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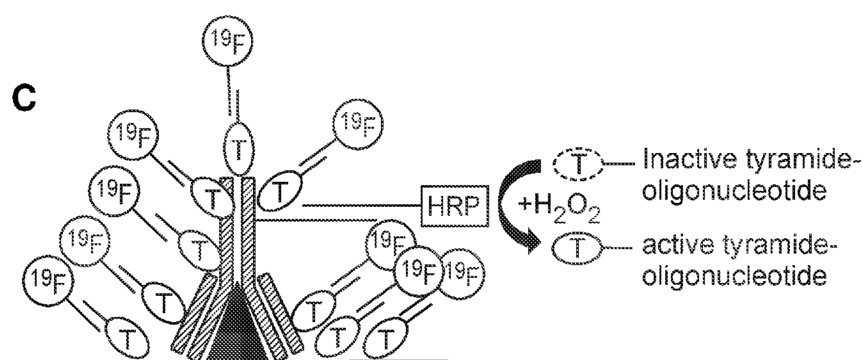

FIGS. 8A-C

(57) Abstract: Provided herein, among other things, is an oligonucleotide-tyramide conjugate, which conjugate can be used in variety of tyramide-signal amplification (TSA)-based detection methods. Some embodiments of the method may comprise incubating a sample that has a binding agent-peroxidase conjugate bound to sites thereon (where the peroxidase may directly linked to the binding agent or indirectly directly linked to the binding agent via a pair of oligonucleotides that are hybridized together) with an oligonucleotide-tyrami de conjugate in the presence of hydrogen peroxide, thereby activating the tyramide and causing deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase of the binding agent-peroxidase conjugate.

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OLIGONUCLEOTIDE-TYRAMIDE CONJUGATE AND USE OF THE SAME IN TYRAMIDE-SIGNAL AMPLIFICATION (TSA)-BASED DETECTION METHODS

CROSS-REFERENCING

This application claims the benefit of provisional application serial no. 63/021,611, filed on May 7, 2020, which application is incorporated by reference herein.

GOVERNMENT RIGHTS

This invention was made with Government support under contract HHSF223201610018C awarded by the Food and Drug Administration. The Government has certain rights in the invention.

BACKGROUND

In tyramide signal amplification (TSA), also known as catalyzed reporter deposition (CARD), a peroxidase (e.g., HRP) converts a tyramide-label conjugate (i.e., tyramide that has been labeled with, e.g., a fluorophore or hapten such as biotin) into a highly reactive product that can covalently bind to tyrosine residues on proteins at or near the peroxidase. Each peroxidase molecule causes several molecules of the tyramide-conjugate to be deposited locally to the enzyme molecule, thereby resulting in dense labeling around the site of the enzyme. This dense labeling makes tyramide signal amplification more sensitive than other commonly used methods. Tyramide signal amplification is described in Bobrow et al. (J. Immunol. Methods 1989 125: 279–285) and Bobrow et al. (J. Immunol. Methods 1991 137 103–112), among other publications.

However, labeling methods that rely on tyramide signal amplification often suffer from similar problems as conventional immunohistochemistry methods in that the number of different epitopes that can be analyzed is limited by the spectral properties of the labels used. Specifically, while tyramide signal amplification has been successfully used to label three epitopes simultaneously using three distinguishable fluorescent labels (see, e.g., Mitchell et al Mod. Pathol. 2014 27:1255-1266 and Toth J. Histochem. Cytochem. 2007 55 545–554), four does not appear to have been achieved. Based on current technology, it would be challenging to

analyze more than three epitopes using tyramide signal amplification labeling system, much less more than 5 or 10 epitopes. This constraint is problematic because it limits the use of tyramide signal amplification in clinical diagnostics, in which field it is very desirable to analyze a much larger number of epitopes.

SUMMARY

Provided herein, among other things, is an oligonucleotide-tyramide conjugate, which conjugate can be used in variety of tyramide-signal amplification (TSA)-based detection methods. Some embodiments of the method may comprise incubating a sample that has a binding agent-peroxidase conjugate bound to sites thereon (where the peroxidase may directly linked to the binding agent or indirectly directly linked to the binding agent via a pair of oligonucleotides that are hybridized together) with an oligonucleotide-tyramide conjugate in the presence of hydrogen peroxide, thereby activating the tyramide and causes deposition of the oligonucleotide of the conjugate onto sites that are proximal to the sites to which the antibody is bound. If the oligonucleotide-tyramide conjugate is not already detectably labeled then the method may comprise detectibly labeling the deposited oligonucleotide-tyramide conjugate. The method may comprise reading the sample by detecting the deposited oligonucleotide-tyramide conjugate.

The sample may be analyzed using any of a variety of different methods, e.g., mass-cytometry, multiplexed ion beam imaging (MIBI), or fluorescence microscopy, for example. Various multiplexed sample analysis method are described herein.

BRIEF DESCRIPTION OF THE FIGURES

The skilled artisan will understand that the drawings described below are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

Fig. 1 schematically illustrates an oligonucleotide-tyramide conjugate.

Fig. 2 schematically illustrates a reagent system comprising an oligonucleotide-tyramide conjugate and a detectably-labeled oligonucleotide. As shown, the oligonucleotides in these conjugates are complementary.

Fig. 3 schematically illustrates a plurality of oligonucleotide-tyramide conjugates, where the sequences of the oligonucleotides in the oligonucleotide-tyramide conjugates vary.

Fig. 4 schematically illustrates a reagent system comprising a plurality of oligonucleotide-tyramide conjugates (A-D, as illustrated in Fig. 3) and a corresponding plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the plurality of oligonucleotide-tyramide conjugates.

Fig. 5 schematically illustrates examples of binding agent conjugates. A binding agent conjugate can be a binding agent peroxidase conjugate or a binding agent oligonucleotide conjugate, for example. As shown, a binding agent-peroxidase conjugate can be composed of a peroxidase that is directly linked to the binding agent or in some embodiments the peroxidase may indirectly directly linked to the binding agent in the binding agent-peroxidase conjugate via a pair of oligonucleotides that are hybridized together

Fig. 6 schematically illustrates a reagent system comprising a set of binding agent-oligonucleotide conjugates, wherein the binding agents have different specificities (for antigens A, B, C and D) and each binding agent is linked to a different oligonucleotide (having sequences A, B, C and D). As shown, this reagent system also comprises a plurality of peroxidase-oligonucleotide conjugates, wherein each of the peroxidase-oligonucleotide conjugates specifically hybridizes with the oligonucleotide of only one of the binding agent oligonucleotides conjugates. As shown the oligonucleotides of the peroxidase-oligonucleotide conjugates have sequences A', B', C' and D').

Fig. 7 schematically illustrates one embodiment of the present method.

Fig. 8A-C schematically illustrates several ways in which the present method may be implemented.

Fig. 9 shows the structure of an exemplary oligonucleotide-tyramide conjugate.

Fig. 10 shows an example of labeling a tissue sample using the present method. In this example. FFPE tonsil tissues was stained with CD3 and E-Cadherin antibodies conjugated to different oligonucleotides. CD3 was detected using an oligonucleotide tyramide-conjugate hybridized with an Alexa 647 labeled oligonucleotide (red). E-Cadherin was detected using an oligonucleotide tyramide-conjugate hybridized with a Cy3-labeled oligonucleotide (green)

before (a-1) and after (a-2) stripping the Alexa 647 labeled oligonucleotide from the sample. The signal intensity of analysis line 1 (b) and analysis line 2 (c) were analyzed by a Fuji line plot profile.

DEFINITIONS

5 Unless defined otherwise herein, all technical and scientific terms used in this specification have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

10 All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

15 The headings provided herein are not limitations of the various aspects or embodiments of the invention. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention 20 belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide one of ordinary skill in the art with the general meaning of many of the terms used herein. Still, certain terms are defined below for the sake of clarity and ease of reference.

25 As used herein, the term “biological feature of interest” refers to any part of a cell that can be indicated by binding to a binding agent. Exemplary biological features of interest include cell walls, nuclei, cytoplasm, membrane, keratin, muscle fibers, collagen, bone, proteins, nucleic acid (e.g., mRNA or genomic DNA, etc.), etc. A binding agent may bind to a corresponding site, e.g., a protein epitope, in the sample.

30 As used herein, the term “multiplexing” refers to the simultaneous detection and/or measurement of multiple biological features of interest, e.g., protein epitopes, in a sample.

As used herein, the terms "antibody" and "immunoglobulin" are used interchangeably herein and are well understood by those in the field. Those terms refer to a protein consisting of one or more polypeptides that specifically binds an antigen. One form of antibody constitutes the basic structural unit of an antibody. This form is a tetramer and consists of two identical pairs of antibody chains, each pair having one light and one heavy chain. In each pair, the light and heavy chain variable regions are together responsible for binding to an antigen, and the constant regions are responsible for the antibody effector functions.

The terms "antibodies" and "immunoglobulin" include antibodies or immunoglobulins of any isotype and fragments of antibodies which retain specific binding to antigen, including, but not limited to, Fab, Fv, scFv, and Fd fragments, chimeric antibodies, humanized antibodies, minibodies, single-chain antibodies, and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein. Also encompassed by the term are Fab', Fv, $F(ab')_2$, and/ or other antibody fragments that retain specific binding to antigen, and monoclonal antibodies. Antibodies may exist in a variety of other forms including, for example, Fv, Fab, and $(Fab')_2$, as well as bi-functional (i.e. bi-specific) hybrid antibodies (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)) and in single chains (e. g., Huston et al., Proc. Natl. Acad. Sci. U.S.A., 85, 5879-5883 (1988) and Bird et al., Science, 242, 423-426 (1988)), which are incorporated herein by reference. (See, generally, Hood et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323, 15-16 (1986)).

The term "specific binding" refers to the ability of a binding agent to preferentially bind to a particular analyte that is present in a homogeneous mixture of different analytes. In certain embodiments, a specific binding interaction will discriminate between desirable and undesirable analytes in a sample. In some embodiments, more than about 10- to 100-fold or more (e.g., more than about 1000- or 10,000-fold).

In certain embodiments, the affinity between a binding agent and analyte when they are specifically bound in a binding agent/analyte complex is characterized by a K_D (dissociation constant) of less than 10^{-6} M, less than 10^{-7} M, less than 10^{-8} M, less than 10^{-9} M, less than 10^{-9} M, less than 10^{-11} M, or less than about 10^{-12} M or less.

A "plurality" contains at least 2 members. In certain cases, a plurality may have at least 2, at least 5, at least 10, at least 100, at least 1000, at least 10,000, at least 100,000, at least 10^6, at least 10^7, at least 10^8 or at least 10^9 or more members. In certain cases, a plurality may have 2 to 100 or 5 to 100 members.

As used herein, the term "labeling" refers to a step that results in binding of a binding agent to specific sites in a sample (e.g., sites containing an epitope for the binding agent (e.g., an antibody) being used, for example) such that the presence and/or abundance of the sites can be determined by evaluating the presence and/or abundance of the binding agent. The term "labeling" refers to a method for producing a labeled sample in which any necessary steps are performed in any convenient order, as long as the required labeled sample is produced. For example, in some embodiments and as will be exemplified below, a sample can be labeled using a plurality of binding agents that are each linked to an oligonucleotide.

As used herein, the term "planar sample" refers to a substantially flat, i.e., two-dimensional, material (e.g. glass, metal, ceramics, organic polymer surface or gel) that comprises cells or any combination of biomolecules derived from cells, such as proteins, nucleic acids, lipids, oligo/polysaccharides, biomolecule complexes, cellular organelles, cellular debris or excretions (exosomes, microvesicles). A planar cellular sample can be made by, e.g., growing cells on a planar surface, depositing cells on a planar surface, e.g., by centrifugation, by cutting a three dimensional object that contains cells into sections and mounting the sections onto a planar surface, i.e., producing a tissue section, adsorbing the cellular components onto a surface that is functionalized with affinity agents (e.g. antibodies, haptens, nucleic acid probes), introducing the biomolecules into a polymer gel or transferring them onto a polymer surface electrophoretically or by other means. The cells or biomolecules may be fixed using any number of reagents including formalin, methanol, paraformaldehyde, methanol:acetic acid, glutaraldehyde, bifunctional crosslinkers such as bis(succinimidyl)suberate, bis(succinimidyl)polyethyleneglycol, etc. This definition is intended to cover cellular samples (e.g., tissue sections, etc.), electrophoresis gels and blots thereof, Western blots, dot-blots, ELISAs, antibody microarrays, nucleic acid microarrays, etc.

As used herein, the term "tissue section" refers to a piece of tissue that has been obtained from a subject, fixed, sectioned, and mounted on a planar surface, e.g., a microscope slide.

As used herein, the term "formalin-fixed paraffin embedded (FFPE) tissue section" refers to a piece of tissue, e.g., a biopsy sample that has been obtained from a subject, fixed in formaldehyde (e.g., 3%-5% formaldehyde in phosphate buffered saline) or Bouin solution, embedded in wax, cut into thin sections, and then mounted on a microscope slide.

As used herein, the term "non-planar sample" refers to a sample that is not substantially flat, e.g., a whole or partial organ mount (e.g., of a lymph node, brain, liver, etc.), that has been

made transparent by means of a refractive index matching technique such as Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging-compatible Tissue-hydrogel (CLARITY). See, e.g., Roberts et al., J Vis Exp. 2016; (112): 54025. Clearing agents such as benzyl-alcohol/benzyl benzoate (BABB) or benzyl-ether may also be used to render a specimen transparent.

As used herein, the term "spatially-addressable measurements" refers to a set of values that are each associated with a specific position on a surface. Spatially-addressable measurements can be mapped to a position in a sample and can be used to reconstruct an image, e.g., a two- or three-dimensional image, of the sample.

A "diagnostic marker" is a specific biochemical in the body which has a particular molecular feature that makes it useful for detecting a disease, measuring the progress of disease or the effects of treatment, or for measuring a process of interest.

A "pathoindicative" cell is a cell which, when present in a tissue, indicates that the animal in which the tissue is located (or from which the tissue was obtained) is afflicted with a disease or disorder. By way of example, the presence of one or more breast cells in a lung tissue of an animal is an indication that the animal is afflicted with metastatic breast cancer.

The term "complementary site" is used to refer to an epitope for an antibody or aptamer, or nucleic acid that has a sequence that is complementary to an oligonucleotide probe. Specifically, if the binding agent is an antibody or aptamer, then the complementary site for the binding agent is the epitope in the sample to which the antibody or aptamer binds. An epitope may be a conformational epitope or it may be a linear epitope composed of, e.g., a sequence of amino acids. If the binding agent is an oligonucleotide probe, then the complementary site for the binding agent is a complementary nucleic acid (e.g., an RNA or region in a genome).

The term "epitope" as used herein is defined as a structure, e.g., a string of amino acids, on an antigen molecule that is bound by an antibody or aptamer. An antigen can have one or more epitopes. In many cases, an epitope is roughly five amino acids or sugars in size. One skilled in the art understands that generally the overall three-dimensional structure or the specific linear sequence of the molecule can be the main criterion of antigenic specificity.

A "subject" of diagnosis or treatment is a plant or animal, including a human. Non-human animals subject to diagnosis or treatment include, for example, livestock and pets.

As used herein, the term "incubating" refers to maintaining a sample and binding agent under conditions (which conditions include a period of time, one or more temperatures, an

appropriate binding buffer and a wash) that are suitable for specific binding of the binding agent to molecules (e.g., epitopes or complementary nucleic acids) in the sample.

As used herein, the term “binding agent” refers to an agent that can specifically bind to complementary sites in a sample. Exemplary binding agents include oligonucleotide probes (RNA or DNA), antibodies, aptamers and peptides (which may specifically bind to a receptor, for example). If antibodies or aptamers are used, in many cases they may bind to protein epitopes.

