. Deoxyinosine can be converted to an abasic site by exposure to AlkA glycosylase. The abasic sites thus generated may then be cleaved, typically by treatment with a suitable endonuclease (e.g. EndoIV or AP lyase).

[0107] Other examples of cleavage sites and methods that can be used to cleave nucleic acids are set forth, for example, in US Pat. No. 7,960,120.

[0108] Modified nucleic acid probes (e.g. extended nucleic acid probes) that are released from a solid support can be pooled to form a fluidic mixture. The mixture can include, for example, at least 10, 100, 1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 or more different modified probes. Alternatively or additionally, a fluidic mixture can include at most 1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 100, 10 or fewer different modified probes. The fluidic mixture can be manipulated to allow detection of the modified nucleic acid probes. For example, the modified nucleic acid probes can be separated spatially on a second solid support (i.e. different from the solid support from which the nucleic acid probes were released after having been contacted with a biological specimen and modified), or the probes can be

separated temporally in a fluid stream.

[0109] Modified nucleic acid probes (e.g. extended nucleic acid probes) can be separated on a solid support in a capture or detection method commonly employed for microarray-based techniques or nucleic acid sequencing techniques such as those set forth previously herein. For example, modified probes can be attached to a microarray by hybridization to complementary nucleic acids. The modified probes can be attached to beads or to a flow cell surface and optionally amplified as is carried out in many nucleic acid sequencing platforms. Modified probes can be separated in a fluid stream using a microfluidic device, droplet manipulation device, or flow cytometer. Typically, detection is carried out on these separation devices, but detection is not necessary in all embodiments.

[0110] A particularly useful droplet manipulation device is a droplet actuator as described for example in US Pat. No. 8,637,242, US Pat. No. 6,911,132, entitled "Apparatus for Manipulating Droplets by Electrowetting-Based Techniques," issued on June 28, 2005; Pamula et al., U.S. Patent Pub. No. 20060194331, entitled "Apparatuses and Methods for Manipulating Droplets on a Printed Circuit Board," published on August 31, 2006; Pollack et al., International Patent Pub. No. WO/2007/120241, entitled "Droplet-Based Biochemistry," published on October 25, 2007; Shenderov, U.S. Patent No. 6,773,566, entitled "Electrostatic Actuators for Microfluidics and Methods for Using Same," issued on August 10, 2004; Shenderov, U.S. Patent No. 25 6,565,727, entitled "Actuators for Microfluidics Without Moving Parts," issued on May 20, 2003; Kim et al., U.S. Patent Pub. No. 20030205632, entitled "Electrowettingdriven Micropumping," published on November 6, 2003; Kim et al., U.S. Patent Pub. No. 20060164490, entitled "Method and Apparatus for Promoting the Complete Transfer of Liquid Drops from a Nozzle," published on July 27, 2006; Kim et al., U.S. Patent Pub. No. 20070023292, entitled "Small Object Moving on Printed Circuit Board," published on February 1, 2007; Shah et al., U.S. Patent Pub. No. 20090283407, entitled "Method for Using Magnetic Particles in Droplet Microfluidics," published on November 19, 2009; Kim et al., U.S. Patent Pub. No. 20100096266, entitled "Method and Apparatus for Real-time Feedback Control of Electrical Manipulation of Droplets on Chip," published on April 22, 2010; Velev, U.S. Patent No. 7,547,380, entitled "Droplet Transportation Devices and Methods Having a Fluid Surface," issued on June 16, 2009; Sterling et al., U.S. Patent No. 7,163,612, entitled "Method, Apparatus and Article for Microfluidic Control via Electrowetting, for Chemical, Biochemical and Biological Assays and the Like," issued on January 16, 2007; Becker et al., U.S. Patent No. 7,641,779, entitled "Method and Apparatus for Programmable Fluidic Processing," issued on January 5, 2010; Becker et al., U.S. Patent No. 6,977,033, entitled "Method and Apparatus for Programmable Fluidic Processing," issued on December 20, 2005; Decre et al., U.S. Patent No. 7,328,979, entitled "System for Manipulation of a Body of Fluid," issued on February 12, 2008; Yamakawa et al., U.S. Patent Pub. No. 15 20060039823, entitled "Chemical Analysis Apparatus," published on February 23, 2006; Wu, U.S. Patent Pub. No. 20110048951, entitled "Digital Microfluidics Based Apparatus for Heat-exchanging Chemical Processes," published on March 3, 2011; Fouillet et al., U.S. Patent Pub. No. 20090192044, entitled "Electrode Addressing Method," published on July 30, 2009; Fouillet et al., U.S. Patent No. 7,052,244, entitled "Device for Displacement of Small Liquid Volumes Along a Micro-catenary Line by

Electrostatic Forces," issued on May 30, 2006; Marchand et al., U.S. Patent Pub. No. 20080124252, entitled "Droplet Microreactor," published on May 29, 2008; Adachi et al., U.S. Patent Pub. No. 20090321262, entitled "Liquid Transfer Device," published on December 31, 2009; Roux et al., U.S. Patent Pub. No. 20050179746, entitled "Device for Controlling the Displacement of a Drop Between Two or Several Solid Substrates," published on August 18, 2005; and Dhindsa et al., "Virtual Electrowetting Channels: Electronic Liquid Transport with Continuous Channel Functionality," Lab Chip, 10:832-836 (2010).

[0111] Modified probes (e.g. extended nucleic acid probes) can be detected, for example, following separation from a fluidic mixture using methods set forth above or known in the art. In particular embodiments, modified probes that are separated on a second solid support (i.e. a solid support that is different from the first solid support where contact was made between probes and biological specimen) can be detected using microarray-based techniques or nucleic acid sequencing techniques such as those set forth previously herein. Probes that are separated in a fluid stream can be detected using optical, electrical or other detectors that are outfitted in known microfluidic devices, droplet manipulation devices, or flow cytometers. A detection method can be used to determine target nucleic acid sequences, barcode sequences or other sequence regions of extended probes.

[0112] Several embodiments have been exemplified with regard to removing modified probes from the solid support where the probes were produced. However, it will be understood that probes on a solid support can be contacted with a biological specimen, modified on the solid support in the presence of target nucleic acids from the specimen and then the modified probes can be detected on the solid support. In such an embodiment, the biological specimen can be removed from the solid support prior to the detection step.

[0113] In particular embodiments the present disclosure provides a method for spatially tagging nucleic acids of a biological specimen that includes the steps of (a) providing a plurality of nucleic acid primers attached to a solid support, wherein the nucleic acid primers in the plurality include a universal primer sequence that is common to the nucleic acid primers in the plurality; (b) binding a population of nucleic acid probes to the plurality of nucleic acid primers, wherein the nucleic acid probes include a universal primer binding sequence that hybridizes to the universal primer sequence, a target capture sequence and a barcode sequence that differs from barcode sequences of other nucleic acid probes in the population, thereby attaching the different nucleic acid probes at randomly located positions on the solid support; (c) amplifying the different nucleic acid probes by extension of the nucleic acid primers, thereby producing nucleic acid clusters having copies of the barcode sequence and target capture sequence at the randomly located positions on the solid support; (d) performing a sequencing reaction to determine the barcode sequences at the randomly located positions on the solid support; (e) contacting a biological specimen with the nucleic acid clusters on the solid support; (f) hybridizing the target capture sequences of the clusters to target nucleic acids from portions of the biological specimen that are proximal to the clusters; and (g) extending the target capture sequences to produce extended probes that include sequences from the target nucleic acids and the copies of the barcode sequences, thereby tagging the nucleic acids of the biological

specimen.

[0114] As exemplified previously herein, a plurality of nucleic acid primers can be attached to a solid support, wherein the nucleic acid primers in the plurality include a universal primer sequence that is common to the nucleic acid primers in the plurality. In this embodiment, a second plurality of nucleic acid primers can be attached to the solid support, and the nucleic acid primers in the second plurality can have a second universal primer sequence that is common to the nucleic acid primers in the second plurality. In this embodiment, a plurality of different nucleic acid probes that is contacted with the support can include a universal primer binding sequence that hybridizes to the universal primer on the solid support, as set forth above, and the different nucleic acid probes can also include a second universal primer binding sequence that hybridizes to the second universal primer sequence. This configuration of universal primers and universal primer binding sites can be particularly useful for amplifying the different nucleic acid probes via bridge amplification, wherein the nucleic acid primers in the first and second plurality are extended.