As used herein, the term “binding agent conjugate” refers to a binding agent, e.g., an antibody, aptamer or oligonucleotide probe, that is non-covalently (e.g., via a streptavidin/biotin interaction) or covalently (e.g., via a “click” reaction (see, e.g., Evans Aus. J. Chem. 2007 60 : 384–395) or the like) linked to a single-stranded oligonucleotide or peroxidase enzyme (e.g., HRP) in a way that the binding agent can still bind to its binding site. The nucleic acid and the binding agent may be linked via a number of different methods, including those that use a cysteine-reactive maleimide or halogen-containing group. The binding agent and the oligonucleotide may be linked proximal to or at the 5’ end of the oligonucleotide, proximal to or at the 3’ end of the oligonucleotide, or anywhere in-between.

The terms “nucleic acid” and “polynucleotide” are used interchangeably herein to describe a polymer of any length, e.g., greater than about 2 bases, greater than about 10 bases, greater than about 100 bases, greater than about 500 bases, greater than 1000 bases, up to about 10,000 or more bases composed of nucleotides, e.g., deoxyribonucleotides, ribonucleotides or a combination thereof, and may be produced enzymatically or synthetically (e.g., PNA as described in U.S. Patent No. 5,948,902 and the references cited therein) and which can hybridize with naturally occurring nucleic acids in a sequence specific manner analogous to that of two naturally occurring nucleic acids, e.g., can participate in Watson-Crick base pairing interactions. Naturally-occurring nucleotides include guanine, cytosine, adenine, thymine, uracil (G, C, A, T and U respectively). DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNAs backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. In PNAs, various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds. A locked nucleic acid (LNA), often referred to as an inaccessible RNA, is an RNA molecule comprising modified RNA nucleotides. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge “locks” the ribose in the 3'-endo (North) conformation, which is often found

in A-form duplexes. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. The term "unstructured nucleic acid", or "UNA", is a nucleic acid containing non-natural nucleotides that bind to each other with reduced stability. For example, an unstructured nucleic acid may contain a G' residue and a C' residue, where these residues correspond to non-naturally occurring forms, i.e., analogs, of G and C that base pair with each other with reduced stability, but retain an ability to base pair with naturally occurring C and G residues, respectively. Unstructured nucleic acid is described in US20050233340, which is incorporated by reference herein for disclosure of UNA.

As used herein, the term "oligonucleotide" refers to a multimer of at least 10, e.g., at least 15 or at least 30 nucleotides. In some embodiments, an oligonucleotide may be in the range of 15-200 nucleotides in length, or more. Any oligonucleotide used herein may be composed of G, A, T and C, or bases that are capable of base pairing reliably with a complementary nucleotide. 7-deaza-adenine, 7-deaza-guanine, adenine, guanine, cytosine, thymine, uracil, 2-deaza-2-thio-guanosine, 2-thio-7-deaza-guanosine, 2-thio- adenine, 2-thio-7-deaza-adenine, isoguanine, 7-deaza-guanine, 5,6-dihydrouridine, 5,6- dihydrothymine, xanthine, 7-deaza-xanthine, hypoxanthine, 7-deaza-xanthine, 2,6 diamino-7- deaza purine, 5-methyl-cytosine, 5-propynyl-uridine, 5-propynyl-cytidine, 2-thio-thymine or 2-thio-uridine are examples of such bases, although many others are known. As noted above, an oligonucleotide may be an LNA, a PNA, a UNA, or a morpholino oligomer, for example. The oligonucleotides used herein may contain natural or non-natural nucleotides or linkages, labels, mass tags, adducts, etc.

As used herein, the term "reading" in the context of reading a fluorescent signal, refers to obtaining an image by scanning or by microscopy, where the image shows the pattern of fluorescence as well as the intensity of fluorescence in a field of view. The term "reading" also encompasses mass spectrometry methods, e.g., multiplexed ion beam imaging (MIBI) and mass cytometry (CyTOF), as well as other types of microscopy (e.g., SEM).

As used herein, the term "signal generated by", in the context of, e.g., reading a fluorescent signal generated by addition of the fluorescent nucleotide, refers to a signal that is emitted directly from the fluorescent nucleotide or a signal that is emitted indirectly via energy transfer to another fluorescent nucleotide (i.e., by fluorescence resonance energy transfer (FRET)).

As used herein, the term "activated tyramide" refers to a reactive form of tyramide that has a radical on the C2 position. Non-activated tyramide can be activated by a variety of

enzymes (e.g., peroxidase) in the presence of hydrogen peroxide (H_2O_2). In this reaction the phenolic part of tyramide is converted to a short-lived quinone-like structure bearing a radical on the C2 position. Activated tyramide covalently binds to nucleophilic residues (e.g., tyrosines) in close proximity to the reaction (see, e.g., Bobrow et al. J. Immunol. Methods 1992 137:103–112; Bobrow et al. J. Immunol Methods 1989 125:279–285; Van Gijlswijk et al. J. Histochem. Cytochem. 1996 44:389–392; and US5,196,306).

Thus, when one incubates a sample that has a peroxidase-linked binding agent that is bound to sites on the sample with an oligonucleotide-tyramide conjugate and hydrogen peroxide, the tyramide of the conjugate is activated and the oligonucleotide of the oligonucleotide-tyramide conjugate reacts with nucleophilic sites (e.g., tyrosines) that are proximal to the peroxidase of the binding agent-peroxidase conjugate. This covalently links the oligonucleotide of the oligonucleotide-tyramide conjugate to those sites. For clarity, the sites to which the oligonucleotide binds can be on the binding agent itself (e.g., the antibody, if the binding agent is an antibody), the sample, and/or any proximal substrate that that may have nucleophilic residues thereon. This concept is illustrated in Figs. 8A-C.

As used herein, the term “oligonucleotide-tyramide conjugate” refers to a molecule containing a tyramide and an oligonucleotide.

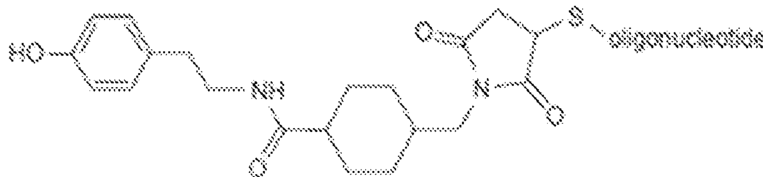
As used herein, the term “cleavable linker” refers to a linker containing a bond that can be selectively cleaved by a specific stimulus, e.g., a reducing agent.

Other definitions of terms may appear throughout the specification.

DETAILED DESCRIPTION

Fig. 1 schematically illustrates an oligonucleotide-tyramide conjugate **2**. As shown, such a conjugate contains an oligonucleotide **4** and a tyramide moiety **6**. The oligonucleotide of the oligonucleotide-tyramide conjugate may be unlabeled or it may be labeled. If the oligonucleotide of the oligonucleotide-tyramide conjugate is labeled, the oligonucleotide may be labeled with any type of detectable label such as a fluorophore, a mass tag or a radioisotope. A labeled oligonucleotide-tyramide conjugate may contain a capture moiety such as a biotin moiety (e.g., biotin or desthiobiotin) or a group that can participate in a click chemistry reaction (e.g., an azide or alkyne). In some embodiments, the oligonucleotide could even be conjugated to an enzyme.

In any embodiment, the oligonucleotide-tyramide conjugate may have the formula of Formula 1:



where the oligonucleotide may be in the range of 2-200 nt in length or longer, although, in many
5 cases, the oligonucleotide may be in the range of 8-100 nt in length. In this formula, the S is linked to the 5' of the oligonucleotide

As shown in Fig. 2, if the oligonucleotide of the oligonucleotide-tyramide conjugate is not labeled, then the system may further comprises a detectably-labeled oligonucleotide **8** composed of an oligonucleotide **10** and a label **12** (e.g., detectable label such as a fluorophore, a
10 mass tag or a radioisotope), wherein where the sequences of the oligonucleotides in the oligonucleotide-tyramide conjugate and the detectably-labeled oligonucleotide are at least partially complementary such that they specifically hybridize t each other in solution. For example, the oligonucleotides can have at least 8, at least 10, at least 12 or at least 15 bases of perfect complementarity. In any embodiment, there may be sites for multiple detectably-labeled
15 oligonucleotides in the oligonucleotide-tyramide conjugate.

As shown in Fig. 3, the system may comprise a plurality of oligonucleotide-tyramide conjugates, wherein the sequences of the oligonucleotides in the oligonucleotide-tyramide conjugates vary in the plurality. Fig. 3 shows four oligonucleotide-tyramide conjugates, where the oligonucleotides have sequences A, B, C and D. In some embodiments, there may be less
20 than 500 (e.g., 3-300) of such conjugates in the system, each having a different sequence. In some embodiments, sequences of the oligonucleotides may be orthogonal to one another, meaning that they do not cross-hybridize to one another or their complements. In addition, the sequences of the oligonucleotides should be designed to minimize binding to other nucleic acids endogenous that may be endogenous to the sample (e.g., RNA or DNA). These conjugates may
25 be in separate containers.

This reagent system may also comprise a corresponding plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the plurality of oligonucleotide-tyramide conjugates. This concept is illustrated in Fig. 4, where detectably-labeled oligonucleotide 1 specifically

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hybridizes to oligonucleotide-tyramide conjugate A and not to any of the other oligonucleotide-tyramide conjugates, detectably-labeled oligonucleotide 2 specifically hybridizes to oligonucleotide-tyramide conjugate B and not to any of the other oligonucleotide-tyramide conjugates, detectably-labeled oligonucleotide 3 specifically hybridizes to oligonucleotide-tyramide conjugate C and not to any of the other oligonucleotide-tyramide conjugates and detectably-labeled oligonucleotide 4 specifically hybridizes to oligonucleotide-tyramide conjugate D and not to any of the other oligonucleotide-tyramide conjugates. In some embodiments, the number of detectably-labeled oligonucleotides in the system may be the same as the number of oligonucleotide-tyramide conjugates in the system. These conjugates may be in separate containers. In these embodiments, the detectably-labeled oligonucleotides may be mass-tagged, fluorescently labeled, or may contain a different label adduct.

As shown in Fig. 5, in some embodiments the labeling system may comprise a binding agent conjugate **20** or **22**. In some embodiments **20** the binding agent conjugate may comprise a binding agent **22** (e.g., an antibody, aptamer, peptide or a sequence that specifically hybridizes with another sequence in the sample being studied, e.g., a human genomic sequence) coupled to a peroxidase **24** (e.g., HRP). In other embodiments **26** the binding agent conjugate comprises a binding agent **22** coupled to an oligonucleotide **28**. In some cases **30** the peroxidase **24** may be indirectly directly linked to the binding agent in the binding agent-peroxidase conjugate via a pair of oligonucleotides (**32** and **34**) that are hybridized together. In some embodiments, the reagent system comprises a set of binding agent conjugates, e.g., 2-200 or more binding agent conjugates, wherein the binding agents have different specificities and each binding agent is linked to a different oligonucleotide. These conjugates, in turn, may be linked to a peroxidases by hybridization of conjugates that are composed of complementary oligonucleotides that are linked to a peroxidase. This concept is illustrated in Fig. 6. As shown in Fig. 6, the binding agent that binds to binds to antigen A is linked to sequence A, the binding agent that binds to binds to antigen B is linked to sequence B, the binding agent that binds to binds to antigen C is linked to sequence C and the binding agent that binds to binds to antigen D is linked to sequence D. As with the oligonucleotide-tyramide conjugates, these oligonucleotides may be orthogonal to one another and designed so that they do not hybridize to sequences that may be native to the sample. As shown, each of the binding agent-oligonucleotide conjugates is linked to a peroxidase via hybridization with a complementary oligonucleotide-peroxidase conjugate.

In certain embodiments, the system may comprise (i) a plurality of (e.g., up to 5, 10, 20, 25, 50, 75, 100, or more) binding agent-peroxidase conjugates; (ii) a corresponding plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides in the conjugates have different sequences; and (iii) a corresponding plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(ii).

In certain embodiments, the system may comprise (i) a plurality of a plurality of (e.g., up to 5, 10, 20, 25, 50, 75, 100, or more) binding agent-oligonucleotide conjugates; (ii) a corresponding plurality of peroxidase-oligonucleotide conjugates, wherein each of the peroxidase-oligonucleotide conjugates specifically hybridizes with only one of the oligonucleotides of (a)(i); (iii) a plurality (e.g., up to 5, 10, 20, 25, 50, 75, 100, or more) of oligonucleotide-tyramide conjugates, wherein the oligonucleotides of the conjugates have different sequences; and (iv) a corresponding plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(iii);

As described above, the label may comprise a mass tag, a heavy metal or a fluorophore, etc. In some embodiments, the labeled oligonucleotide or the oligonucleotide-tyramide conjugate may be linked to a metal chelator (e.g., DTPA or DOTA) so that mass tags can be chelated as desired. In these embodiments, the system may further comprise one or more mass tags, e.g., a lanthanide, that is either separate or in a complex with the chelator.

Oligonucleotide-tyramide conjugates may be employed in a variety of methods. In some embodiments, the method may comprise incubating a sample that has a binding agent-peroxidase conjugate bound to sites thereon with an oligonucleotide-tyramide conjugate in the presence of hydrogen peroxide, thereby activating the tyramide and causing deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase (which is linked to the binding agent). . As shown in Fig. 7, the peroxidase **40** of the binding agent-peroxidase conjugate may directly linked to the binding agent in the binding agent-peroxidase conjugate, or it may be indirectly linked to the binding agent in the binding agent-peroxidase conjugate via a pair of oligonucleotides that are hybridized together. In the latter embodiments, the method may comprise, binding a binding agent-oligonucleotide conjugate with the sample and then hybridizing a peroxidase-oligonucleotide conjugate to the

sample, wherein the oligonucleotide of the peroxidase-oligonucleotide conjugate hybridizes to the oligonucleotide of the binding agent-oligonucleotide.

The sample **42** may be planar or non-planar, but it should have sites **44** (e.g., antigens) to which the binding agent binds. As would be apparent, non-deposited oligonucleotide-conjugate conjugate can be washed away. If the oligonucleotide-tyramide conjugate **46** is detectably labeled (e.g., with a fluorophore or mass-tag, for example) then the deposition pattern could be read without any further steps. In some embodiments, however, if the oligonucleotide-tyramide conjugate **46** is detectably labeled then the method may comprise inactivating or removing the peroxidase, inactivating or removing the label from the sample, and labeling different sites on the sample by repeating the labeling using a different peroxidase-oligonucleotide conjugate and a different oligonucleotide-tyramide conjugate. In these embodiments, the labels used in the different labeling cycles should be distinguishable so that they can be independently detected by scanning the sample.

If the oligonucleotide-tyramide conjugate is not already detectably-labeled then the method may further comprise detectibly labeling the deposited oligonucleotide-tyramide conjugate. This may be done a variety of different ways, depending on the conjugate. For example, if the oligonucleotide-tyramide conjugate comprises a capture moiety, then the deposited oligonucleotide can be labeled by using a partner for the capture moiety. For example, if the oligonucleotide-tyramide conjugate is biotinylated, then the deposited oligonucleotide could be labeled with a labeled streptavidin. Likewise, if the oligonucleotide-tyramide conjugate is labeled with a chemistry group (e.g., an azide or an alkyl), then the deposited oligonucleotide could be labeled using click reactive label (e.g., a fluorophore that has the other click reactive group on it). Alternatively, the oligonucleotide could be labeled enzymatically, e.g., using a terminal transferase and fluorescent dNTPs.

As such, in some embodiments, the method may comprise (a) incubating a sample that has a binding agent-peroxidase conjugate bound to sites thereon with an oligonucleotide-tyramide conjugate in the presence of hydrogen peroxide, thereby activating the tyramide and causes deposition of the oligonucleotide in the oligonucleotide-tyramide conjugate on the sample near to the sites to which the antibody is bound; (b) if the oligonucleotide-tyramide conjugate is not already detectably labeled then detectibly labeling the deposited oligonucleotide-tyramide conjugate; and (c) reading the sample by detecting the labeled deposited oligonucleotide-tyramide conjugate.

This method may be multiplexed in a variety of different ways. For example, in some embodiments, the method may comprise inactivating or removing the peroxidase after step (a). For example, the peroxidase can be inactivated (e.g., by treatment with 3–30% hydrogen peroxide w/v for 1 min to 1 hr; see, e.g., Sennepin et al. Analytical Biochemistry 2009 393: 129-131 and Arnao et al. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1990 1038: 85-89) or, if the peroxidase is attached to the binding agent indirectly via hybridization, then the peroxidase may be removed by denaturation and washed away. In these embodiments before step (c) the method may comprise repeating steps (a) and (b) using a different peroxidase-oligonucleotide conjugate and a different oligonucleotide-tyramide conjugate. In these embodiments, the label used in the initial step (a) or (b) should be distinguishable from the repeat steps, thereby allowing several labels to be detected in the reading step.

With reference to Fig. 7, in some embodiments the oligonucleotide-tyramide conjugate may be unlabeled, and the deposited oligonucleotides may be detected by: (b) hybridizing the sample with a detectably-labeled oligonucleotide **48** that is complementary to the deposited oligonucleotide, wherein the detectably-labeled oligonucleotide hybridizes to the deposited oligonucleotide; and (c) reading the sample by detecting the label of the detectably-labeled oligonucleotide hybridized with the sample in step (b). The reactions that occur during this method are illustrated in Fig 8A. In these embodiments, the method may be multiplexed by, e.g., inactivating the peroxidase after step (a), inactivate the label or removing the hybridized labeled oligonucleotide from the sample after step (c); and labeling different sites on the sample by repeating steps (a), (b) and (c) using a different peroxidase-oligonucleotide conjugate and a different detectably-labeled oligonucleotide. Alternatively, before step (c) the method may comprise repeating steps (a) and (b) using a different peroxidase-oligonucleotide conjugate and a different oligonucleotide-tyramide conjugate. In these embodiments, the label used in the initial step (a) or (b) should be distinguishable from the labels used in the repeat steps, thereby allowing several labels to be detected in the reading step.

In any embodiments, the oligonucleotides may be designed so that multiple labeled oligonucleotides hybridize to a deposited oligonucleotide-tyramide conjugate. In these embodiment, the oligonucleotide-tyramide conjugate may hybridize to at least 2, at least 3, at least 4, at least 5 labeled oligonucleotides, each labeled with a mass tag or fluorophore, etc..

In some embodiments, the method may be a multiplex method that comprises (a) obtaining: (i) a plurality of binding agent-peroxidase conjugates (as illustrated in Fig. 6); (ii) a plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides in the oligonucleotide-tyramide conjugates have different sequences (as illustrated in Fig. 6); and (iii) a plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(ii). This method may comprise: (b) labeling the sample with a single binding agent-peroxidase conjugate of (a)(i), wherein the binding agent-peroxidase conjugate binds to sites on the sample, (c) treating the sample with an oligonucleotide-tyramide conjugate of (a)(ii), wherein the peroxidase of the binding agent-peroxidase conjugates bound to the sample in (b) activates the tyramide in the oligonucleotide-tyramide conjugate and causes deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase of the binding agent-peroxidase conjugate, (d) hybridizing a detectably labeled oligonucleotide of (a)(iii) with the sample, thereby producing complexes that comprise the detectably-labeled oligonucleotide at or near the sites to which the binding agent-peroxidase conjugate is bound; and (e) reading the sample to obtain data on the binding of the label of the detectably-labeled oligonucleotide.

In some embodiments, the detectably labeled oligonucleotides of (a)(iii) are distinguishable and the method comprises: (f) inactivating or removing (e.g., by denaturation) the peroxidase after step (c); and, between steps (d) and (e), (h) repeating step (b), (c) and (d) multiple times followed by a peroxidase inactivation step after step (c), each time using a different binding agent of (a)(i), a different oligonucleotide-tyramide conjugate of (a)(ii) and a detectably-labeled oligonucleotides labeled oligonucleotide of (a)(iii). In these embodiments, the reading step may comprise reading the sample to obtain data on the binding of all of the labels added in each step (d). An alternative version of this method that provides a similar result can be implemented by (f) inactivating the peroxidase after step (c); and, between steps (c) and (d), (g) repeating steps (b) and (c) multiple times followed by a peroxidase inactivation step after step (c), each time using a different binding agent-peroxidase conjugate of (a)(i) and a different oligonucleotide-tyramide conjugate of (a)(ii). In this embodiment, step (d) comprises hybridizing multiple detectably labeled oligonucleotide of (a)(iii) with the sample, thereby producing complexes that comprise the detectably labeled oligonucleotides, and step (e) comprises reading the sample to obtain data on the binding of all of the labels added in step (d).

In another embodiment, the method may comprise: (a) obtaining: (i) a plurality of binding agent-oligonucleotide conjugates; (ii) a plurality of peroxidase-oligonucleotide-conjugates, wherein each of the peroxidase-oligonucleotide conjugates specifically hybridizes with only one of the oligonucleotides of (a)(i); (iii) a plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides of the conjugates have different sequences; and (iv) a plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(iii). In this embodiment, the method may comprise; (b) labeling the sample with the plurality of binding agents of (a)(i); (c) specifically hybridizing a single peroxidase-linked oligonucleotide of the plurality of peroxidase-linked oligonucleotides of (a)(ii) with the sample, thereby producing complexes that comprise the peroxidase; (d) treating the sample with an oligonucleotide-tyramide conjugate of (a)(iii), wherein the peroxidase in the complexes produced in (c) activate the tyramide in the conjugate and cause covalent binding of the oligonucleotide in the conjugate to the sample near the complexes; (e) specifically hybridizing a detectably labeled oligonucleotide of (a)(iv) with the sample, thereby producing complexes that comprise the detectably labeled oligonucleotide; and (f) reading the sample to determine the abundance and/or location of the detectably labeled oligonucleotide hybridized in (e). As illustrated in Fig. 8B, in some implementations of this method the detectably-labeled oligonucleotides of (a)(iv) may be fluorescently labeled In these embodiments, the reading of (f) may be done by fluorescence-based imaging. As illustrated in Fig. 8C, in some implementations the detectably-labeled oligonucleotides of (a)(iv) may be mass tagged. In these embodiments, the reading step (f) may be done by a mass spectrometry method, e.g., by multiplexed ion beam imaging (MIBI) or mass cytometry.

In some multiplex embodiments of this implementation of the method, the method may comprise inactivating or removing the peroxidase after step (d). In these embodiments, the method may comprise, between steps (d) and (e) and without removing the oligonucleotides that become associated with the sample in step (d), repeating steps (c) and (d) multiple times, each repeat followed by a peroxidase inactivation or removal step, each repeat using a different peroxidase-linked oligonucleotide of (a)(ii) and a different oligonucleotide-tyramide conjugate of (a)(iii). In this embodiment, step (e) may comprise hybridizing multiple detectably-labeled oligonucleotides of (a)(iv) that are distinguishably labeled with the sample, thereby producing complexes that comprise the distinguishable detectably labeled oligonucleotides. In this

embodiment, step (f) may comprise reading the sample to determine the abundance and/or location of the multiple distinguishable detectably labeled oligonucleotide hybridized in (e). In fluorescence-based implementations, the detectably-labeled oligonucleotides of (a)(iv) are fluorescently labeled and up to seven (e.g., 2, 3, 4, 5, 6, or 7) distinguishable detectably labeled oligonucleotides are used in step (e). In this embodiment, the reading step of (f) may comprise determining the abundance and/or location of the detectably labeled oligonucleotides hybridized in (e) by fluorescence-based imaging.

In another multiplex implementation of this method, the method may comprise inactivating or removing the peroxidase after step (d) and between steps (e) and (f) and without removing or inactivating the detectably labeled oligonucleotide that become associated with the sample in step (c), repeating steps (c), (d) and (e) multiple times with a peroxidase removal or inactivation step after step (d), each repeat using a different peroxidase-oligonucleotide-conjugate of (a)(ii), a different oligonucleotide-tyramide conjugate of (a)(iii) and a different detectably-labeled oligonucleotide of (a)(iv). In these embodiments, the labels are distinguishable and, as such, the reading step (f) may comprise reading the sample to determine the abundance and/or location of the multiple detectably labeled oligonucleotide hybridized in (e). In some embodiments, the detectably-labeled oligonucleotides of (a)(iv) may be fluorescently labeled and up to seven (e.g., 2, 3, 4, 5, 6, or 7) distinguishable detectably labeled oligonucleotides are used in step (e). As would be apparent, in this embodiment he reading step of (f) comprises determining the abundance and/or location of the detectably labeled oligonucleotides hybridized in (e) by fluorescence-based imaging.

In another exemplary method, the sample may be analyzed by: (a) obtaining: (i) a plurality of binding agent-oligonucleotide conjugates; (ii) a plurality of peroxidase-oligonucleotide conjugates, wherein each of the peroxidase-oligonucleotide conjugates specifically hybridizes with only one of the oligonucleotides of (a)(i); (iii) a plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides of the conjugates have different sequences; and (iv) a plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(iii). This method may comprise: (b) labeling the sample with the plurality of binding agents of (a)(i); (c) hybridizing a single peroxidase-oligonucleotide conjugates of the plurality of peroxidase-oligonucleotide conjugates of (a)(ii) with the sample, thereby producing complexes that comprise the

peroxidase; (d) treating the sample with an oligonucleotide-tyramide conjugate of (a)(iii), wherein the peroxidase in the complexes produced in (c) activates the tyramide in the oligonucleotide-tyramide conjugate and the oligonucleotide-tyramide conjugate covalently binds to the sample at sites that are near the complexes; (e) inactivating or removing the peroxidase added in step (c); (f) repeating (c), (d) and (e) (e.g. 2-200 times) each repeat with a different peroxidase-oligonucleotide conjugate of (a)(ii) and different oligonucleotide-tyramide conjugate of (a)(iii); (g) hybridizing a set of fluorescently labeled oligonucleotide of (a)(iv) with the sample, wherein the members of the set are distinguishably labeled, to produce complexes that comprise the distinguishable fluorescently labeled oligonucleotides; (h) reading the sample to determine the abundance and/or location of the set of fluorescently labeled oligonucleotide hybridized in (g); (i) inactivating or removing the fluorescently labeled oligonucleotide hybridized in (g); and (j) repeating steps (g), (h) and (i) multiple times, each time with a different set of set of distinguishably fluorescently labeled oligonucleotide. In these embodiments the set of fluorescently labeled oligonucleotide hybridized in step (g) may comprises 2-7 (e.g., 2, 3, 4, 5, 6 or 7) distinguishable fluorescently labeled oligonucleotides.

In these embodiments, the method may comprise storing the sample after step (f) and before step (g), for a period of days, at least one week or at least one month at a temperature in the range of -20 °C to 25 °C, e.g., at -20°C, 4°C or room temperature. In addition, the steps (g)-(j) may be automated.

The number of binding agent-peroxidase conjugates used in the method may vary. In some embodiments, the method may be performed using at least 10 or at least 20 binding agents, up to 50 or up 100 or more binding agents. As noted above, peroxidase may be directly or indirectly linked to the binding agent (via a pair of complementary oligonucleotides).

In some embodiments, the oligonucleotides used in the method may be, independently, 8 nucleotides in length to as long as 150 nucleotides in length (e.g., in the range of 8 to 100 nucleotides in length). However, in many embodiments the oligonucleotides are 8 to 50 nucleotides in length, e.g., 10 to 30 nucleotides or 11 to 25 nucleotides in length although oligonucleotides having a length outside of these ranges can be used in many cases. In some embodiments, an oligonucleotide may have a calculated T_m in the range of 15°C to 70°C (e.g., 20°C-60°C or 35°C-50°C). In some embodiments, the oligonucleotides may be T_m-matched, where the term "T_m-matched" refers to sequences that have melting temperatures that are within a defined range, e.g., within less than 15°C, less than 10°C or less than 5°C of a defined

temperature. T_m matching allows the hybridization steps to be performed under the same conditions in each cycle. In some embodiments, the sequences of the oligonucleotides of the oligonucleotide-tyramide conjugates and the labeled oligonucleotides may be the same length as and may be perfectly complementary.

5 Oligonucleotides and/or peroxidase may be linked to binding agents or peroxidase using any convenient method (see, e.g., Gong et al., Bioconjugate Chem. 2016 27: 217–225 and Kazane et al. Proc Natl Acad Sci 2012 109: 3731-3736). For example, a oligonucleotide may be linked to a binding agents or peroxidase directly using any suitable chemical moiety on the binding agents or peroxidase (e.g., a cysteine residue or via an engineered site). In some 10 embodiments, an oligonucleotide may be linked to the binding agents or peroxidase directly or indirectly via a non-covalent interaction. In some embodiments, the binding agents and the peroxidase may be linked to their respective oligonucleotides by reacting an oligonucleotide-maleimide conjugate with the binding agent or peroxidase, thereby joining those molecules together.

15 In some embodiments, the method may comprise labeling the sample with a plurality of binding agents. This step may involve contacting the sample (e.g., an FFPE section mounted on a planar surface such as a microscope slide, but it could be any sample such as a western blot or a non-planar tissue) with a single binding agent or several binding binding agents, *en masse* under conditions by which the binding agents bind to complementary sites (e.g., protein 20 epitopes or nucleotide sequences) in the sample. Methods for binding antibodies and aptamers to complementary sites in the sample and methods for hybridizing nucleic acids probes to a sample *in situ* are well known. In some embodiments, the binding agents may be cross-linked to the sample, thereby preventing the binding agents from disassociating during subsequent steps. This crosslinking step may be done using any amine-to-amine crosslinker although a variety of 25 other chemistries can be used to cross-link the binding agents to the sample if desired. In some embodiments, the binding agents are not cross-linked to the sample.

After the sample has been bound to the binding agents, in some embodiments, the method may further comprise specifically hybridizing a single peroxidase-linked oligonucleotide of the plurality of peroxidase-linked oligonucleotides with the sample, thereby 30 producing complexes that comprise the peroxidase. As such, in some embodiments, the method may further comprise specifically hybridizing one of the peroxidase-linked oligonucleotides

with the binding agent-labeled sample, thereby producing peroxidase-containing complexes that are bound to specific sites in the sample.

After the sample has been washed to remove peroxidase-linked oligonucleotides that have not hybridized to the sample, the method may further comprise treating the sample with an oligonucleotide-tyramide conjugate, as described above in the presence of hydrogen peroxide (e.g., about 1 mM H_2O_2). In this step, the peroxidase in the complexes produced in the earlier step activates the tyramide in the conjugate and causes covalent binding of the label to the sample near the complexes. This reaction is similar to the tyramide signal amplification reaction described in Bobrow et al. (J. Immunol. Methods 1992 137:103–112), Bobrow et al. (J. Immunol Methods 1989 125:279–285) and Van Gijlswijk et al. (J. Histochem. Cytochem. 1996 44:389–392) and results in deposition of the oligonucleotide at sites in the sample that are proximal to the binding complex.

After unreacted oligonucleotide-tyramide conjugate has been washed away, the peroxidase can be removed by denaturation or otherwise inactivated (e.g., by treatment with 3–30% hydrogen peroxide w/v for 1 min to 1 hr; see, e.g., Sennepin et al. Analytical Biochemistry 2009 393: 129-131 and Arnao et al. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1990 1038: 85-89) prior to reading the sample to obtain data on the binding of the label.