[0115] Typically, when a nucleic acid probe contains first and second universal primer binding sites, they will be located at the ends of the probe. In some embodiments it may be desirable to remove at least one of the primer binding sites from the nucleic acid probe or from amplicons produced from the probe. Accordingly, the nucleic acid probes can optionally include a cleavage site between the target capture sequence and one of the universal primer binding sequence. In this case, a cleavage reaction can be performed to separate the universal primer binding site from the target capture sequence. Generally, the portion of the probe (or its amplicons) that contains the target capture sequence will be attached to the solid support resulting in removal of the primer binding site from the solid support and retention of the target capture sequence. Thus, the cleaved probe can be used for hybridizing target nucleic acids and the cleaved probe can be extended using method set forth previously herein.

[0116] In some embodiments, a nucleic acid probe will include two different cleavage sites. A first cleavage site will be located between a first primer binding site and one or more other sequence elements of the probe. A second cleavage site can be located between a second primer binding site and the one or more other sequence elements of the probe. The cleavage sites can be reactive to different cleavage reactions such that each one can be selectively cleaved without necessarily cleaving the other. Accordingly, the first cleavage site can be cleaved prior to modifying the probe (for example, prior to producing an extended probe), thereby separating the first primer binding site from the one or more other sequence elements that remain attached to a solid support. The second cleavage site can be cleaved after modifying the probe (for example, after producing the extended probe), thereby releasing the modified probe for subsequent detection.

[0117] Alternatively, a nucleic acid probe can include the first cleavage site and a primer that is used to capture or amplify the nucleic acid probe can include the second cleavage site. In this configuration, the first cleavage site can be located between a first primer binding site and one or more other sequence elements of the probe such that cleavage separates the first primer

binding site from one or more other sequence elements of the probe that remain attached to a solid support. Again, this first cleavage step will typically be carried out prior to modifying the probe (for example, prior to producing an extended probe). A second cleavage step can be carried out to cleave the second cleavage site after modifying the probe (for example, after producing the extended probe), thereby releasing the modified probe for subsequent detection.

[0118] The two embodiments above exemplify a cleavage site located between a point of attachment of a nucleic acid probe (or modified nucleic acid probe) and one or more sequences of the probe (or modified probe) that contain information such as a spatial barcode or target sequence. Thus, this cleavage site is useful for release of modified probes (e.g. extended probes) to detect the sequence information and determine what sequences are present in a biological specimen and where the sequences are present in the specimen.

[0119] In some embodiments, one or more probes that are contacted with a solid support in a method set forth herein can include a sequencing primer binding site. Accordingly, a modified probe (e.g. extended probe) can be detected in a sequencing technique that includes a step of hybridizing a sequencing primer to the sequencing primer binding site. The sequencing primer binding site can be located in the probe such that cleavage of a modified version of the probe (e.g. an extended probe) will yield a released probe that includes the sequencing primer binding site. The sequencing primer binding site can be a universal sequencing primer binding site such that a plurality of different probes (e.g. having different barcode and/or target sequences) will have the same sequencing primer binding site.

[0120] This disclosure further provides a method for spatially tagging nucleic acids of a biological specimen, the method including steps of (a) providing an array of beads on a solid support, wherein different nucleic acid probes are attached to different beads in the array, wherein the different nucleic acid probes each include a barcode sequence, wherein each bead includes a different barcode sequence from other beads on the solid support, and wherein each of the different nucleic acid probes includes a target capture sequence; (b) performing a decoder probe hybridization reaction on the solid support to determine the barcode sequences at the randomly located probes on the solid support; (c) contacting a biological specimen with the array of beads; (d) hybridizing the different nucleic acid probes to target nucleic acids from portions of the biological specimen that are proximal to the beads; and (e) extending the different nucleic acid probes to produce extended probes that include sequences from the target nucleic acids and the barcode sequences, thereby tagging the nucleic acids of the biological specimen.

[0121] It will be understood that manipulations of solid supports or of nucleic acids attached to solid supports can be carried out using beads as solid supports. The beads can be attached to a surface (e.g. an array of wells as in a BeadArray™ from Illumina) before or after such manipulations are carried out. For example, nucleic acid probes can be captured on beads before or after the beads are distributed on an array, nucleic acid probes can be amplified to create amplicons on beads before or after the beads are distributed on an array etc.

EXAMPLE I

Spatially Tagging mRNA from a Tissue Sample Using Illumina Flow Cells

[0122] A method for generating barcoded oligo-dT containing clusters, then revealing the barcoded oligo-dT with a restriction enzyme digest followed by sequencing is described in Fig. 1. A library of fragments containing a single stranded, barcoded oligo-dA, P5',P7, SBS3 sequencing primer binding site and a BspHI restriction enzyme site (shown in the top panel of Fig. 1) were prepared by oligo synthesis (Integrated DNA Technologies). The barcodes were 27mers and were randomly generated during synthesis. The binding site for the SBS3 sequencing primer was included for decoding of the barcode by sequencing. An oligo-dA stretch was included to generate an oligo dT site upon clustering and linearization. Bridge amplification and clustering were performed according to standard cluster chemistry (Illumina TruSeq PE Cluster Kit v3 cBot P/N: 15037931) on an Illumina GA flow cell using manufacture's recommended protocol.

[0123] Following bridge amplification and clustering the clusters were linearized by cleavage of 8-oxo-G in P7 primer using Formamidopyrimidine DNA glycosylase (Fpg) enzyme provided in the TruSeq PE Cluster kit. This was followed by restriction enzyme digest with 200 Units/mL BspH1 (NEB Cat # R0517L at 37°C for 15min to remove P7' from the P5 adapter anchored strand of the cluster to unveil the oligo-dT stretch for subsequent extension in the presence of an mRNA. Enzyme concentrations in the range of 100-400U/mL have been tested for 15 or 30min. The de-coding of the barcode was initiated by the SBS3 sequencing primer.

[0124] As shown in the bottom panel of Fig. 1, oligo-dT sequences in the cluster were used to capture poly A+ RNA after decoding of the barcode. Barcoded cDNA was produced by extension of the oligo-dT strand of the cluster using TruSeq RNA Sample Prep Kit (Illumina P/N: 15012997) and MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre P/N: MM070150) according to the manufacturer's recommended conditions. The captured RNA was used as a template. Barcoded cDNA was released from the P5 sequence of the flow cell using Illumina's Uracil Specific Excision reagents (USER) (Illumina's TruSeq PE cluster kit) liberating a barcoded cDNA library that was used for sequencing on a second Illumina flow cell.

[0125] The availability of oligo dT capture sequence after the restriction enzyme digest with BspH1 was confirmed by hybridizing the linearized clusters with a Cy5 labeled poly A (24mer) as diagrammed in panel A of Fig. 2. Briefly, after the restriction enzyme digestion, the clusters were treated with 0.1 N NaOH and washed with HT2 low salt buffer to remove the second strand on the flow cell. Then, 500nM of Cy5 oligo-dA (24mer) was flowed over the linearized and denatured clusters at 30µl / min rate and incubated at 40°C for 5min and then imaged. Hybridization of Cy5 labeled poly A to the oligo dT was detected in lanes 2-7 of the GA flow cell where the oligo dT containing BODT-1 libraries were present (see the image of the flow cell

shown in Fig. 2, Panel B). As evident from the flow cell image (Panel B), and the bargraph (Panel C), the control PhiX libraries (lanes 1 and 8 of the flow cell) were shown to have very low fluorescence in the Cy5 signal. These results demonstrated that an oligo-dT site can be created in the cluster that upon linearization can bind specifically to Cy5 poly A (24mer).

[0126] The sequencing metrics of the flow cell described above with 3.2pM of BODT-1 library is given in the table shown in Fig. 3. Millions of reads were detected in 21 tiles from GA sequencing. Following sequencing, the number of unique barcodes were determined as plotted in Fig. 4. This was done by assuming that every passing filter (PF) read was a barcode and determining the number of unique reads (barcodes) in each lane. Between 5 and 11 million unique barcoded clusters were detected after sequencing tiles compared to the PhiX control libraries. These results demonstrated that sequence decoding of a library of barcoded oligo-dT sequences is feasible and generates millions of unique barcodes.