In embodiments in which the reading is done by mass spectrometry (e.g., multiplexed ion beam imaging (MIBI) or mass cytometry (CyTOF)), the labeled oligonucleotides used in the method may be mass tagged (which, in many embodiments, is a mass-tag/chelator-tyramide conjugate complex). In these embodiments, the term "mass tag" refers to an isotope of any element, including transition metals, post-transition metals, halides, noble metals or lanthanides, that is identifiable by its mass, distinguishable from other mass tags, and used to tag a biologically active material or analyte. A mass tag has an atomic mass that is distinguishable from the atomic masses present in the analytical sample and in the particle of interest. The term "monoisotopic" means that a tag contains a single type of metal isotope (although any one tag may contain multiple metal atoms of the same type). Lanthanides are elements having atomic numbers 58 to 71 and can be readily used herein because they can be chelated by diethylene triamine penta-acetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA).

In some embodiments, the peroxidase can be inactivated or removed and, before reading the sample and without removing or inactivating the label, the method further comprises repeating the hybridizing, treating and inactivating steps multiple times, each time using a different oligonucleotide-tyramide conjugate and a different labeled oligonucleotide. In some embodiments, these steps may be repeated at least 2, at least 5, at least 10 or at least 50 (e.g., 5 to 100) times to produce a sample that is labeled with multiple mass tags. As there are more than 80 naturally occurring elements having more than 250 stable isotopes, the cells may be labeled using at least 5, at least 10, at least 20, at least 30, at least 50, or at least 100, up to 150 mass tags, or more if the mass tags are combined in at least some of the cycles.

In some embodiments, the method further comprises a step of reading the sample by multiplexed ion beam imaging (MIBI). This embodiment method may involve scanning the sample by secondary ion mass spectrometry (SIMS) using a positively or negatively charged ion beam to generate a data set that comprises spatially-addressed measurements of the identity and abundance of the mass tags across the sample. Because ionization removes a layer from the top of the sample and the ion beam can raster through the sample several times, the spatially-addressed measurements can be used to reconstruct a two-dimensional or three-dimensional image of the sample. The general principles of MIBI, including methods by which samples may be made, methods for ionizing the tags, and methods for analyzing the data, as well as hardware that can be employed in MIBI, including but not limited to, mass spectrometers and computer control systems are known and are reviewed in a variety of publications including, but not limited to Angelo et al. Nature Medicine 2014 20:436, Rost et al. Lab. Invest. 2017 97: 992-1003, US 9,766,224, US 9,312,111 and US2015/0080233, among many others, which patents and publications are incorporated by reference herein for disclosure of those methods and hardware.

Alternatively, in some embodiments, the method further comprises a step of reading the sample by mass cytometry (CyTOF). In these embodiments, the sample may comprise a suspension of disassociated cells that are separated from another and capable of being sorted in a flow cytometer. As such, in these embodiments, the cells may be labeled in solution and washed after each step. In these embodiments, the population of cells may be obtained from blood (e.g., peripheral blood mononuclear cells (PBMC) such as lymphocytes, monocytes, macrophages, etc., red blood cells, neutrophils, eosinophils, basophils, etc., or other cells that are circulating in peripheral blood), cells that are grown in culture such as a suspension of single

cells, and single cell organisms. In some cases, the sample may be made from a tissue sample (particularly of a soft tissue such as, e.g., spleen, liver or brain) or cultured cells (e.g., human embryonic kidney cells, COS cells, HeLa cells, Chinese hamster ovary cells, cancer cell lines; stem cell lines, such as embryonic stem cells and induced pluripotent stem cells, etc.) that have been trypsinized to physically disassociate the cells from one another.

Mass cytometry makes uses a plasma beam to atomize mass-tag labeled cells in a sample and generate a data set that comprises temporally-addressable measurements of the abundance of the mass tags in or on each of the analyzed cells. In mass cytometry, mass-tag labeled cells are introduced into a fluidic system and hydrodynamically focused one cell at a time through a flow cell using a sheath fluid prior to being vaporized, atomized and ionized by plasma (e.g., an inductively coupled plasma) to produce ions that are subsequently analyzed by spectrometry (using, e.g., a mass spectrometer or an emission spectrometer) to determine the identity and/or relative abundance of the mass tags associated with the cell. The general principles of mass cytometry, including methods by which single cell suspensions can be made, methods by which cells can be labeled using, e.g., mass-tagged antibodies, methods for atomizing particles and methods for performing elemental analysis on particles, as well as hardware that can be employed in mass cytometry, including flow cells, ionization chambers, reagents, mass spectrometers and computer control systems are well-known and have been amply reviewed in a variety of publications including, but not limited to Bandura et al. Analytical Chemistry 2009 81: 6813-6822), Tanner et al. (Pure Appl. Chem 2008 80: 2627-2641), U.S. Patent Nos. 7,479,630 (Method and apparatus for flow cytometry linked with elemental analysis) and 7,135,296 (Elemental analysis of tagged biologically active materials); and published U.S. patent application 20080046194, for example, which publications are incorporated by reference herein for disclosure of those methods and hardware.

MIBI and/or CyTOF embodiments can implemented using an oxygen beam or cesium beam, and other sources of plasma.

In some embodiments, the mass tags may be composed of a tyramide-chelator conjugate and a stable metal isotope that is bound by the chelator. The chelator may be, e.g., DTPA or DOTA. The stable metal isotope used in the method may be any stable isotope that is not commonly found in the sample under analysis. These may include, but are not limited to, the high molecular weight members of the transition metals (e.g., Rh, Ir, Cd, Au), post-transition metals (e.g., Al, Ga, In, Tl), metalloids (e.g., Te, Bi), alkaline metals, halogens, and actinides,

although others may be used in some circumstances. A mass tag may have an atomic number in the range of 21 to 238. In certain embodiments, a lanthanide may be used. The lanthanide series of the periodic table comprises 15 elements, 14 of which have stable isotopes (La, Ce, Pr, Nd, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu). Lanthanides can be readily used because of their rarity in the biosphere. There are more than 100 stable isotopes of elements having an atomic number between 1 and 238 that are not commonly found in biological systems. In some embodiments, tagging isotopes may comprise non-lanthanide elements that can form stable metal chelator tags for the applications described herein. When using a SIMS-based measurement method, in contrast to some inductively coupled plasma mass spectrometry (ICP-MS)-based methods, the elemental reporter could also comprise lower MW transition elements not common in biological systems (e.g. Al, W, and Hg). Elements suitable for use in this method in certain embodiments include, but are not limited to, lanthanides and noble metals such as gold, silver or platinum. In certain cases, an elemental tag may have an atomic number of 21-92. In particular embodiments, the elemental tag may contain a transition metal, i.e., an element having the following atomic numbers, 21-29, 39-47, 57-79, and 89. Transition elements include the lanthanides and noble metals. See, e.g., Cotton and Wilkinson, 1972, pages 528-530. The elemental tags employed herein are not commonly present in typical biological samples, e.g., cells, unless they are provided exogenously.

In certain embodiments, the reading is done by fluorescence-based imaging (FBI) and the labels may be fluorophores. Fluorophores of interest include but are not limited to xanthene dyes, e.g., fluorescein and rhodamine dyes, such as fluorescein isothiocyanate (FITC), 6-carboxyfluorescein (commonly known by the abbreviations FAM and F), 6-carboxy-
5 2',4',7',4,7-hexachlorofluorescein (HEX), 6-carboxy-4', 5'-dichloro-2', 7'-dimethoxyfluorescein (JOE or J), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA or T), 6-carboxy-X-rhodamine (ROX or R), 5-carboxyrhodamine-6G ($R6G^5$ or G^5), 6-carboxyrhodamine-6G ($R6G^6$ or G^6), and rhodamine 110; cyanine dyes, e.g., Cy3, Cy5 and Cy7 dyes; coumarins, e.g., umbelliferone; benzimide dyes, e.g. Hoechst 33258; phenanthridine
10 dyes, e.g., Texas Red; ethidium dyes; acridine dyes; carbazole dyes; phenoxazine dyes; porphyrin dyes; polymethine dyes, e.g., BODIPY dyes and quinoline dyes.

In some embodiments, the reading is done by fluorescence-based imaging (FBI) to detect samples labeled with two, three, or four distinguishable fluorophores and the method comprises repeating the hybridization, treatment and inactivation steps multiple times (at least

one or twice, up to the number of distinguishable fluorophores), each time using a different conjugates, prior to reading the sample by fluorescence microscopy to produce an image showing the pattern of binding of the label to the sample. This step may be done using any convenient reading method and, in some embodiments, e.g., hybridization of the different probes can be separately read using a fluorescence microscope equipped with an appropriate filter for the fluorophore used, or by using dual or triple band-pass filter sets to observe multiple fluorophores (see, e.g., U.S. Pat. No. 5,776,688), as appropriate.

If any of the conjugates contains a cleavable linker, then the cleavable linker should be capable of being selectively cleaved using a stimulus (e.g., a chemical, light or a change in its environment) without breaking any bonds in the oligonucleotides. In some embodiments, the cleavable linkage may be a disulfide bond, which can be readily broken using a reducing agent (e.g., β-mercaptoethanol, TCEP or the like). Suitable cleavable bonds that may be employed include, but are not limited to, the following: base-cleavable sites such as esters, particularly succinates (cleavable by, for example, ammonia or trimethylamine), quaternary ammonium salts (cleavable by, for example, diisopropylamine) and urethanes (cleavable by aqueous sodium hydroxide); acid-cleavable sites such as benzyl alcohol derivatives (cleavable using trifluoroacetic acid), teicoplanin aglycone (cleavable by trifluoroacetic acid followed by base), acetals and thioacetals (also cleavable by trifluoroacetic acid), thioethers (cleavable, for example, by HF or cresol) and sulfonyls (cleavable by trifluoromethane sulfonic acid, trifluoroacetic acid, thioanisole, or the like); nucleophile-cleavable sites such as phthalamide (cleavable by substituted hydrazines), esters (cleavable by, for example, aluminum trichloride); and Weinreb amide (cleavable by lithium aluminum hydride); and other types of chemically cleavable sites, including phosphorothioate (cleavable by silver or mercuric ions) and diisopropyldialkoxysilyl (cleavable by fluoride ions).

In some embodiments, the sample may be treated with a single labeled oligonucleotide-tyramide conjugate (or single labeled oligonucleotide, if the oligonucleotide-tyramide conjugate is unlabeled) in each cycle, thereby labeling the sample with a single label in each cycle. In other embodiments, the sample may treated with multiple (e.g., up to two, three, four or five) distinguishable labeled oligonucleotides in each cycle, thereby labeling the sample with multiple labels in each cycle. These latter embodiments can be used to increase the level of multiplexing since sites that are labeled with a particular combination of labels are distinguishable from sites that are labeled with a single label or other combinations of labels.

In embodiments in which the sample is read by multiplexed ion beam imaging (MIBI), each reading step may produce an image of the sample showing the pattern of binding of multiple binding agents. In particular embodiments, in any one pixel of the image, the intensity of the color of the pixel correlates with the magnitude of the signals obtained for a mass tag obtained in the original scanning. In these embodiments, the resulting false color image may show color-code cells in which the intensity of the color in any single pixel of a cell correlates with the amount of specific binding reagent that is associated with the corresponding area in the sample.

In embodiments in which the sample is read by fluorescence, each reading step may produce an image of the sample showing the pattern of binding of a single binding agent. In some embodiments, the method may further comprise analyzing, comparing or overlaying, at least two of the images. In some embodiments, the method may further comprise overlaying all of the images to produce an image showing the pattern of binding of all of the binding agents to the sample. The image analysis module used may transform the signals from each fluorophore to produce a plurality of false color images. The image analysis module may overlay the plurality of false color images (e.g., superimposing the false colors at each pixel) to obtain a multiplexed false color image. Multiple images (e.g., unweighted or weighted) may be transformed into a single false color, e.g., so as to represent a biological feature of interest characterized by the binding of specific binding agent. False colors may be assigned to specific binding agents or combinations of binding agents, based on manual input from the user. In certain aspects, the image may comprise false colors relating only to the intensities of labels associated with a feature of interest, such as in the nuclear compartment. The image analysis module may further be configured to adjust (e.g., normalize) the intensity and/or contrast of signal intensities or false colors, to perform a deconvolution operation (such as blurring or sharpening of the intensities or false colors), or perform any other suitable operations to enhance the image. The image analysis module may perform any of the above operations to align pixels obtained from successive images and/or to blur or smooth intensities or false colors across pixels obtained from successive images.

In some embodiments, images of the sample may be taken at different focal planes, in the z direction. These optical sections can be used to reconstruct a three-dimensional image of the sample. Optical sections may be taken using confocal microscopy, or by any other method known to an artisan of ordinary skill in the biological arts.

In addition to the labeling methods described above, the sample may be stained using a cytological stain, either before or after performing the method described above. In these embodiments, the stain may be, for example, phalloidin, gadodiamide, acridine orange, bismarck brown, barmine, Coomassie blue, bresyl violet, brystal violet, DAPI, hematoxylin, eosin, ethidium bromide, acid fuchsine, haematoxylin, hoechst stains, iodine, malachite green, methyl green, methylene blue, neutral red, Nile blue, Nile red, osmium tetroxide (formal name: osmium tetraoxide), rhodamine, safranin, phosphotungstic acid, osmium tetroxide, ruthenium tetroxide, ammonium molybdate, cadmium iodide, carbohydrazide, ferric chloride, hexamine, indium trichloride, lanthanum nitrate, lead acetate, lead citrate, lead(II) nitrate, periodic acid, phosphomolybdic acid, potassium ferricyanide, potassium ferrocyanide, ruthenium red, silver nitrate, silver proteinate, sodium chloroaurate, thallium nitrate, thiosemicarbazide, uranyl acetate, uranyl nitrate, vanadyl sulfate, or any derivative thereof. The stain may be specific for any feature of interest, such as a protein or class of proteins, phospholipids, DNA (e.g., dsDNA, ssDNA), RNA, an organelle (e.g., cell membrane, mitochondria, endoplasmic recticulum, golgi body, nuclear envelope, and so forth), or a compartment of the cell (e.g., cytosol, nuclear fraction, and so forth). The stain may enhance contrast or imaging of intracellular or extracellular structures. In some embodiments, the sample may be stained with haematoxylin and eosin (H&E).

Kits

Also provided by this disclosure are kits that contain any of the reagent systems described, optionally with any further components (e.g., labeled nucleotides, enzymes, etc.) for practicing the subject methods, as described above. These various components of a kit may be in separate vessels or mixed in the same vessel.

The various components of the kit may be present in separate containers or certain compatible components may be pre-combined into a single container, as desired.

In addition to the above-mentioned components, the subject kit may further include instructions for using the components of the kit to practice the subject method.

Utility

The methods and compositions described herein find general use in a wide variety of applications for analysis of any sample (e.g., in the analysis of tissue sections, sheets of cells,

spun-down cells, cell suspensions, blots of electrophoresis gels, Western blots, dot-blots, ELISAs, antibody microarrays, nucleic acid microarrays, whole tissues or parts thereof, or non-planar pieces of tissue etc.). The method may be used to analyze any tissue, including tissue that has been clarified, e.g., through lipid elimination, for example. The sample may be prepared using expansion microscopy methods (see, e.g., Chozinski et al. Nature Methods 2016 13: 485–488), which involves creating polymer replicas of a biological system created through selective co-polymerization of organic polymer and cell components. The method can be used to analyze spreads of cells, exosomes, extracellular structures, biomolecules deposited on a solid support or in a gel (Elisa, western blot, dot blot), whole organism, individual organs, tissues, cells, extracellular components, organelles, cellular components, chromatin and epigenetic markers, biomolecules and biomolecular complexes, for example. The binding agents may bind to any type of molecule, including proteins, lipids, polysaccharides, proteoglycans, metabolites, or artificial small molecules or the like. The method may have many biomedical applications in screening and drug discovery and the like. Further, the method has a variety of clinical applications, including, but not limited to, diagnostics, prognostics, disease stratification, personalized medicine, clinical trials and drug accompanying tests.

In particular embodiments, the sample may be a section of a tissue biopsy obtained from a patient. Biopsies of interest include both tumor and non-neoplastic biopsies of skin (melanomas, carcinomas, etc.), soft tissue, bone, breast, colon, liver, kidney, adrenal, gastrointestinal, pancreatic, gall bladder, salivary gland, cervical, ovary, uterus, testis, prostate, lung, thymus, thyroid, parathyroid, pituitary (adenomas, etc.), brain, spinal cord, ocular, nerve, and skeletal muscle, etc.

The binding agents may specifically bind to specific target molecules, including genomic DNA, RNA, or proteins. In some embodiments, the protein are intracellular, but in other embodiments the proteins may be secreted, such as cytokines, hormones and growth factors. In certain embodiments, the binding agents specifically bind to biomarkers, including cancer biomarkers, that may be proteinaceous. Exemplary cancer biomarkers, include, but are not limited to carcinoembryonic antigen (for identification of adenocarcinomas), cytokeratins (for identification of carcinomas but may also be expressed in some sarcomas), CD15 and CD30 (for Hodgkin's disease), alpha fetoprotein (for yolk sac tumors and hepatocellular carcinoma), CD117 (for gastrointestinal stromal tumors), CD10 (for renal cell carcinoma and acute lymphoblastic leukemia), prostate specific antigen (for prostate cancer), estrogens and

progesterone (for tumour identification), CD20 (for identification of B-cell lymphomas) and CD3 (for identification of T-cell lymphomas).

The above-described method can be used to analyze cells from a subject to determine, for example, whether the cell is normal or not or to determine whether the cells are responding to a treatment. In one embodiment, the method may be employed to determine the degree of dysplasia in cancer cells. In these embodiments, the cells may be a sample from a multicellular organism. A biological sample may be isolated from an individual, e.g., from a soft tissue. In particular cases, the method may be used to distinguish different types of cancer cells in FFPE samples.

Immunological markers to identify cancer cells are generally well known and available for many types of cancers (see, generally, Painter et al, Toxicol. Pathol. 2010 38: 131–141 and Bahrami et al Arch Pathol Lab Med. 2008 132:326–48, among many others). For example, the cancer cells identified in step (a) may be i. melanoma cells identified by expression of one or more of the following markers: S-100, Melan-A, Sox10, MITF, tyrosinase, and HMB45 (e.g., S-100, Melan-A, Sox10 and HMB45); ii. carcinoma cells identified by the expression of one or more of the following markers: pan-cytokeratin (CK), CK7, CK20, CK5/6, CK8/18, napsin A, TTF-1, PSA, PSMA, CDX2, GATA3, synaptophysin, chromogranin A, NSE, EpCAM, and MUC-1 (e.g., CK7, CK20, TTF-1, PSA, CDX2, GATA3); iii. lymphoma/leukemia cells identified by the expression of one or more of the following markers: CD45, CD3, PAX5, CD20, Myc, CyclinD1, BCL-2, BCL-6, IRF4, CD138, CD30, kappa, lambda, TdT, CD10, ALK, and lysoszyme (e.g., CD45, PAX5, CD20, Myc, CyclinD1, BCL-2, BCL-6, IRF4, CD138, and CD30); iv. sarcoma/mesothelioma cells identified by the expression of one or more of the following markers: vimentin, SMA, desmin, caldesmin, MyoD1, CD34, calretinin, podoplanin, and CD47 (e.g., vimentin, SMA, desmin, CD34); v. glioma cells/neural tumor cells identified by the expression of one or more of the following markers: GFAP, IDH-1(R132H), neurofilament, and NeuN (e.g, GFAP, IDH-1(R132H)); or vi. germ cell tumor cells identified by the expression of one or more of the following markers: beta-HCG, OCT4, SALL4, PLAP, inhibin A, HPL and AFP.

Exemplary panels of markers for identifying various cancer cells are shown in the following table, although there are many alternatives that can be used.

Acute Leukemia IHC Panel	CD3, CD7, CD10, CD20, CD34, CD45, CD56, CD61, CD71, CD117, MPO, PAX-5, and TdT.

Adenocarcinoma vs. Mesothelioma IHC Panel	Pan-CK, CEA, MOC-31, BerEP4, TTF1, calretinin, and WT-1.
Bladder vs. Prostate Carcinoma IHC Panel	CK7, CK20, PSA, CK 903, uroplakin, thrombomodulin, p53 and p63.
Breast IHC Panel	ER, PR, Ki-67, and HER2. Reflex to HER2 FISH after HER2 IHC is available.
Burkitt vs. DLBC Lymphoma IHC panel	BCL-2, c-MYC, Ki-67.
Carcinoma Unknown Primary Site, Female (CUPS IHC Panel - Female)	CK7, CK20, mammaglobin, GATA-3, ER, TTF1, CEA, CA19-9, S100, synaptophysin, and WT-1.
Carcinoma Unknown Primary Site, Male (CUPS IHC Panel - Male)	CK7, CK20, TTF1, PSA, CEA, CA19-9, S100, and synaptophysin.
GIST IHC Panel	CD117, DOG-1, CD34, and desmin.
Hepatoma/Cholangio vs. Metastatic Carcinoma IHC Panel	HSA (HepPar 1), CDX2, CK7, CK20, CAM 5.2, TTF-1, and CEA (polyclonal).
Hodgkin vs. NHL IHC Panel	BOB-1, BCL-6, CD3, CD10, CD15, CD20, CD30, CD45 LCA, CD79a, MUM1, OCT-2, PAX-5, and EBER ISH.
Lung Cancer IHC Panel	chromogranin A, synaptophysin, CK7, p63, and TTF-1.
Lung vs. Metastatic Breast Carcinoma IHC Panel	TTF1, mammaglobin, GCDFP-15 (BRST-2), and ER.
Lymphoma Phenotype IHC Panel	BCL-2, BCL-6, CD3, CD4, CD5, CD7, CD8, CD10, CD15, CD20, CD30, CD79a, CD138, cyclin D1, Ki67, MUM1, PAX-5, TdT, and EBER ISH.
Lymphoma vs. Carcinoma IHC Panel	CD30, CD45, CD68, CD117, pan-keratin, MPO, S100, and synaptophysin.
Lymphoma vs. Reactive Hyperplasia IHC Panel	BCL-2, BCL-6, CD3, CD5, CD10, CD20, CD23, CD43, cyclin D1, and Ki-67.
Melanoma vs. Squamous Cell Carcinoma IHC Panel	CD68, Factor XIIIa, CEA (polyclonal), S-100, melanoma cocktail (HMB-45, MART-1/Melan-A, tyrosinase) and Pan-CK.
Mismatch Repair Proteins IHC Panel (MMR/Colon Cancer)	MLH1, MSH2, MSH6, and PMS2.
Neuroendocrine Neoplasm IHC Panel	CD56, synaptophysin, chromogranin A, TTF-1, Pan-CK, and CEA (polyclonal).
Plasma Cell Neoplasm IHC Panel	CD19, CD20, CD38, CD43, CD56, CD79a, CD138, cyclin D1, EMA, IgG kappa, IgG lambda, and MUM1.

Prostate vs. Colon Carcinoma IHC Panel	CDX2, CK 20, CEA (monoclonal), CA19-9, PLAP, CK 7, and PSA.
Soft Tissue Tumor IHC Panel	Pan-CK, SMA, desmin, caldesmon, MyoD1, myogenin, S100, CD34, vimentin, and CD68.
T-Cell Lymphoma IHC panel	ALK1, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD20, CD21, CD30, CD56, TdT, and EBER ISH.
T-LGL Leukemia IHC panel	CD3, CD8, granzyme B, and TIA-1.
Undifferentiated Tumor IHC Panel	Pan-CK, CK8/18, S100, CD45, and vimentin.

In some embodiments, the method may involve obtaining data (an image) as described above (an electronic form of which may have been forwarded from a remote location), and the image may be analyzed by a doctor or other medical professional to determine whether a patient has abnormal cells (e.g., cancerous cells) or which type of abnormal cells are present. The image may be used as a diagnostic to determine whether the subject has a disease or condition, e.g., a cancer. In certain embodiments, the method may be used to determine the stage of a cancer, to identify metastasized cells, or to monitor a patient's response to a treatment, for example.

The compositions and methods described herein can be used to diagnose a patient with a disease. In some cases, the presence or absence of a biomarker in the patient's sample can indicate that the patient has a particular disease (e.g., a cancer). In some cases, a patient can be diagnosed with a disease by comparing a sample from the patient with a sample from a healthy control. In this example, a level of a biomarker, relative to the control, can be measured. A difference in the level of a biomarker in the patient's sample relative to the control can be indicative of disease. In some cases, one or more biomarkers are analyzed in order to diagnose a patient with a disease. The compositions and methods of the disclosure are particularly suited for identifying the presence or absence of, or determining expression levels, of a plurality of biomarkers in a sample.

In some cases, the compositions and methods herein can be used to determine a treatment plan for a patient. The presence or absence of a biomarker may indicate that a patient is responsive to or refractory to a particular therapy. For example, a presence or absence of one or more biomarkers may indicate that a disease is refractory to a specific therapy, and an alternative therapy can be administered. In some cases, a patient is currently receiving the

therapy and the presence or absence of one or more biomarkers may indicate that the therapy is no longer effective.

In some cases, the method may be employed in a variety of diagnostic, drug discovery, and research applications that include, but are not limited to, diagnosis or monitoring of a disease or condition (where the image identifies a marker for the disease or condition), discovery of drug targets (where the a marker in the image may be targeted for drug therapy), drug screening (where the effects of a drug are monitored by a marker shown in the image), determining drug susceptibility (where drug susceptibility is associated with a marker) and basic research (where is it desirable to measure the differences between cells in a sample).

In certain embodiments, two different samples may be compared using the above methods. The different samples may be composed of an “experimental” sample, i.e., a sample of interest, and a “control” sample to which the experimental sample may be compared. In many embodiments, the different samples are pairs of cell types or fractions thereof, one cell type being a cell type of interest, e.g., an abnormal cell, and the other a control, e.g., normal, cell. If two fractions of cells are compared, the fractions are usually the same fraction from each of the two cells. In certain embodiments, however, two fractions of the same cell may be compared. Exemplary cell type pairs include, for example, cells isolated from a tissue biopsy (e.g., from a tissue having a disease such as colon, breast, prostate, lung, skin cancer, or infected with a pathogen, etc.) and normal cells from the same tissue, usually from the same patient; cells grown in tissue culture that are immortal (e.g., cells with a proliferative mutation or an immortalizing transgene), infected with a pathogen, or treated (e.g., with environmental or chemical agents such as peptides, hormones, altered temperature, growth condition, physical stress, cellular transformation, etc.), and a normal cell (e.g., a cell that is otherwise identical to the experimental cell except that it is not immortal, infected, or treated, etc.); a cell isolated from a mammal with a cancer, a disease, a geriatric mammal, or a mammal exposed to a condition, and a cell from a mammal of the same species, preferably from the same family, that is healthy or young; and differentiated cells and non-differentiated cells from the same mammal (e.g., one cell being the progenitor of the other in a mammal, for example). In one embodiment, cells of different types, e.g., neuronal and non-neuronal cells, or cells of different status (e.g., before and after a stimulus on the cells) may be employed. In another embodiment of the invention, the experimental material contains cells that are susceptible to infection by a pathogen such as a virus, e.g., human immunodeficiency virus (HIV), etc., and the control material contains cells

that are resistant to infection by the pathogen. In another embodiment, the sample pair is represented by undifferentiated cells, e.g., stem cells, and differentiated cells.

The images produced by the method may be viewed side-by-side or, in some embodiments, the images may be superimposed or combined. In some cases, the images may be in color, where the colors used in the images may correspond to the labels used.

Cells from any organism, e.g., from bacteria, yeast, plants and animals, such as fish, birds, reptiles, amphibians and mammals may be used in the subject methods. In certain embodiments, mammalian cells, i.e., cells from mice, rabbits, primates, or humans, or cultured derivatives thereof, may be used.

EXAMPLES

In order to further illustrate some embodiments of the present invention, the following specific examples are given with the understanding that they are being offered to illustrate examples of the present invention and should not be construed in any way as limiting its scope.

Materials and Methods

Tyramide-Oligonucleotide conjugation: Tyramide and oligonucleotide was conjugated by two step reaction using sulfo-succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) (Thermo Scientific). First step, 0.8 mg tyramide hydrochloride (Sigma Aldrich) and 1.8 mg sulfo-SMCC were rotated in pH 7.2 phosphate bufferd saline (PBS) overnight at room temperature in the dark. Second step, 100 ug tyramide-oligonucleotide-1 (to-1, Table 1) was added into first step reaction tube and were rotated 2 hours at room temperature in the dark. Any suitable amount of tyramide-oligonucleotide could be used in this step, since another amount may be optimal. After filtration with 7K zeba desalting column (Thermo Fisher Scientific) Tyramide-Oligonucleotide-1 (TO-1) was stored at -20 °C. Working concentration was determined by titration of Tyramide-Oligonucleotide for appropriate application each time.

Table 1. Sequences of oligonucleotide

tyramide-oligonucleotide-1(to-1)	5’-SH-ATTAAAGCTGATGGAGTTCGTGACTGG-3’ SEQ ID NO: 1
Tyramide-Oligonucleotide-1(TO-1)	Tyramide::ATTAAAGCTGATGGAGTTCGTGACTGG SEQ ID NO: 2

#23 antibody-oligonucleotide	5'-Maleimide-GGTTTCCTCAGACAC-3' SEQ ID NO: 3
#55 antibody-oligonucleotide	5'-Maleimide-AGGTCAACTCGCAC-3' SEQ ID NO: 4
#23 AS-oligonucleotide	5'-GTGTCTGAGGAAACC-3' SEQ ID NO: 5
#55 AS-oligonucleotide	5'-GTGCGAGTTGACCT-3' SEQ ID NO: 6
#23 HRP-oligonucleotide	5'-HRP-GTGTCTGAGGAAACC-3' SEQ ID NO: 7
#55 HRP-oligonucleotide	5'-HRP-GTGCGAGTTGACCT-3' SEQ ID NO: 8
Alexa 647-anti-sense to-1	5'-Alexa647-CCAGTCACGAACTCCATCAGC-3' SEQ ID NO: 9

Antibody-oligonucleotide conjugation: CD3 zeta (6B10.2, Santa Cruz Biotechnology) and E-cadherin (4A2C7, Thermo Fisher Scientific) were conjugated specific oligonucleotide (Table 1) using standard Maleimide-based conjugation method. We obtained maleimide-modified oligonucleotide from TriLink. Meleimide group on oligonucleotides was deprotected by heating in toluene at 90 °C for 4 hours. After deprotection, oligonucleotides were washed with 100% ethanol twice and resuspended with buffer C (150nM NaCl, 2mM Tris-HCl pH 7.2, 1mM EDTA, 0.02% (w/v) NaN_3) and aliquoted 100 ug in 0.2 ml PCR tubes (E&K Scientific). After lyophilization, oligonucleotides were stored at -20 °C until conjugation. Disulfide bonds in antibody (50 ug) were partially cleaved by 2 mM Tris[2-carboxyethyl] phosphine hydrochloride (TCEP) with 2mM ethylendiaminetetraacetic acid (EDTA) in pH 7.2 PBS for 30 min at room temperature. After TCEP treatment antibody was resuspended with buffer C using 7K zeba desalting column (Thermo Fisher Scientific). The partially reduced antibody was incubated with 100 ug meleimide-oligonucleotide containing NaCl at a final concentration of 350 mM in buffer C for 2 hours at room temperature. After antibody-oligonucleotide conjugation, reaction buffer was exchanged with storage buffer (100 mM phosphate, 150 mM NaCl, 1 mM EDTA, 0.1% NaN_3 pH 7.4) using 40K zeba desalting column (Thermo Fisher Scientific). The protein concentration of oligonucleotide-conjugated antibody was measured by Bradford assay using IgG protein standard (Bio Rad).