EXAMPLE II

Cell Adhesion on Illumina Flow Cells

[0127] Single cells were captured on a patterned flow cell (HiSeq X10 flow cell, Illumina). All reagent flow steps were performed using a peristaltic pump or the cBOT cluster generation instrument (Illumina). Briefly, nuclease free water was flowed on all lanes of the patterned flow cell followed by 30-70K Poly D Lysine Solution (100 µg/ml and 20 µg/ml) at a flow rate of 100 µl/min for 8 min. Heat inactivated Fetal Bovine Serum (Life Technologies #10082-139) was also tested as an adhesive. The adhesives were incubated on the flow cell lanes for 1 hr, followed by a 1X PBS + 0.5% Pluronic F-68 (Life Technologies #24040-032) wash. Next, the cells were adhered to the coated flow cells by flowing 5 to 50 cells / µl or approximately 100 - 1000 cells per lane at a rate of 100 µl / min, followed by an incubation step for 60min to bind the cells. The flow cell was washed with 1X PBS/0.5% pluronic at a rate of 75 µl / min. If cells were fixed on the flow cell, 1% Paraformaldehyde (PFA) was flowed on the flow cell after flowing the cells as described above and incubated for 15min followed by the 1X PBS/0.5% pluronic was step. The flow cell was removed and the number of cells per lane counted using a microscope.

[0128] Fig. 5, Panel A shows an image of cells captured on the patterned flow cell. The cell count data shown in Fig. 5, Panel B confirmed that the poly D Lysine coated flow cells aided cell adherence compared to the BSA coated or no adhesive treated control. As shown in Fig. 6, the adhered cells can be successfully fixed with 1% PFA.

EXAMPLE III

Spatially Localized Capture of Target mRNA by Probes Attached to a Gel Surface

[0129] This example describes creation of a lawn of poly T probes on a gel coated slide, placement of tissue slices on top of a lawn of poly T probes, release of RNA from the tissue sections, capture of the released mRNA by the poly T probes, reverse transcription to Cy 3 label the poly T probes, removal of the tissue and imaging of the slide.

[0130] Fig. 7, Panel A shows a diagrammatic representation of steps and reagents used to create probes attached to a gel. Briefly, a microscope slide was coated with silane free acrylamide (SFA), P5 and P7 primers were attached (see US Pat. App. Pub. No. 2011/0059865 A1), probes having a poly A sequence and either a P5 or P7 complementary sequence were hybridized to the P5 and P7 primers, respectively, and the P5 and P7 primers were extended to produce poly T sequence extensions. A quality control step was performed by hybridizing Cy5 labeled polyA oligonucleotides to the extended primers and imaging the surface using an Axon Imager.

[0131] As shown in Panel B of Fig. 7, a tissue section was placed on the gel having the polyT extended primers. The tissue was treated to release mRNA and poly A tails of the released mRNA were hybridized to poly T sequences of the extended primers. The poly T sequences were extended using the captured mRNAs as templates and the extended primers were selectively labeled with Cy3. The tissue was removed from the gel and the surface was imaged to detect Cy3 flourescence.

[0132] As shown in the image of Fig. 7, areas of the gel that were proximal to areas of the tissue that released mRNA species appeared fluorescent while areas that did not release mRNA appeared dark in the image. Thus, the captured mRNA created a fingerprint-like image of the tissue.

EXAMPLE IV

Spatially Localized Capture of Target mRNA by Probes Attached to a BeadArray™ Surface

[0133] This example describes placement of tissue slices on top of a BeadArray™ having poly T probes, release of RNA from the tissue sections, capture of the released mRNA by the poly T probes, reverse transcription to Cy5 label the poly T probes, removal of the tissue and imaging of the BeadArray™.

[0134] As shown in Panel A of Fig. 8, a mouse olfactory tissue section was placed on a BeadArray™ having polyT probes. The tissue was treated to release mRNA and poly A tails of

the released mRNA were hybridized to poly T sequences of the probes. The poly T sequences were extended using the captured mRNAs as templates and the extended primers were selectively labeled with Cy5. The tissue was removed from the BeadArray™ and the BeadArray™ was imaged to detect Cy5 flourescence.

[0135] As shown in Panel B of Fig. 7, areas of the BeadArray™ that were proximal to areas of the tissue that released mRNA species appeared fluorescent while areas that did not release mRNA appeared dark in the image. Thus, the captured mRNA created a fingerprint-like image of the tissue.

[0136] The term "comprising" is intended herein to be open-ended, including not only the recited elements, but further encompassing any additional elements.

REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Fremgangsmåde til rumlig tagging af nukleinsyrer i en biologisk prøve, som omfatter:

(a) tilvejebringelse af et array af korn på en fast understøtning, hvor forskellige nukleinsyreprober er bundet til forskellige korn i arrayet, hvor de forskellige nukleinsyreprober hver omfatter en stregkodesekvens, hvor hvert korn omfatter en stregkodesekvens, der er forskellig fra andre korn på den faste understøtning, og hvor hver af de forskellige nukleinsyreprober omfatter en målindfangningssekvens;

(b) udførelse af en afkoder-probehybridiseringsreaktion på den faste understøtning til bestemmelse af stregkodesekvenserne i de vilkårligt placerede prober på den faste understøtning;

(c) etablering af kontakt mellem en biologisk prøve og arrayet af korn;

(d) hybridisering af de forskellige nukleinsyreprober til målnukleinsyrer fra dele af den biologiske prøve, der er proksimalt for kornene;

(e) forlængelse af de forskellige nukleinsyreprober til forbringelse af forlængede prober, der omfatter sekvenser fra målnukleinsyrerne og stregkodesekvenserne til derved tagging af nukleinsyrerne i den biologiske prøve;

(f) fjernelse af de forlængede prober fra arrayet af korn, som eventuelt yderligere omfatter puljning af de forlængede prober til frembringelse af en blanding af de forlængede prober, der er blevet fjernet fra arrayet af korn; og

(g) binding af de forlængede prober, der er blevet fjernet fra den faste understøtning, til en anden fast understøtning.

2. Fremgangsmåde ifølge krav 1, hvor trin (a) omfatter vilkårlig fordeling af de forskellige korn på den faste understøtning.

3. Fremgangsmåde ifølge krav 1 eller 2, hvor den faste understøtning omfatter brønde med dimensioner, der højst rummer et enkelt korn af de forskellige korn, eventuelt hvor antallet af forskellige stregkodesekvenser overstiger antallet af brønde.

4. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 3, hvor arrayet af korn omfatter mindst 1×10^6 af de forskellige korn, der omfatter de forskellige nukleinsyreprober.

5. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 4, hvor arrayet af korn har en gennemsnitlig afstand på mindre end 10 µm, og/eller hvor de forskellige korn har en gennemsnitlig diameter på mindre end 10 µm.

6. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 5, hvor de forskellige nukleinsyreprober omfatter forskellige målindfangningssekvenser, der hybridiserer til forskellige målnukleinsyresekvenser fra den biologiske prøve.

7. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 5, hvor de forskellige nukleinsyreprober omfatter en målindfangningssekvens, der er fælles for de forskellige nukleinsyreprober i arrayet af korn, eventuelt hvor målindfangningssekvensen omfatter en poly-T- eller poly-A-sekvens.

8. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 7, som yderligere omfatter et trin med erhvervelse af et billede af den biologiske prøve, der er i kontakt med arrayet af korn, og eventuelt yderligere omfatter et trin med korrelering af stregkodesekvenserne af de forskellige korn med lokationer i billedet af den biologiske prøve.

9. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 8, som yderligere omfatter bestemmelse af sekvenserne fra målnukleinsyrerne og stregkodesekvenserne for de forlængede prober, der er blevet fjernet fra den faste understøtning.

10. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 9, hvor sekvenserne fra målnukleinsyrerne og stregkodesekvenserne bestemmes ved hjælp af en teknik med sekventering ved hjælp af syntese.

11. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 10, hvor den biologiske prøve, der bringes i kontakt med den faste understøtning, er en blanding af celler, eventuelt hvor trin (c) yderligere omfatter binding af cellerne til den faste understøtning og/eller lysering af cellerne til frigivelse af målnukleinsyrerne fra cellerne.

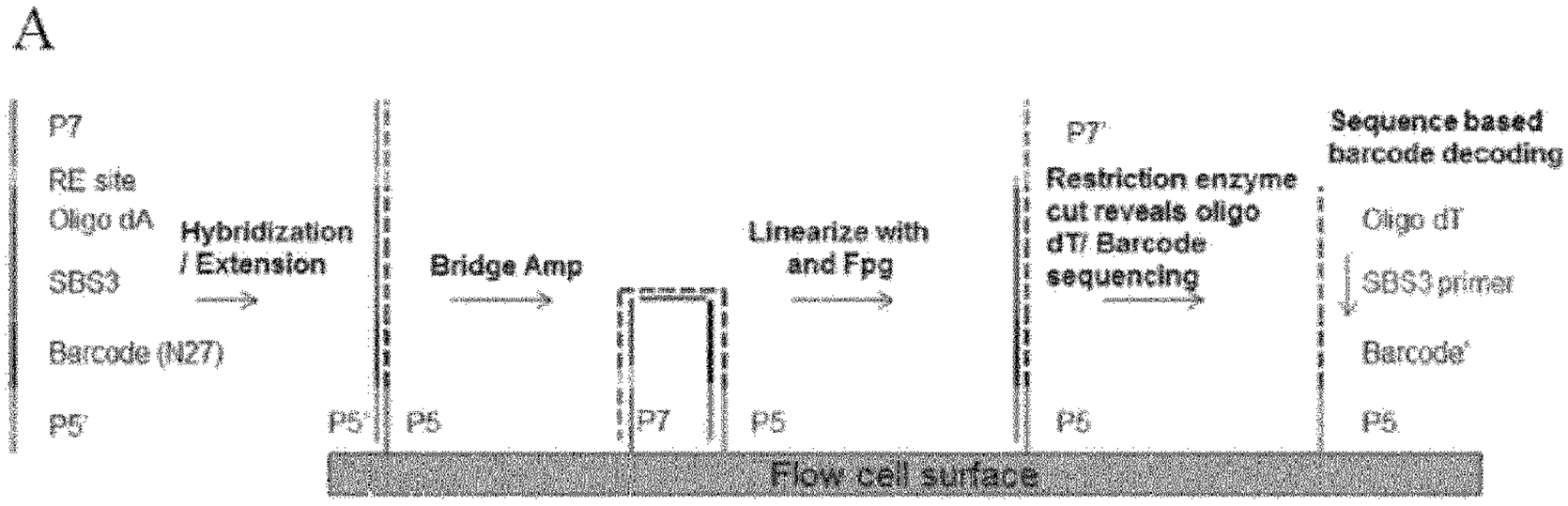
12. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 11, hvor den biologiske prøve, der bringes i kontakt med den faste understøtning, er et væv, eventuelt hvor trin (c) yderligere omfatter binding af vævet til den faste understøtning og/eller permeabilisering af vævet til frigivelse af målnukleinsyrerne fra vævet.

13. Fremgangsmåde ifølge et hvilket som helst af kravene 1 til 12, hvor målnukleinsyren er valgt fra gruppen, der består af mRNA, gDNA, rRNA eller tRNA.

DRAWINGS

Figure 1

A



B

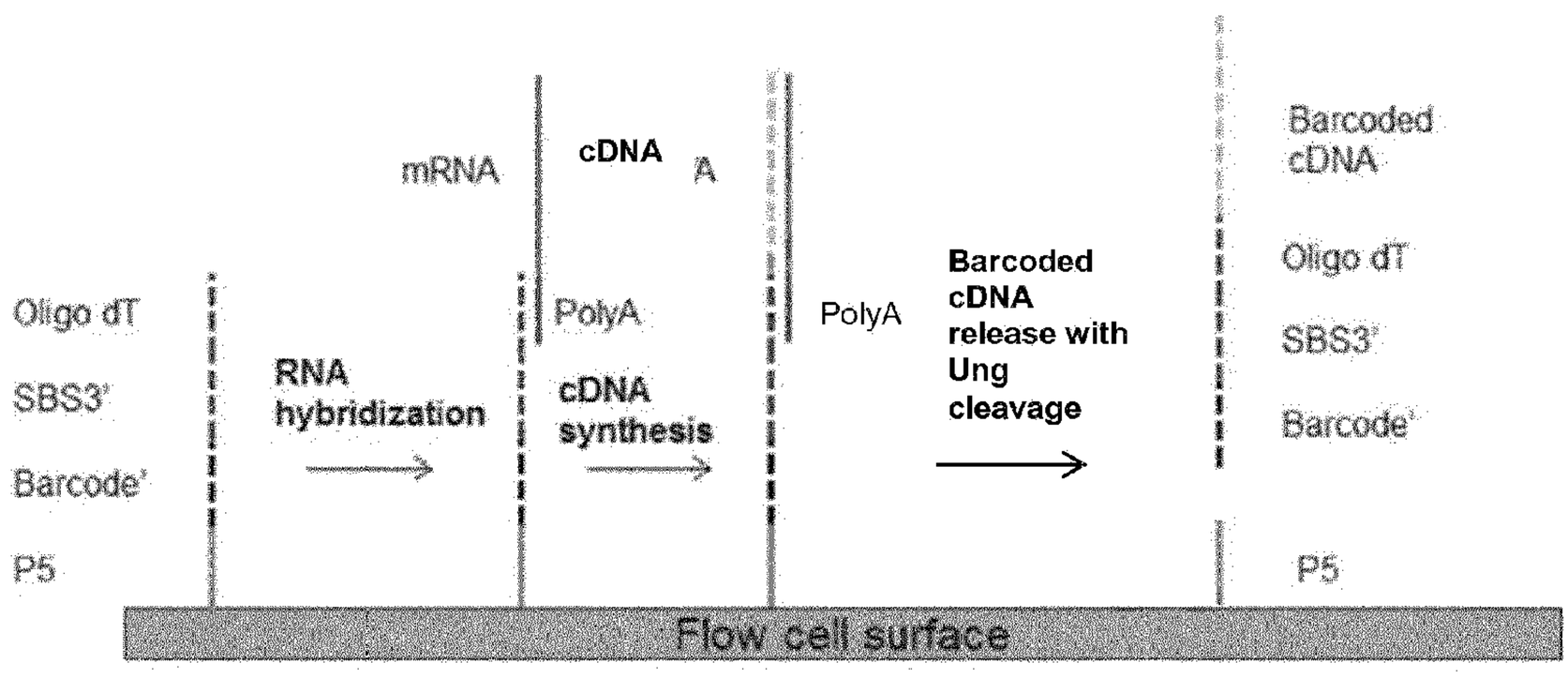
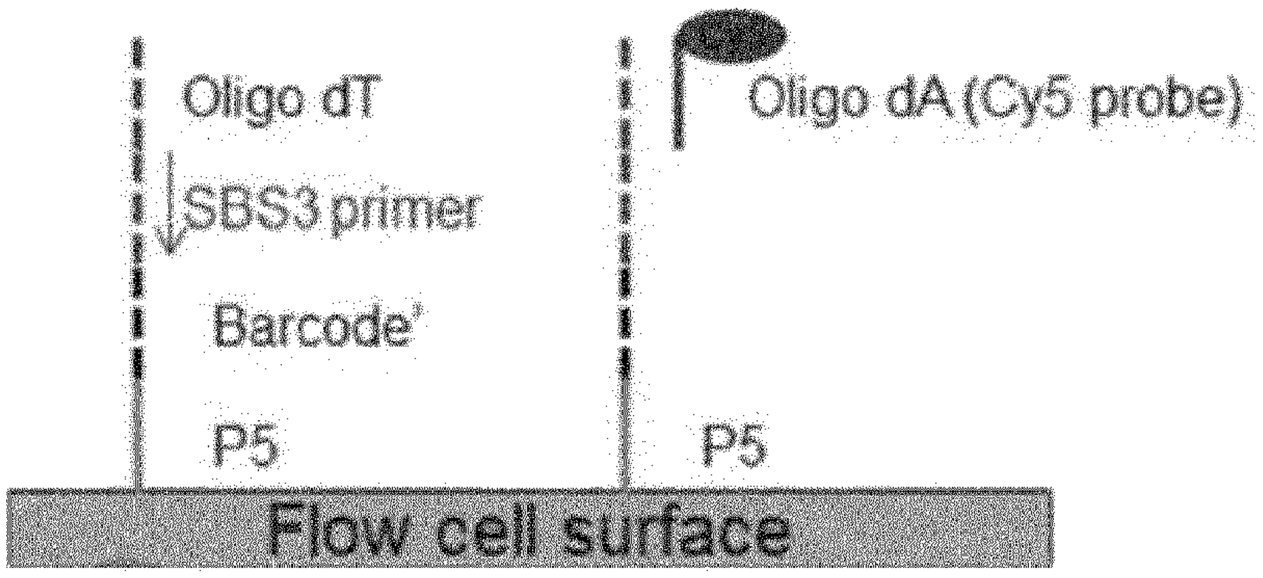
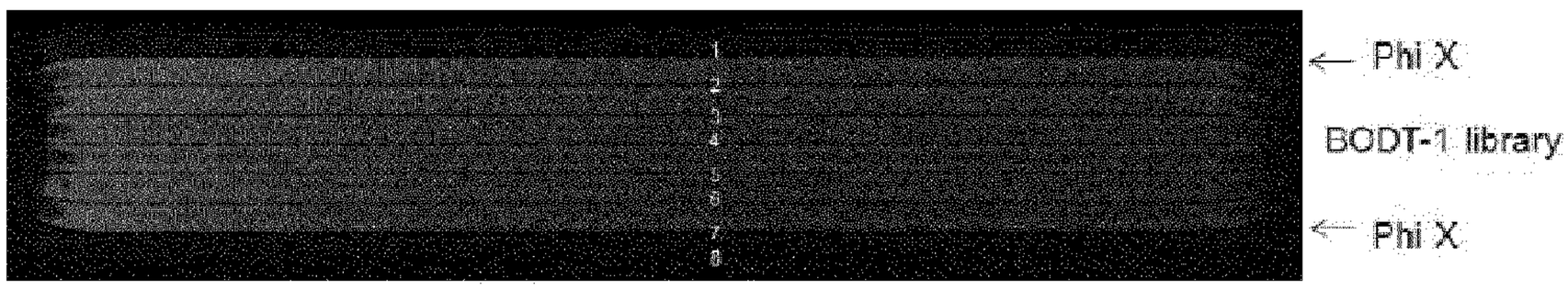