Tyramide CODEX-2 staining

1) Antibody staining: A formalin-fixed paraffin-embedded (FFPE) tonsil specimen from a healthy donor was used for CODEX-2 staining. The tissue was cut to 4 um thickness and the sections were placed on poly-Lysine coated 22x22 glass coverslip (Electron Microscopy Sciences). Paraffin was removed from tissue specimen by consecutive washes with xylene and

ethanol (100% xylene triple and 100% ethanol twice, each step for 3 min) after baked of coverslips at 60 °C for 20 min. After deparaffinization the specimens were hydrated in descending concentrations of ethanol in water (90% 80% 70%, each step for 3 min) and final rinse in ddH_2O twice for 1 min. Heat-induced epitope retrieval was performed in R-UNIVERSAL Epitope Recovery Buffer (Electron Microscopy Sciences) using a pressure cooker (121 °C for 15 min). After 40 min of pressure reaction coverslips were taken out from a pressure cooker and put it on the lab bench to cool for 20 min. After cooling, coverslips were washed in TBS IHC wash buffer with Tween 20 (Cell Marque) twice for 3 min and they were treated with 0.3 % hydrogen peroxide for 15 min for quenching endogenous peroxidase due to reducing background. For nonspecific protein blocking, tissue specimen was blocked with 0.2 mg/mL salmon sperm DNA (ssDNA) and 750 nM anti-sense oligonucleotide for CD3 (#55 AS-oligonucleotide) and E-cadherin (#23 AS-oligonucleotide) (Table 1) in TNB Blocking Buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl 0.5% Blocking Reagent) (Perkin Elmer) for 30 min at room temperature. After blocking, specimen was stained with #55 antibody-oligonucleotide-conjugated-CD3 and #23 antibody-oligonucleotide-conjugated E-cadherin antibodies each 20 ng and 0.2 mg/mL ssDNA in TNB Blocking Buffer overnight at 4 °C in humidity chamber. After staining, coverslips were washed with TNT Wash Buffer (0.05% (v/v) Tween 20 in PBS) three times for 5 min each. After washing, coverslips were fixed with 1.6 % paraformaldehyde in PBS for 10 min at room temperature and were washed with PBS three times for 5 min each. Then, coverslips were incubated in 100 % ice cold methanol for 5 min on ice and were washed with PBS three times for 5 min each. After fixation, coverslips were cross-linked with 3 mg/mL bis(sulfosuccinimidyl)suberate (BS3) for 30 min at room temperature and the reaction was stopped by final concentration of 20 mM Tris-HCl pH 7.5 for 15 min at room temperature.

2) Hybridization of Horseradish Peroxidase (HRP) oligonucleotide: Three hundred nM HPR-conjugated oligonucleotide was hybridized to antibody-oligonucleotide with antibody in 20% dimethyl sulfoxide (DMSO) (v/v) in KRB (10 mM Tris-HCl pH 7.5, 10mM $NaCl_2$, 150 mM NaCl, 0.1% TritonX-100 (v/v) and 0.02% (w/v) NaN_3) for 5 min at room temperature. After hybridization, coverslip was washed with TNT Wash Buffer three times for 5 min each.

3) Stripping of HRP-conjugated oligonucleotide: HPR-conjugated oligonucleotide was stripped from antibody-oligonucleotide with antibody by repeating three times, a set of washing steps using the different concentration of DMSO KRB (80% DMSO KRB, 2 min and 20% DMSO KRB 1 min).

4) Tyramide reaction and imaging: The coverslip was incubated with Cyanine 3 (Cy3)-labeled tyramide (Perkin Elmer) or Tyramide-Oligonucleotide-1 (TO-1) (Table 1) in TSA Plus Working Solution (Perkin Elmer) for 10 min and was washed with TNT Wash Buffer three times for 5 min each. For imaging of TO-1, coverslip was hybridized with 300 nM Alexa 647-anti-sense for to-1 (Table 1) and 2.5 ug/mL Hoechst 34580 (Thermo Fisher Scientific) in 20% DMSO KRB for 5 min at room temperature and was washed with 20% DMSO KRB six times. After the tyramide reaction, HPR-conjugated oligonucleotide was stripped from tissue by repeating three times, a set of washing steps using the different concentration of DMSO KRB (80% DMSO KRB, 2 min and 20% DMSO KRB 1 min). The antibody signal was visualized using the BZ-X800 fluorescence microscope (Keyence) and image was analyzed by Fuji-ImageJ (NIH).

5) The ablation of tyramide-fluorophore signal: The tyramide-fluorophore signal was removed from tissue by repeating three times, a set of washing steps using the different concentration of DMSO KRB (80% DMSO KRB, 2 min and 20% DMSO KRB 1 min).

Results

Tyramide Signal Amplification (TSA) can be used in a variety of applications, such as imaging, enzyme-linked immunosorbent assay (ELISA), the detection of protein and gene expression. To expand the applications for TSA we created removable and more flexible detection system for TSA using an oligonucleotide-tyramide conjugate, i.e. an oligonucleotide with a tyramide adduct. The system is flexible in that one can detect any signals as tyramide signal using specific anti-sense oligonucleotide for tyramide-oligonucleotide with any detectors such as an enzyme, fluorophore, biotin, metal isotope, or radioisotope labeled nucleotide and these signals are able to remove from samples fast and easily. In these cases, the TSA product is "reversible" since the hybridized anti-sense oligonucleotides can be removed by denaturation and thus the present system should open new fields of use.

A covalent conjugation between tyramide and oligonucleotide (Fig. 9) was synthesized using a heterobifunctional crosslinker that contain N-hydroxysuccinimide (NHS) ester and a maleimide group.

The tonsil tissue specimen was stained with CD3-#55 and E-cadherin-#23 antibodies (Table 1). After standard CODEX2 post staining fixation, an HPR-conjugated oligonucleotide for E-cadherin (#23 HPR-oligonucleotide, Table1) was hybridized to #23 antibody-

oligonucleotide with E-cadherin in 20% DMSO in KRB. After hybridization, coverslip was washed with TNT Wash Buffer and was incubated with Cyanine 3 (Cy3)-labeled tyramide and was washed with TNT Wash Buffer. Then, #23-HPR-oligonucleotide was stripped from tissue by repeating the combination was with the different concentration of DMSO in KRB.

5 Second, HPR-conjugated oligonucleotide for CD3 (#55 HPR-oligonucleotide, Table 1) was hybridized to #55 antibody-oligonucleotide with CD3 in 20% DMSO in KRB. After hybridization, coverslip was washed with TNT Wash Buffer and was incubated with Tyramide-Oligonucleotide-1 (TO-1, Table 1) and was washed with TNT Wash Buffer. Then, coverslip was incubated with Alexa 647-congugated oligonucleotide (Alexa 647-anti-sense for to-1, Table 10 1) and Hoechst 34580 for nuclear stain in 20% DMSO in KRB.

We took the image for E-cadherin and CD3 using a BZ-X800 fluorescence microscope. The E-cadherin tyramide signal was detected using commercially available Cy3-tyramide and CD3 tyramide signal using TO-1 (Fig. 10a-1). After imaging, coverslip was washed by repeating the combination wash with the different concentration of DMSO in KRB. The 15 tyramide signal of CD3 was clearly disappeared from tissue specimen by this combination wash (Fig. 10a-2).

To confirm the tyramide signal ablation using Tyramide-Oligonucleotide, we analyzed the image before and after ablation of CD3 signal by Fuji-ImageJ. CD3 tyramide signal was significantly disappeared after the combination wash (Fig. 10b) but E-cadherin tyramide signal 20 was no difference before and after combination wash (Fig. 10c). This data indicates that Tyramide-Oligonucleotide signal can be ablated from tissue specimen by mild condition at room temperature.

CODEX staining

The following example illustrates how to use oligonucleotide-tyramide conjugates in a 25 "CODEX"-type method.

In this example, 50 different antibodies are used, each conjugated to a different oligonucleotide. In this example, there are also 50 different HRP conjugated oligonucleotides, where each HRP conjugated oligonucleotide hybridizes to only one of the oligonucleotides that are conjugated to the antibodies. These reagents are listed below:

30 Ab-1 with oligo-1, hybridizes with HRP-anti-sense oligo-1

Ab-2 with oligo-2, hybridizes with HRP-anti-sense oligo-2

...

Ab-50 with oligo-50, hybridizes with HRP-anti-sense oligo-50

The sample is stained with all antibodies together, i.e., *en masse*.

In this example, the reaction undergoes multiple cycles and four channel fluorescence microscopy is used. In each cycle the signals of three antibodies and a nuclear stain are detected. The method can be implemented with using more or less fluorescence channels, depending on the fluorophores used.

In the first cycle HRP-anti-sense oligo-1 is hybridized with the sample. This oligonucleotide hybridizes to Ab-1 oligonucleotide. After washing, Tyramide-oligonucleotide 1 (TO-1) is then added to the sample and is deposited near to the HRP. After this, HRP-anti-sense oligo-1 is stripped from the Ab-1 oligonucleotide and washed away.

Next, HRP-anti-sense oligo-2 is hybridized with the sample. This oligonucleotide hybridizes with Ab-2 oligonucleotide. After washing, Tyramide-oligonucleotide 2 (TO-2) is then added to the sample and is deposited near the HRP. After this, HRP-anti-sense oligo-2 is stripped from the Ab-2 oligonucleotide and washed away.

At the end of first cycle, HRP-anti-sense oligo-3 is hybridized with the sample. This oligonucleotide hybridizes with Ab-3 oligonucleotide. After washing, Tyramide-oligonucleotide 3 (TO-3) is then added to the sample and is deposited near the HRP.

After this step, three distinguishably labeled fluorescent oligonucleotides are hybridized at the same time: Alexa 488-anti-sense to-1 (which is complementary to TO-1, which marks the sites to which Ab-1 binds), ATTO 550-anti-sense to-2 (which is complementary to TO-2, which marks the sites to which Ab-3 binds) and Alexa 647-antisence to-3 (which is complementary to TO-3, which marks the sites to which Ab-3 binds).

The sample is then imaged in order to read the signals from Alexa 488, ATTO 550 and Alexa 647. After imaging, the fluorophore labeled oligonucleotides and the HRP-anti-sense oligo-3 are stripped from the sample.

In the second cycle, the steps of the first cycle are repeated, with another set of oligonucleotides, i.e.:

HRP-anti-sense oligo-4 is hybridized with the sample and hybridizes to the Ab-4 oligonucleotide. After washing, Tyramide-oligonucleotide 4 (TO-4) is then added to the sample and is deposited near to the HRP. After this, HRP-anti-sense oligo-4 is stripped from the Ab-4 oligonucleotide and washed away.

Next, HRP-anti-sense oligo-5 is hybridized with the sample and hybridizes to the Ab-5 oligonucleotide. After washing, Tyramide-oligonucleotide 5 (TO-5) is then added to the sample and is deposited near to the HRP. After this, HRP-anti-sense oligo-5 is stripped from the Ab-5 oligonucleotide and washed away.

5 At the end of second cycle, HRP-anti-sense oligo-6 is hybridized with the sample. This oligonucleotide hybridizes with Ab-6 oligonucleotide. After washing, Tyramide-oligonucleotide 6 (TO-6) is then added to the sample and is deposited near the HRP.

After this step, three distinguishably labeled fluorescent oligonucleotides are hybridized at the same time: Alexa 488-anti-sense to-4 (which is complementary to TO-4, which marks the 10 sites to which Ab-4 binds), ATTO 550-anti-sense to-5 (which is complementary to TO-5, which marks the sites to which Ab-5 binds) and Alexa 647-antisence to-6 (which is complementary to TO-6, which marks the sites to which Ab-6 binds).

The sample is then imaged in order to read the signals from Alexa 488, ATTO 550 and Alexa 647. After imaging, the fluorophore labeled oligonucleotides and the HRP-anti-sense 15 oligo-6 are stripped from the sample.

The cycles are repeated using different oligonucleotides until the last cycle.

In the last cycle, HRP-anti-sense oligo-48 is hybridized with the sample and hybridizes to the Ab-48 oligonucleotide. After washing, Tyramide-oligonucleotide 48 (TO-48) is then added to the sample and is deposited near to the HRP. After this, HRP-anti-sense oligo-48 is 20 stripped from the Ab-48 oligonucleotide and washed away.

Next, HRP-anti-sense oligo-49 is hybridized with the sample and hybridizes to the Ab-49 oligonucleotide. After washing, Tyramide-oligonucleotide 49 (TO-49) is then added to the sample and is deposited near to the HRP. After this, HRP-anti-sense oligo-49 is stripped from the Ab-49 oligonucleotide and washed away.

25 At the end of last cycle, HRP-anti-sense oligo-50 is hybridized with the sample. This oligonucleotide hybridizes with Ab-50 oligonucleotide. After washing, Tyramide-oligonucleotide 50 (TO-50) is then added to the sample and is deposited near the HRP.

After this step, three distinguishably labeled fluorescent oligonucleotides are hybridized at the same time: Alexa 488-anti-sense to-48 (which is complementary to TO-48, which marks 30 the sites to which Ab-48 binds), ATTO 550-anti-sense to-49 (which is complementary to TO-49, which marks the sites to which Ab-49 binds) and Alexa 647-antisence to-50 (which is complementary to TO-50, which marks the sites to which Ab-50 binds).

The sample is then imaged in order to read the signals from Alexa 488, ATTO 550 and Alexa 647.

As would be apparent, the detection oligonucleotides used in the same cycle should be distinguishable, e.g., Alexa 488-anti-sense to-1, ATTO 550-anti-sense to-2 and Alexa 647-antisence to-3.

If the number of detection channels increases one can increase the number of antibodies detected in each cycle. For example, if there are five detection channel (not include nuclear stain) they one can detect five different antibodies using five different flourophores in each cycle.

MIBI and CyTOF staining

In this example, different antibodies are used, each conjugated to a different oligonucleotide, as described above. For example one could use 50 different antibodies, each conjugated to a different oligonucleotide. In this example, there is a corresponding number of HRP conjugated oligonucleotides. For example, there may be 50 different HRP conjugated oligonucleotides, where each HRP conjugated oligonucleotide hybridizes to only one of the antibody-oligonucleotides conjugates.

As with the prior example, the sample is stained with all antibodies together, i.e., *en masse*.

The sample is then processed as follows:

In the first cycle HRP-anti-sense oligo-1 is hybridized with the sample. This oligonucleotide hybridizes to Ab-1 oligonucleotide. After washing, Tyramide-oligonucleotide 1 (TO-1) is then added to the sample and is deposited near to the HRP. After this, the HRP-anti-sense oligo-1 is stripped from the Ab-1 oligonucleotide and washed away.

In the second cycle HRP-anti-sense oligo-2 is hybridized with the sample. This oligonucleotide hybridizes to Ab-2 oligonucleotide. After washing, Tyramide-oligonucleotide 2 (TO-2) is then added to the sample and is deposited near to the HRP. After this, the HRP-anti-sense oligo-2 is stripped from the Ab-2 oligonucleotide and washed away.

In the third cycle HRP-anti-sense oligo-3 is hybridized with the sample. This oligonucleotide hybridizes to Ab-3 oligonucleotide. After washing, Tyramide-oligonucleotide 3 (TO-3) is then added to the sample and is deposited near to the HRP. After this, the HRP-anti-sense oligo-3 is stripped from the Ab-3 oligonucleotide and washed away.

These reactions are repeated using different oligonucleotides until the last cycle (cycle 50 in this example).

In cycle 50, HRP-anti-sense oligo-50 is hybridized with the sample. This oligonucleotide hybridizes to Ab-50 oligonucleotide. After washing, Tyramide-oligonucleotide 50 (TO-50) is
5 then added to the sample and is deposited near to the HRP. After this, the HRP-anti-sense oligo-50 is stripped from the Ab-50 oligonucleotide and washed away.

Next, 50 different mass-tagged (metal-isotope labeled) anti-sense oligonucleotides, are hybridized to the sample, where one each tyramide oligonucleotide hybridizes to only one of the mass-tagged oligonucleotides.