Figure 2

A


Oligo dT
SBS3 primer
Barcode
P5
Oligo dA (Cy5 probe)
P5
Flow cell surface

B


1
2
3
4
5
6
7
8
← Phi X
BODT-1 library
← Phi X

C

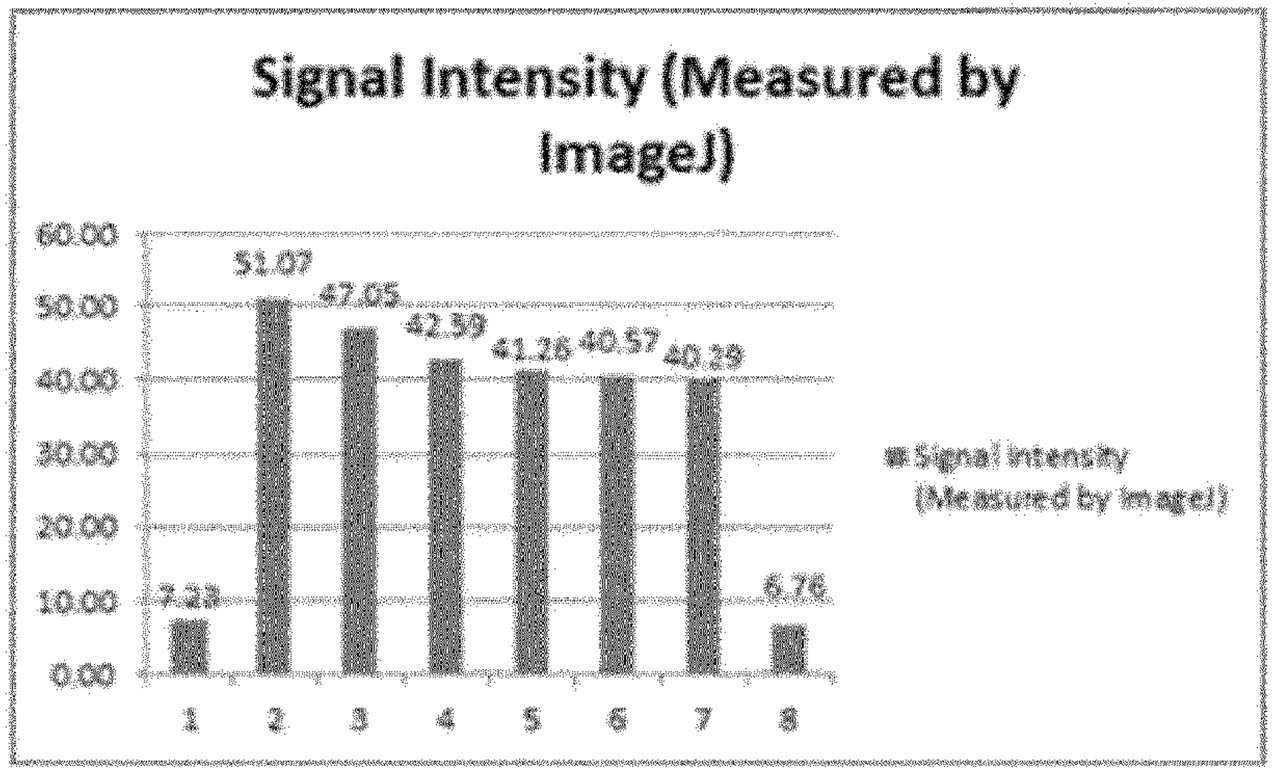

Signal Intensity (Measured by ImageJ)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
7.38
51.07
47.05
42.50
41.26
40.57
40.29
6.76
1
2
3
4
5
6
7
8
Signal Intensity (Measured by ImageJ)

Figure 3

Lane	Tiles	Density (K/mm2)	Clusters PF (%)	Phas/Prephas (%)	Reads (M)	Reads PF (M)	% >= Q30
1) Phi X	21	904 +/- 62	92.1 +/- 1.0	0.797 / 0.097	9.83	9.06	94.6
2) 3.2 nM BODT -1	21	1026 +/- 171	48.2 +/- 36.6	0.948 / 0.151	11.15	5.59	72.9
3) 3.2 nM BODT -1	21	975 +/- 186	41.9 +/- 43.2	0.964 / 0.154	10.6	5.03	70.8
4) 3.2 nM BODT -1	21	1144 +/- 24	85.3 +/- 14.3	0.969 / 0.163	12.45	10.62	76.4
5) 3.2 nM BODT -1	21	1136 +/- 37	90.6 +/- 0.8	0.977 / 0.173	12.35	11.19	80.5
6) 3.2 nM BODT -1	21	1128 +/- 28	90.5 +/- 0.7	0.953 / 0.182	12.27	11.1	80.6
7) 3.2 nM BODT -1	21	1131 +/- 28	90.3 +/- 0.8	0.955 / 0.170	12.3	11.1	82.6
8) Phi X	21	763 +/- 41	91.3 +/- 0.4	0.756 / 0.135	8.3	7.58	94