10 The sample can by analyzed by CyTOF or MIBI.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and
15 modifications may be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

What is claimed is:

1. A reagent system comprising an oligonucleotide-tyramide conjugate.

2. The reagent system of claim 1, wherein the oligonucleotide of the oligonucleotide-tyramide conjugate is labeled with a fluorophore, a capture moiety, a mass tag, or a radioisotope.

3. The reagent system of claim 1 or 2, wherein the oligonucleotide of the oligonucleotide-tyramide conjugate is not labeled, and the system further comprises a detectably-labeled oligonucleotide, wherein the sequences of the oligonucleotides in the oligonucleotide-tyramide conjugate and the detectably-labeled oligonucleotide are at least partially complementary.

4. The reagent system of claim 1 or 2, wherein the system comprises a plurality of oligonucleotide-tyramide conjugates, wherein the sequences of the oligonucleotides in the oligonucleotide-tyramide conjugates vary in the plurality.

5. The reagent system of claim 4, wherein the sequences of the oligonucleotides are orthogonal to one another.

6. The reagent system of claim 4 or 5, wherein the reagent system comprises a corresponding plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the plurality of oligonucleotide-tyramide conjugates.

7. The reagent system of claim 6, wherein the detectably-labeled oligonucleotides are mass-tagged.

8. The reagent system of claim 6, wherein the detectably-labeled oligonucleotides are fluorescently labeled.

9. The reagent system of any of claims 1-8, further comprising a binding agent conjugate, wherein the conjugate comprises a binding agent and a peroxidase moiety or an oligonucleotide.

10. The reagent system of claim 9, wherein the binding agent conjugate comprises binding agent coupled to peroxidase moiety.

11. The reagent system of claim 10, wherein the binding agent conjugate comprises binding agent coupled to an oligonucleotide.

12. The reagent system of claim 11, wherein the reagent system comprises a set of binding agent conjugates, wherein the binding agents have different specificities and each binding agent is linked to a different oligonucleotide.

13. The reagent system of any of claims 9-12, wherein the binding agent is an antibody, aptamer, peptide or oligonucleotide.

14. A method for analyzing a sample, comprising;

(a) incubating a sample that has a binding agent-peroxidase conjugate bound to sites thereon with an oligonucleotide-tyramide conjugate in the presence of hydrogen peroxide, thereby activating the tyramide and causing deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase of the binding agent-peroxidase conjugate ;

(b) optionally detectibly labeling the deposited oligonucleotide-tyramide conjugate if the oligonucleotide-tyramide conjugate is not already detectably labeled; and

(c) reading the sample by detecting the deposited oligonucleotide-tyramide conjugate.

15. The method of claim 14, wherein the peroxidase is directly linked to the binding agent in the binding agent-peroxidase conjugate.

16. The method of claim 14, wherein the wherein the peroxidase is indirectly linked to the binding agent in the binding agent-peroxidase conjugate via a pair of oligonucleotides that are hybridized together.

17. The method of claim 16, wherein the method comprises:

wherein the method comprises, prior to step (a) binding a binding agent-oligonucleotide conjugate with the sample and then hybridizing a peroxidase-oligonucleotide conjugate to the sample, wherein the oligonucleotide of the peroxidase-oligonucleotide conjugate hybridizes to the oligonucleotide of the binding agent-oligonucleotide.

18. The method of any of claims 14-17, further comprising:

inactivating or removing the peroxidase after step (a); and, before step (c),

repeating steps (a) and optional step (b) using a different peroxidase-oligonucleotide conjugate and a different oligonucleotide-tyramide conjugate.

19. A method for analyzing a sample, comprising:

(a) obtaining:

(i) a plurality of binding agent-peroxidase conjugates;

(ii) a plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides in the conjugates have different sequences; and

(iii) a plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(ii);

(b) labeling the sample with a single binding agent-peroxidase conjugate of (a)(i), wherein the binding agent-peroxidase conjugate binds to sites on the sample;

(c) treating the sample with an oligonucleotide-tyramide conjugate of (a)(ii), wherein the peroxidase of the binding agent-peroxidase conjugates bound to the sample in (b) activates the tyramide in the oligonucleotide-tyramide conjugate and causing deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase of the binding agent-peroxidase conjugate;

(d) hybridizing a detectably labeled oligonucleotide of (a)(iii) with the sample, thereby producing complexes that comprise the detectably-labeled oligonucleotide at or near the sites to which the binding agent-peroxidase conjugate is bound; and

(e) reading the sample to obtain data on the binding of the label of the detectably-labeled oligonucleotide.

20. The method of claim 19, wherein the detectably labeled oligonucleotides of (a)(iii) are distinguishable and the method comprises:

(f) inactivating the peroxidase after step (c); and, between steps (d) and (e),

(h) repeating step (b), (c) and (d) multiple times followed by a peroxidase inactivation step after step (c), each time using a different binding agent of (a)(i), a different oligonucleotide-tyramide conjugate of (a)(ii) and a detectably-labeled oligonucleotides labeled oligonucleotide of (a)(iii); and

wherein step (e) comprises reading the sample to obtain data on the binding of all of the labels added in each step (d).

21. The method of claim 19, wherein the detectably labeled oligonucleotides of (a)(iii) are distinguishable and the method comprises:

(f) inactivating the peroxidase after step (c); and, between steps (c) and (d),

(g) repeating steps (b) and (c) multiple times followed by a peroxidase inactivation step after step (c), each time using a different binding agent-peroxidase conjugate of (a)(i) and a different oligonucleotide-tyramide conjugate of (a)(ii);

wherein step (d) comprises hybridizing multiple detectably labeled oligonucleotide of (a)(iii) with the sample, thereby producing complexes that comprise the detectably labeled oligonucleotides, and

wherein step (e) comprises reading the sample to obtain data on the binding of all of the labels added in step (d).

22. A method for analyzing a sample, comprising:

(a) obtaining:

(i) a plurality of binding agent-oligonucleotide conjugates;

(ii) a plurality of peroxidase-oligonucleotide conjugates, wherein each of the peroxidase-oligonucleotide conjugates specifically hybridizes with only one of the oligonucleotides of (a)(i);

(iii) a plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides of the conjugates have different sequences; and

(iv) a plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(iii);

(b) labeling the sample with the plurality of binding agents of (a)(i);

(c) specifically hybridizing a single peroxidase-linked oligonucleotide of the plurality of peroxidase-linked oligonucleotides of (a)(ii) with the sample, thereby producing complexes that comprise the peroxidase;

(d) treating the sample with an oligonucleotide-tyramide conjugate of (a)(iii), wherein the peroxidase in the complexes produced in (c) activates the tyramide in the conjugate and causes deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase of the binding agent-peroxidase conjugate;

(e) specifically hybridizing a detectably labeled oligonucleotide of (a)(iv) with the sample, thereby producing complexes that comprise the detectably labeled oligonucleotide; and

(f) reading the sample to determine the abundance and/or location of the detectably labeled oligonucleotide hybridized in (e).

23 The method of claim 22, wherein the detectably-labeled oligonucleotides of (a)(iv) are fluorescently labeled, and the reading of (f) is done by fluorescence-based imaging.

24. The method of claim 22, wherein the detectably-labeled oligonucleotides of (a)(iv) are mass tagged, and wherein the reading step (f) is done by a mass spectrometry method.

25. The method of claim 24, wherein the reading is done by multiplexed ion beam imaging (MIBI) or mass cytometry (Cy-TOF).

26. The method of any of claims 22-25, further comprising:

inactivating or removing the peroxidase after step (d);

between steps (d) and (e) and without removing the oligonucleotides that become associated with the sample in step (d), repeating steps (c) and (d) multiple times, each repeat followed by a peroxidase inactivation or removal step, each repeat using a different peroxidase-linked oligonucleotide of (a)(ii) and a different oligonucleotide-tyramide conjugate of (a)(iii);

wherein step (e) comprises hybridizing multiple detectably-labeled oligonucleotides of (a)(iv) that are distinguishably labeled with the sample, thereby producing complexes that comprise the distinguishable detectably labeled oligonucleotides, and

wherein step (f) comprises reading the sample to determine the abundance and/or location of the distinguishable detectably labeled oligonucleotide hybridized in (e).

27. The method of claim 26, wherein the detectably-labeled oligonucleotides of (a)(iv) are fluorescently labeled, up to seven distinguishable detectably labeled oligonucleotides are used in step (e), and the reading of (f) comprises determining the abundance and/or location of the detectably labeled oligonucleotides hybridized in (e) by fluorescence-based imaging,

28. The method of any of claims 22-25, further comprising:

inactivating or removing the peroxidase after step (d);

between steps (e) and (f) and without removing or inactivating the detectably labeled oligonucleotide that become associated with the sample in step (c), repeating steps (c), (d) and (e) multiple times with a peroxidase removal or inactivation step after step (d), each repeat using a different peroxidase-oligonucleotide-conjugate of (a)(ii), a different oligonucleotide-tyramide conjugate of (a)(iii) and a different detectably-labeled oligonucleotide of (a)(iv), wherein the labels are distinguishable; and

wherein step (f) comprises reading the sample to determine the abundance and/or location of the multiple detectably labeled oligonucleotide hybridized in (e).

29. The method of claim 28, wherein the detectably-labeled oligonucleotides of (a)(iv) are fluorescently labeled, up to seven distinguishable detectably labeled oligonucleotides are used in step (e), and the reading of (f) comprises determining the abundance and/or location of the detectably labeled oligonucleotides hybridized in (e) by fluorescence-based imaging.

30. A method for analyzing a sample, comprising:

(a) obtaining:

(i) a plurality of binding agent-oligonucleotide conjugates;

(ii) a plurality of peroxidase-oligonucleotide conjugates, wherein each of the peroxidase-oligonucleotide conjugates specifically hybridizes with only one of the oligonucleotides of (a)(i);

(iii)a plurality of oligonucleotide-tyramide conjugates, wherein the oligonucleotides of the conjugates have different sequences; and

(iv) a plurality of detectably-labeled oligonucleotides, wherein each of the detectably-labeled oligonucleotides specifically hybridizes with only one of the oligonucleotides of the oligonucleotide-tyramide conjugates of (a)(iii);

(b) labeling the sample with the plurality of binding agents of (a)(i);

(c) hybridizing a single peroxidase-oligonucleotide conjugates of the plurality of peroxidase-oligonucleotide conjugates of (a)(ii) with the sample, thereby producing complexes that comprise the peroxidase;

(d) treating the sample with an oligonucleotide-tyramide conjugate of (a)(iii), wherein the peroxidase in the complexes produced in (c) activates the tyramide in the oligonucleotide-tyramide conjugate and causes deposition of the oligonucleotide of the oligonucleotide-tyramide conjugate onto sites that are proximal to the peroxidase in the complexes;

(e) inactivating or removing the peroxidase added in step (c);

(f) repeating (c), (d) and (e) each repeat with a different peroxidase-oligonucleotide conjugate of (a)(ii) and different oligonucleotide-tyramide conjugate of (a)(iii);

(g) hybridizing a set of fluorescently labeled oligonucleotide of (a)(iv) with the sample, wherein the members of the set are distinguishably labeled, to produce complexes that comprise the distinguishable fluorescently labeled oligonucleotides;

(h) reading the sample to determine the abundance and/or location of the set of fluorescently labeled oligonucleotide hybridized in (g);

(i) inactivating or removing the fluorescently labeled oligonucleotide hybridized in (g); and

(j) repeating steps (g), (h) and (i) multiple times, each time with a different set of set of distinguishably fluorescently labeled oligonucleotide.

31. The method of claim 30, wherein step (f) comprises repeating (c), (d) and (e) done 2-200 times.

32. The method of claim 30 or 31, wherein the set of fluorescently labeled oligonucleotide hybridized in step (g) comprises 2-7 distinguishable fluorescently labeled oligonucleotides.

33. The method of any of claims 30-32, wherein the method comprises storing the sample after step (f) and before step (g), for a period of days, at least one week or at least one month.

34. The method of any of claims 30-33, wherein steps (g)-(j) are automated.

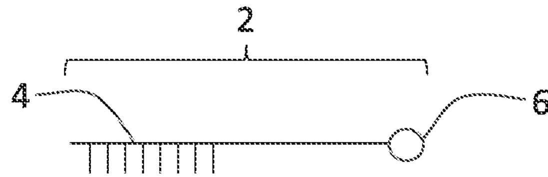


FIG. 1

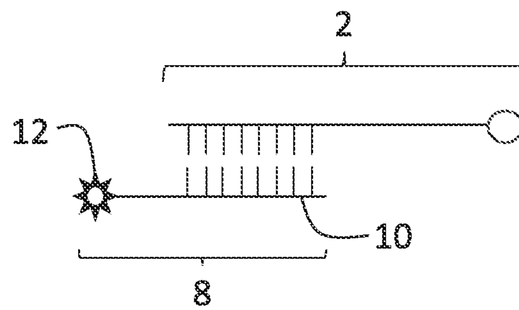
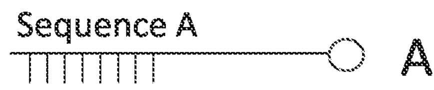
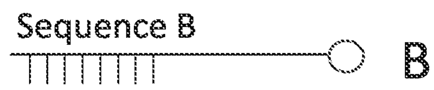
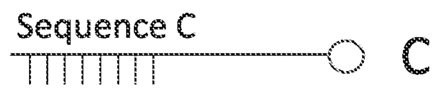