Figure 4

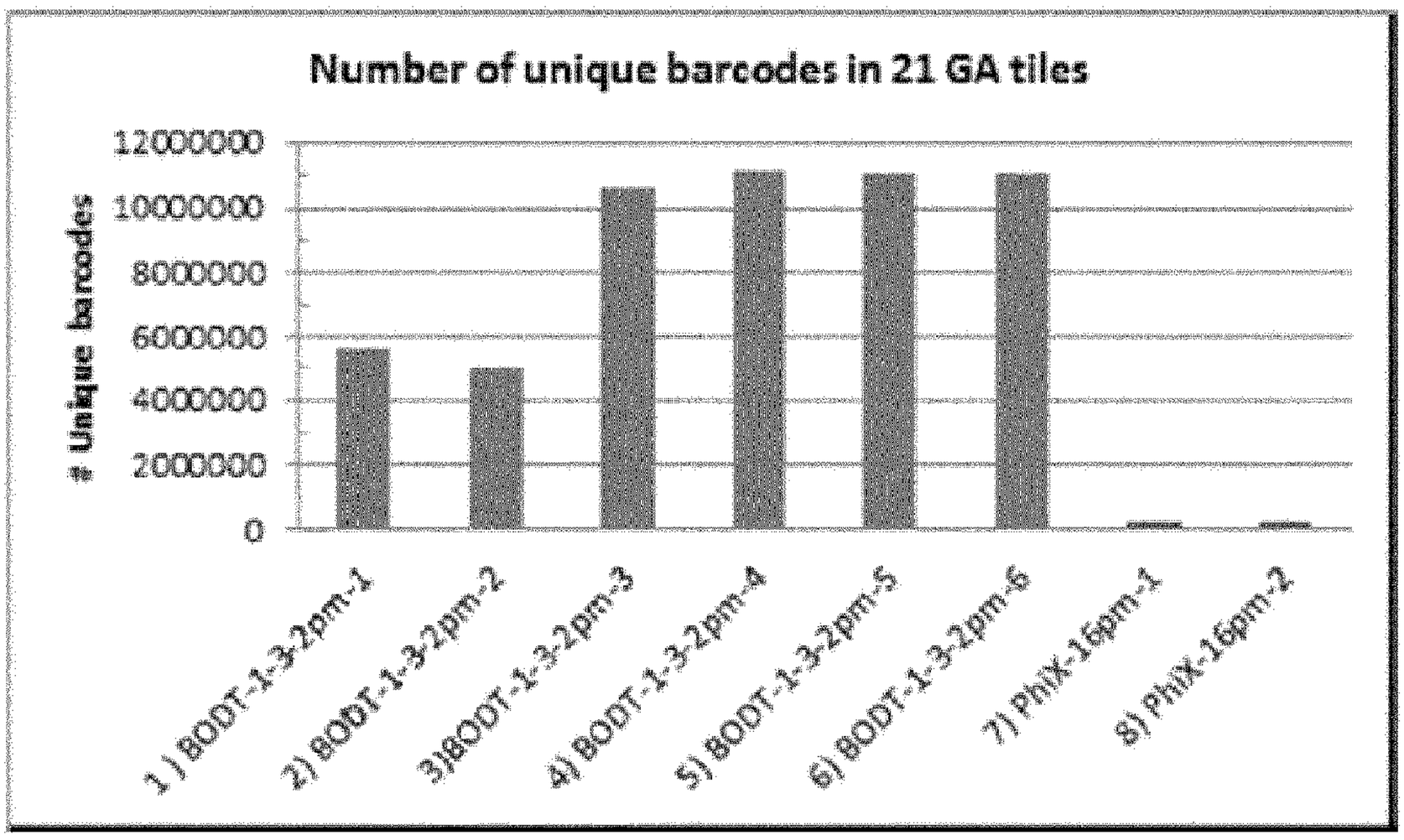
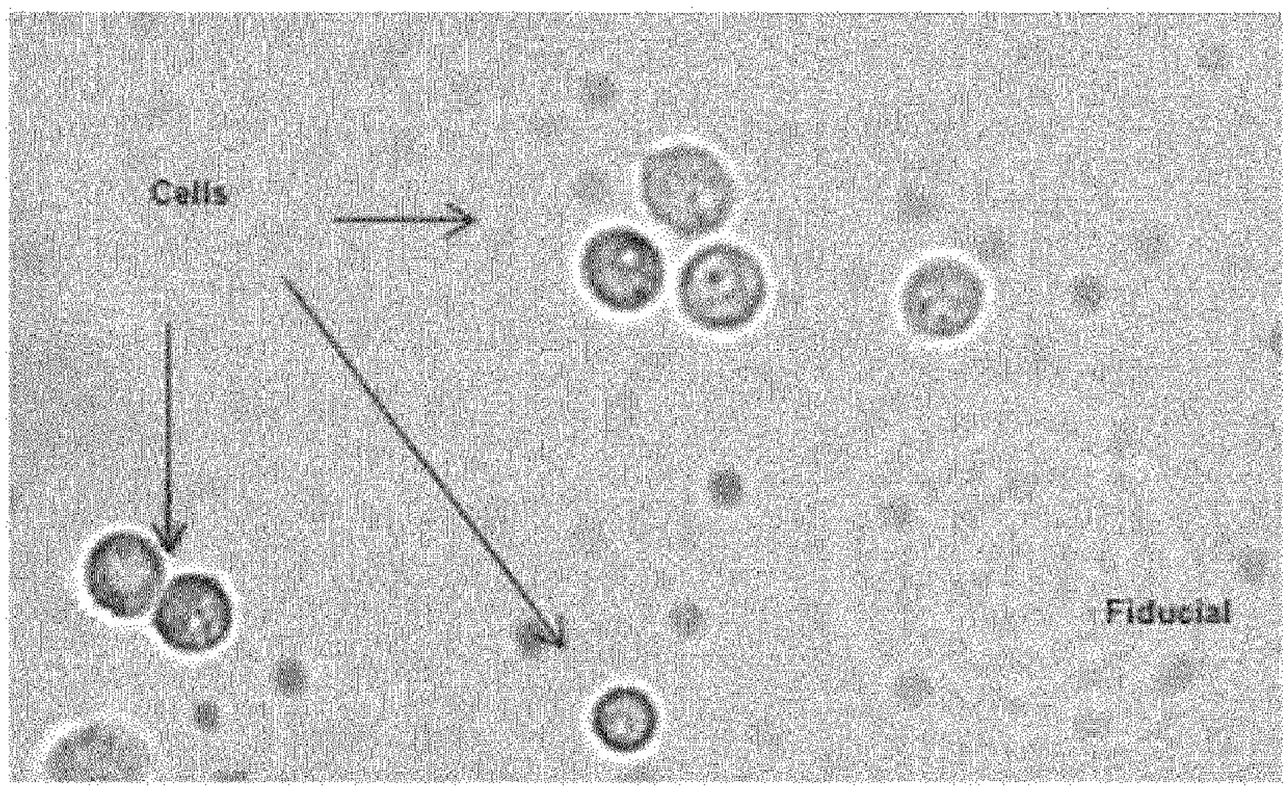

Number of unique barcodes in 21 GA tiles
Unique barcodes
12000000
10000000
8000000
6000000
4000000
2000000
0
1) BODT-1-3-2pm-1
2) BODT-1-3-2pm-2
3)&ODT-1-3-2pm-3
4) BODT-1-3-2pm-4
5) BODT-1-3-2pm-5
6) BODT-1-3-2pm-6
7) PhiX-16pm-1
8) PhiX-16pm-2

Figure 5

A


Cells
Fiducial

B

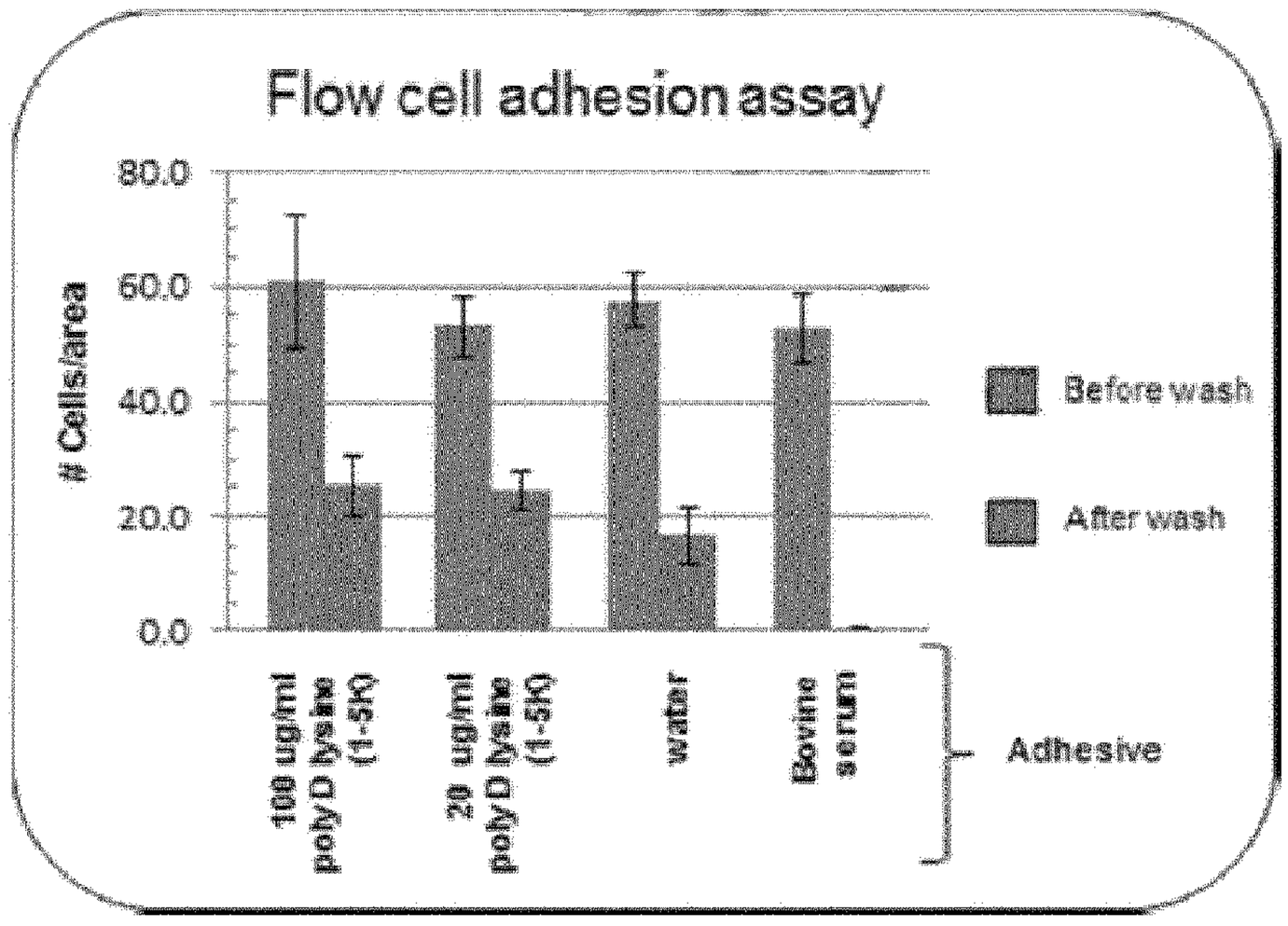

Flow cell adhesion assay
Cells/area
80.0
60.0
40.0
20.0
0.0
100 ug/ml polyD lysine (1-5K)
20 ug/ml polyD lysine (1-5K)
water
Bovine serum
Before wash
After wash
Adhesive

Figure 6

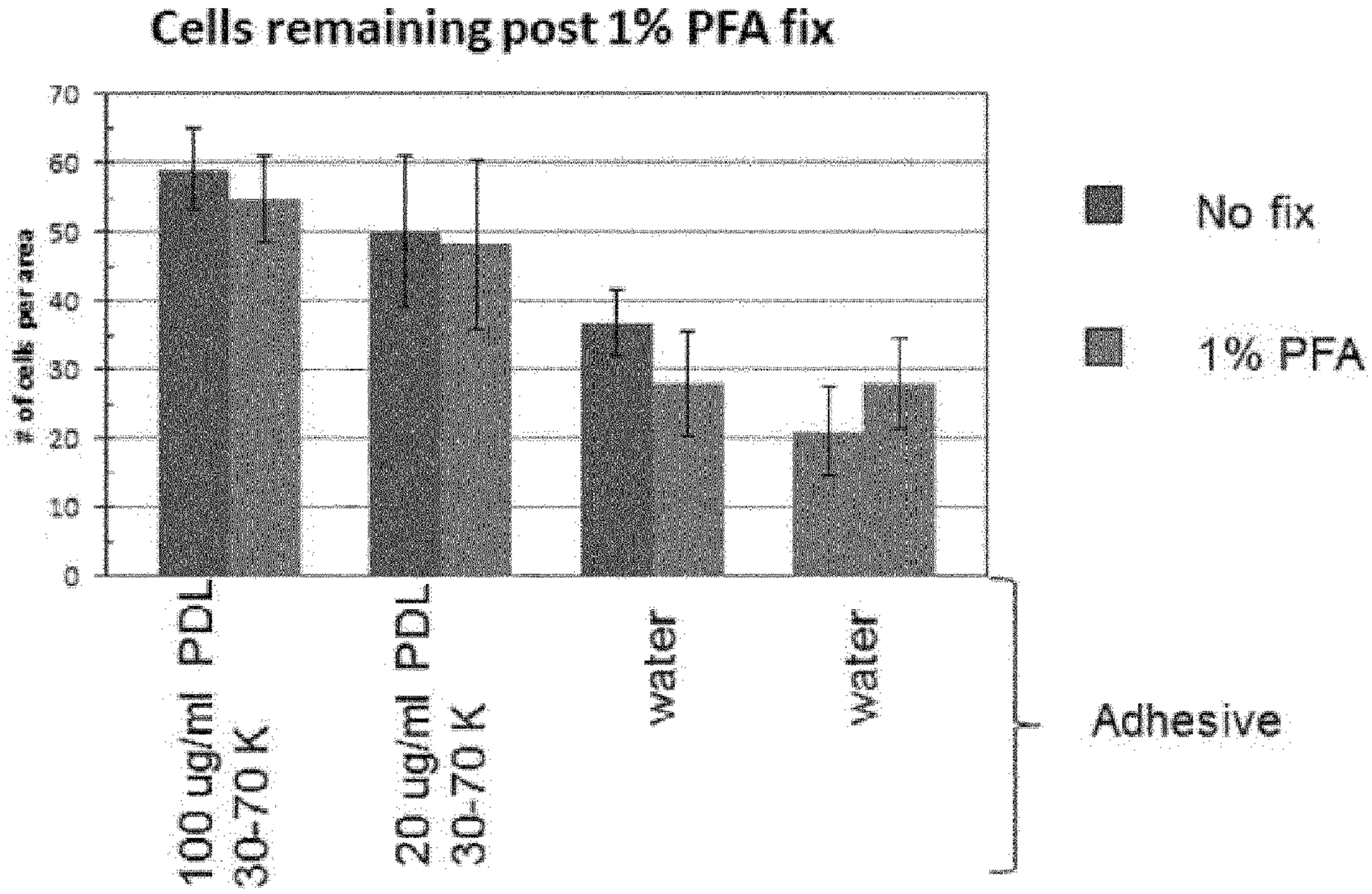
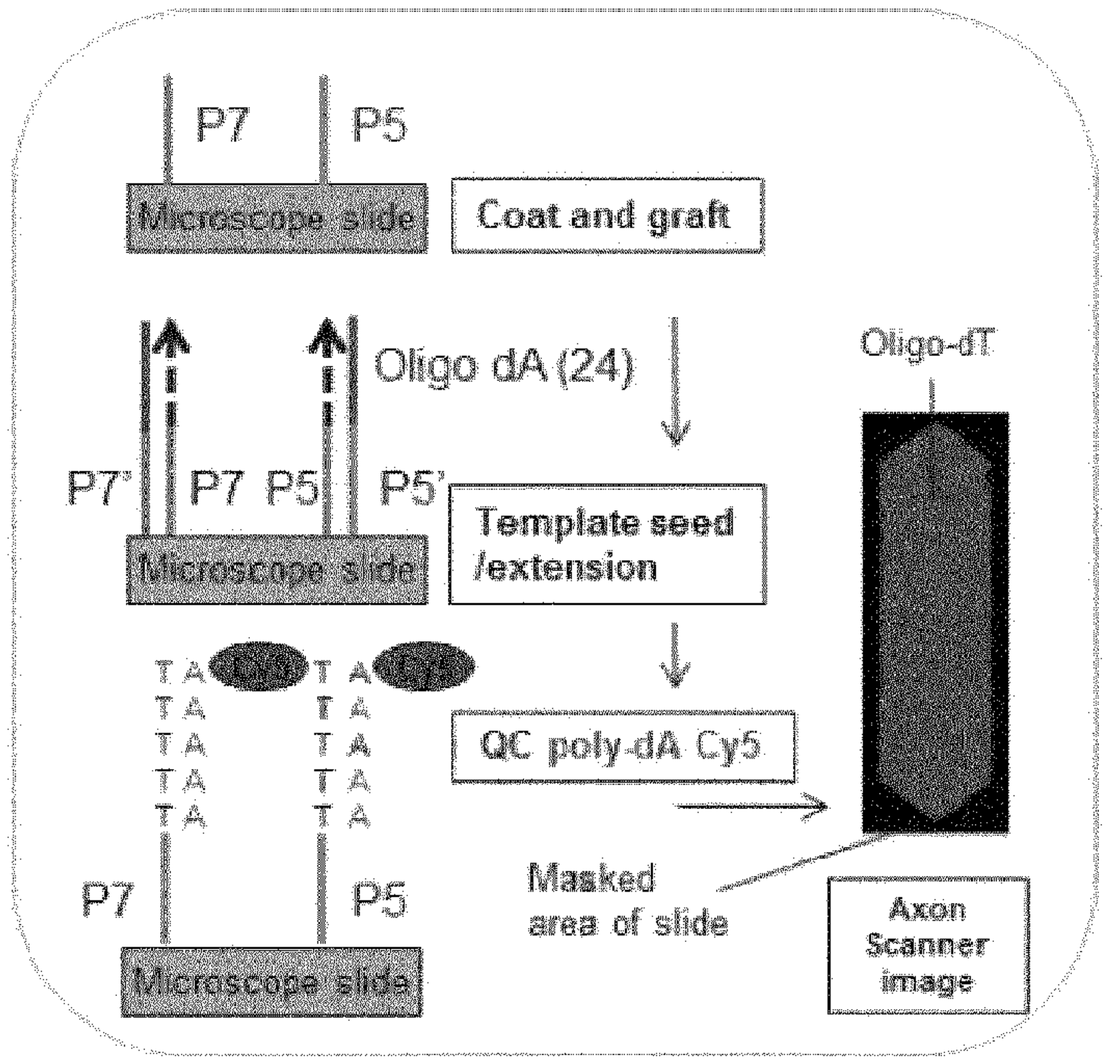

Cells remaining post 1% PFA fix
# of cells per area
70
60
50
40
30
20
10
0
100 ug/ml PDL 30-70 K
20 ug/ml PDL 30-70 K
water
water
No fix
1% PFA
Adhesive

Figure 7

A


P7
P5
Microscope slide
Coat and graft
Oligo dA (24)
Oligo-dT
P7'
P7
P5
P5'
Template seed /extension
Cy3
Cy5
QC poly-dA Cy5
Masked area of slide
Axon Scanner image
P7
P5
Microscope slide

B

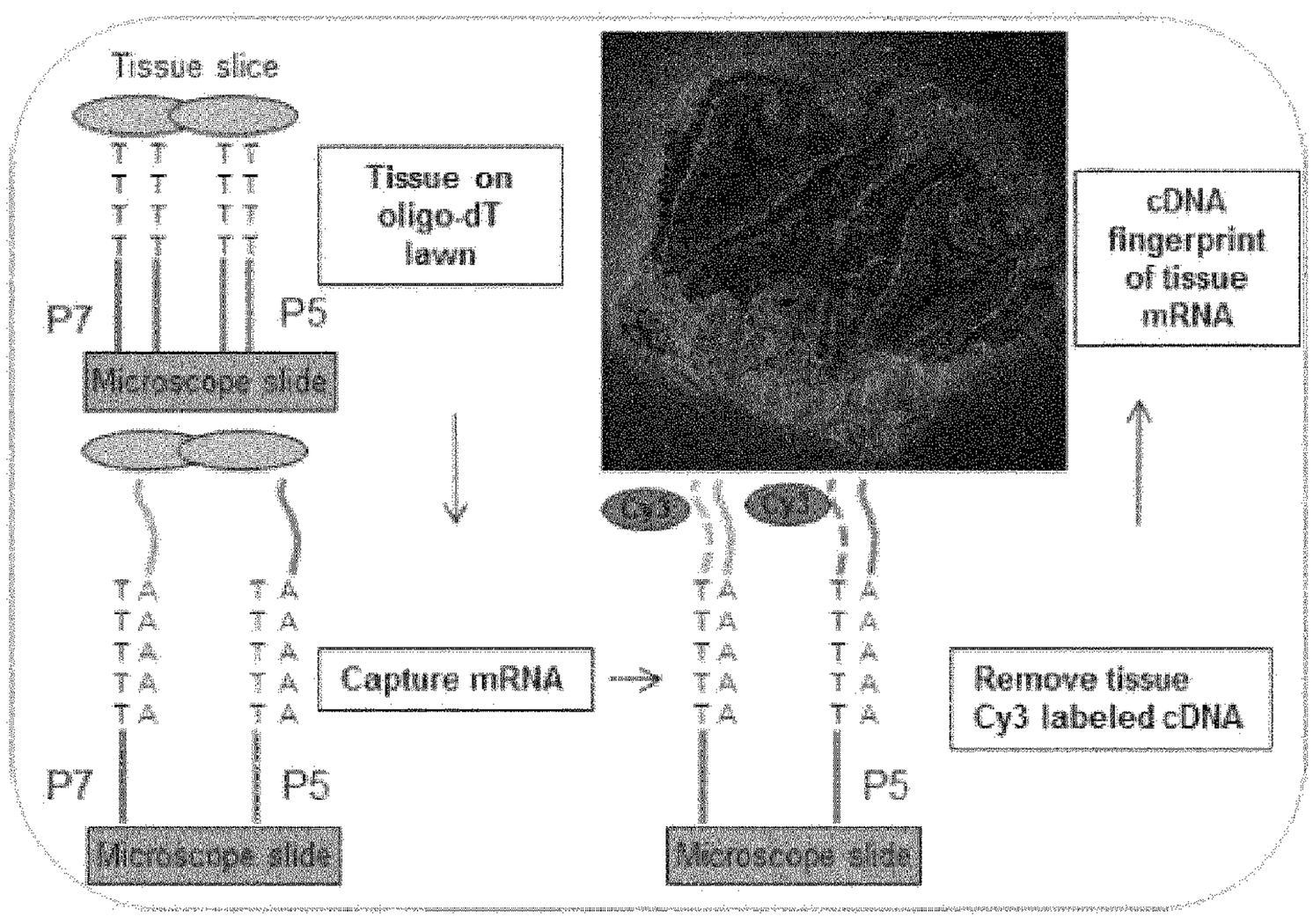
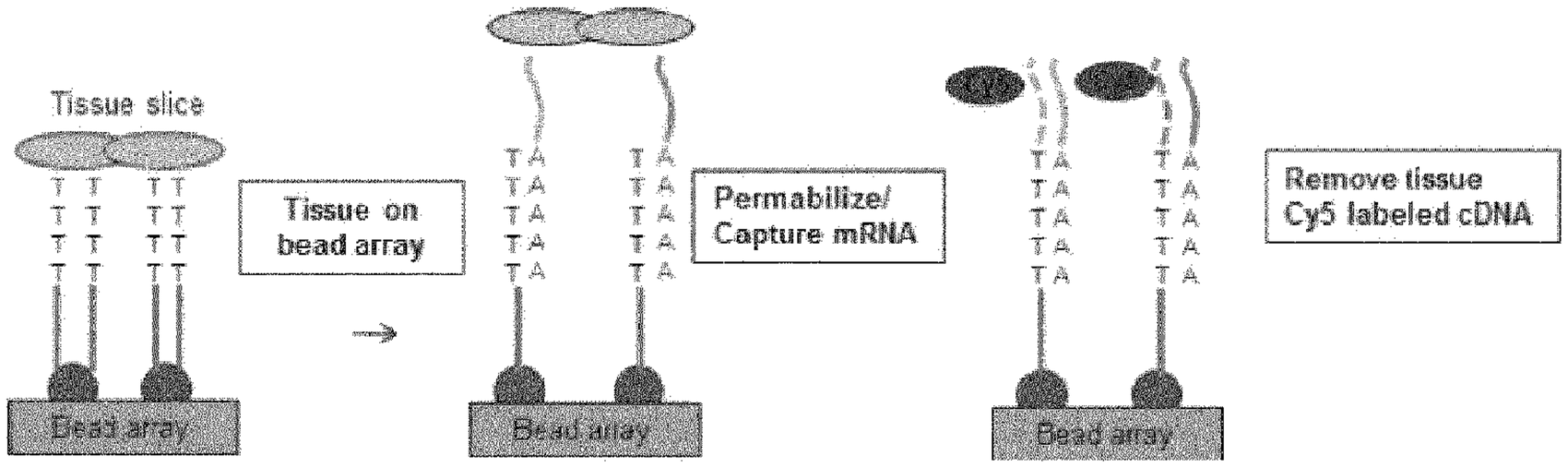

Tissue slice
Tissue on oligo-dT lawn
P7
P5
Microscope slide
cDNA fingerprint of tissue mRNA
Capture mRNA
Cy3
Remove tissue Cy3 labeled cDNA
P7
P5
Microscope slide

Figure 8

A


Tissue slice
Bead array
Tissue on bead array
Bead array
Permabilize/ Capture mRNA
Bead array
Remove tissue Cy5 labeled cDNA

B