FIG. 2







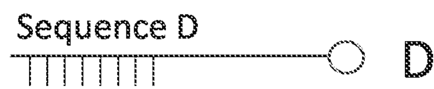


FIG. 3

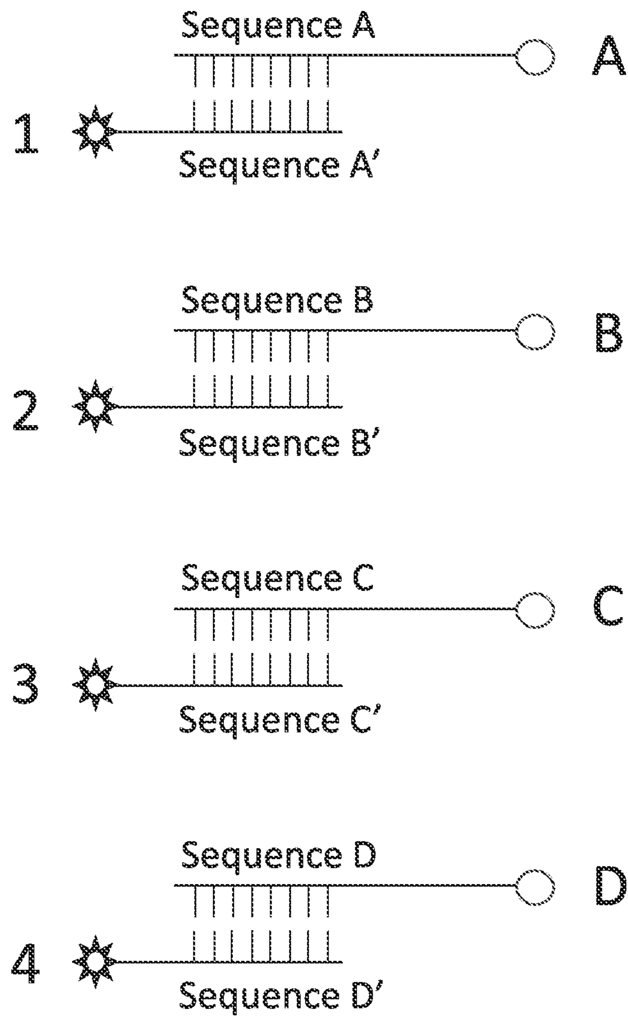


FIG. 4

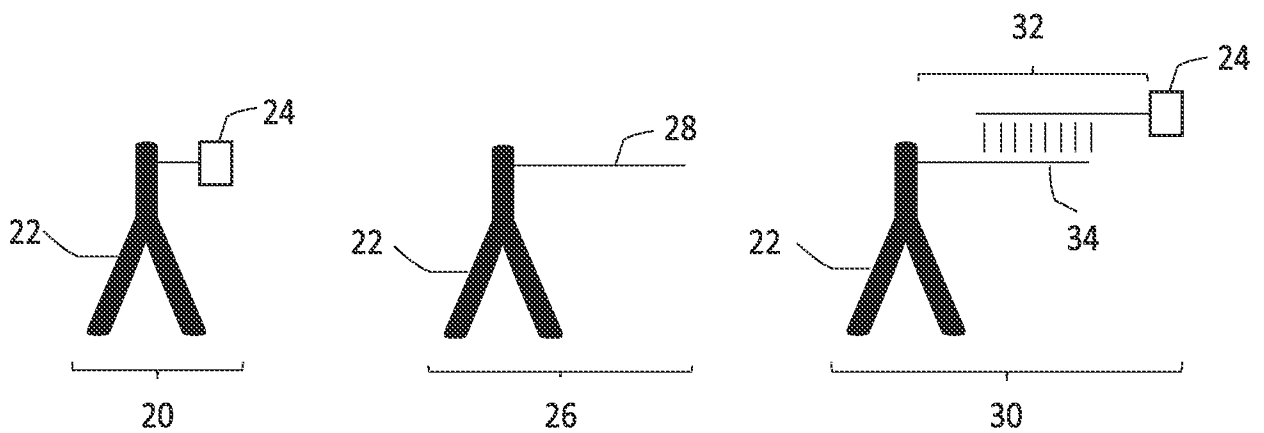
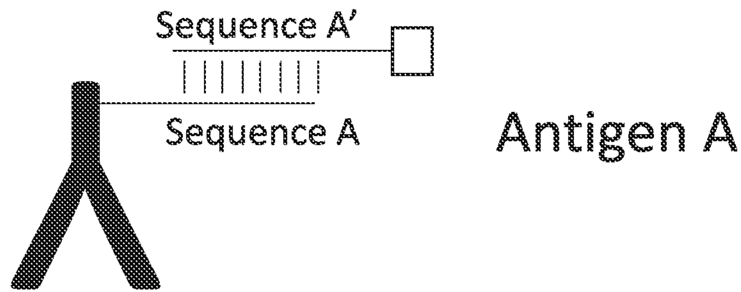
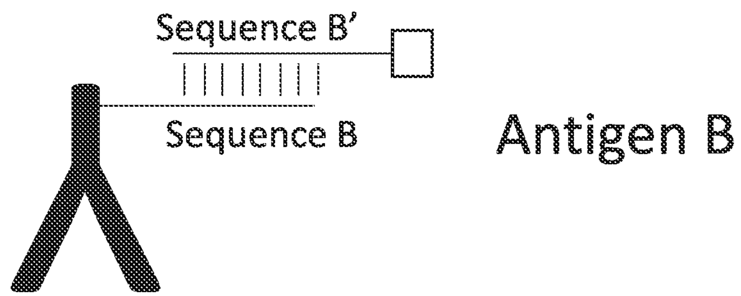
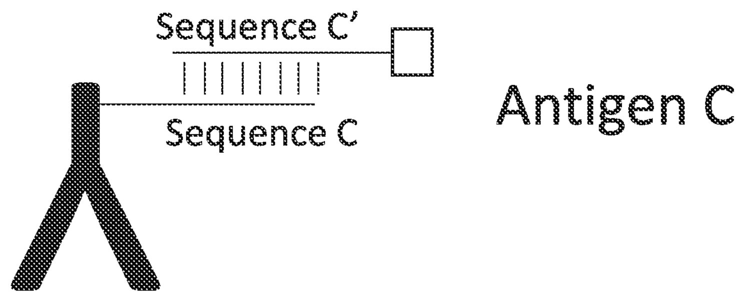


FIG. 5







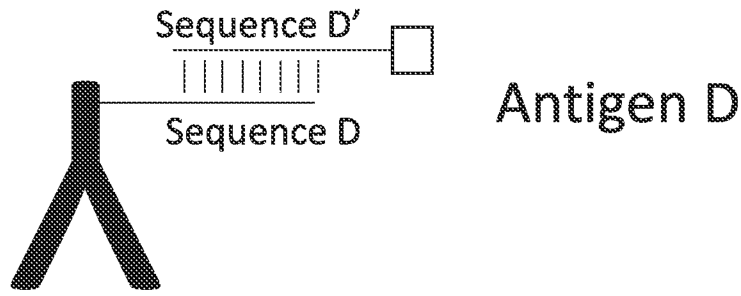


FIG. 6

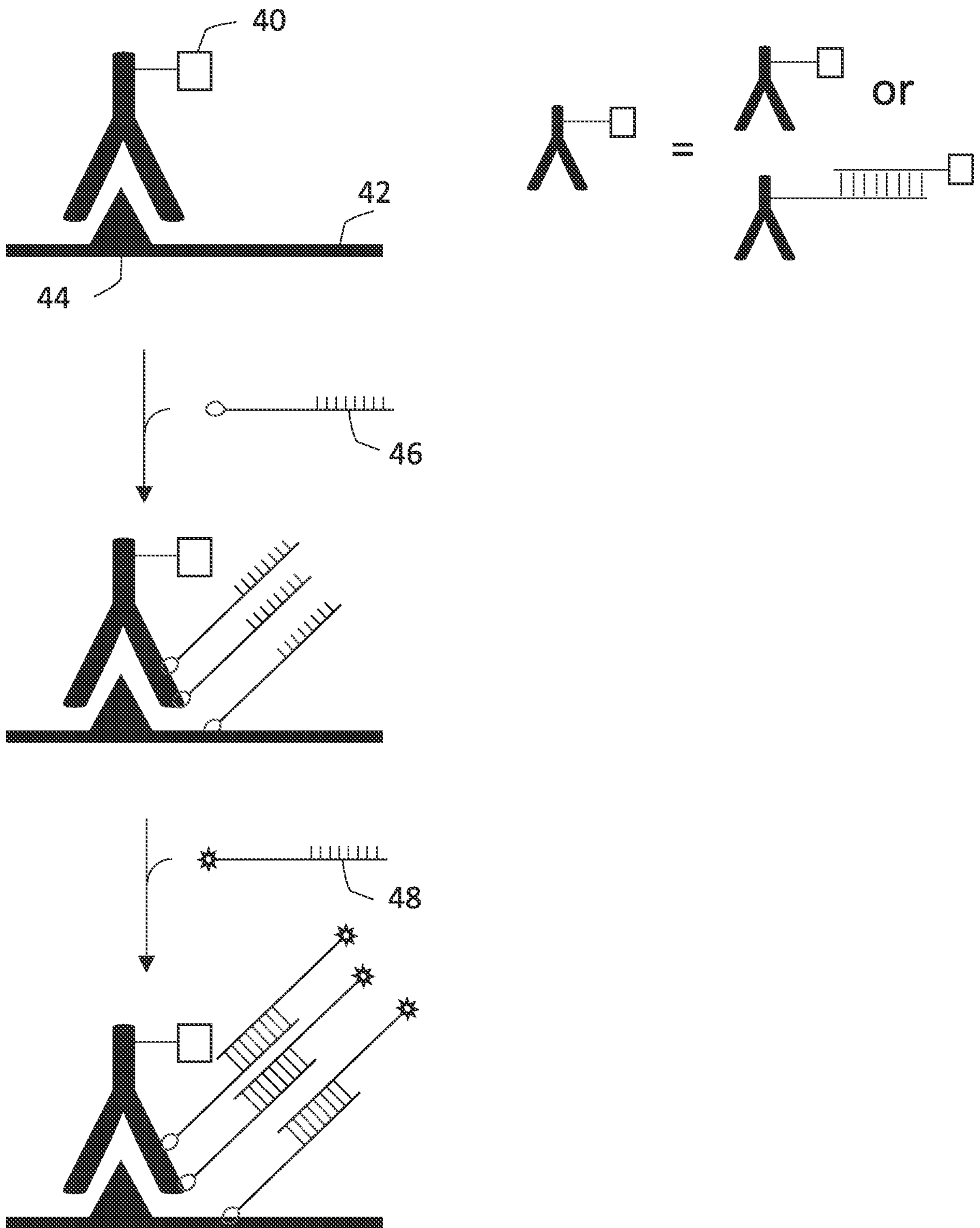
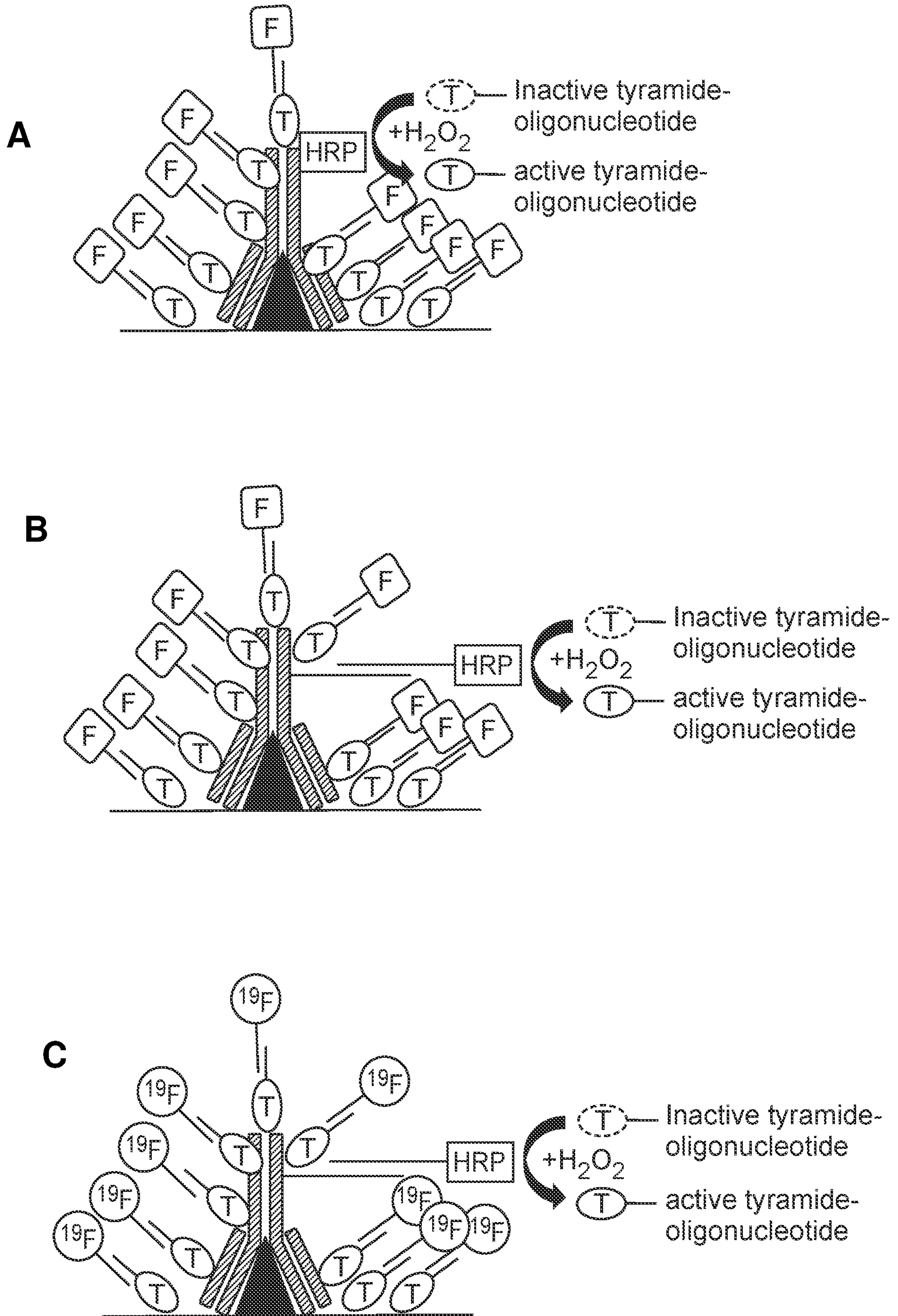


FIG. 7



FIGS. 8A-C

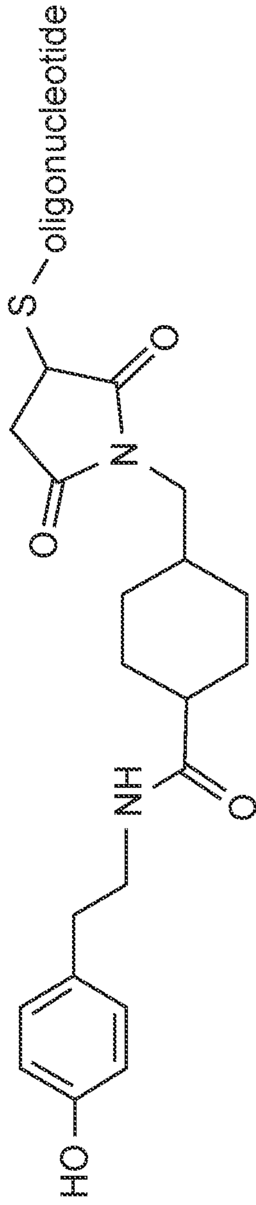


FIG. 9

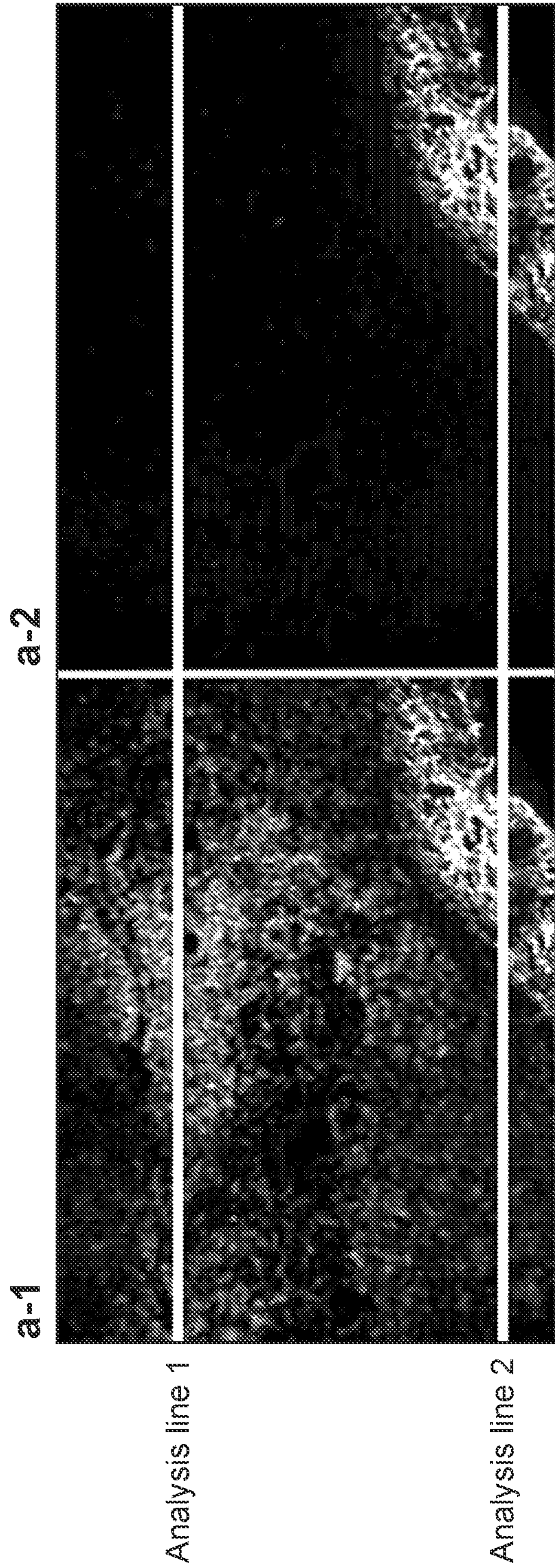


FIG. 10

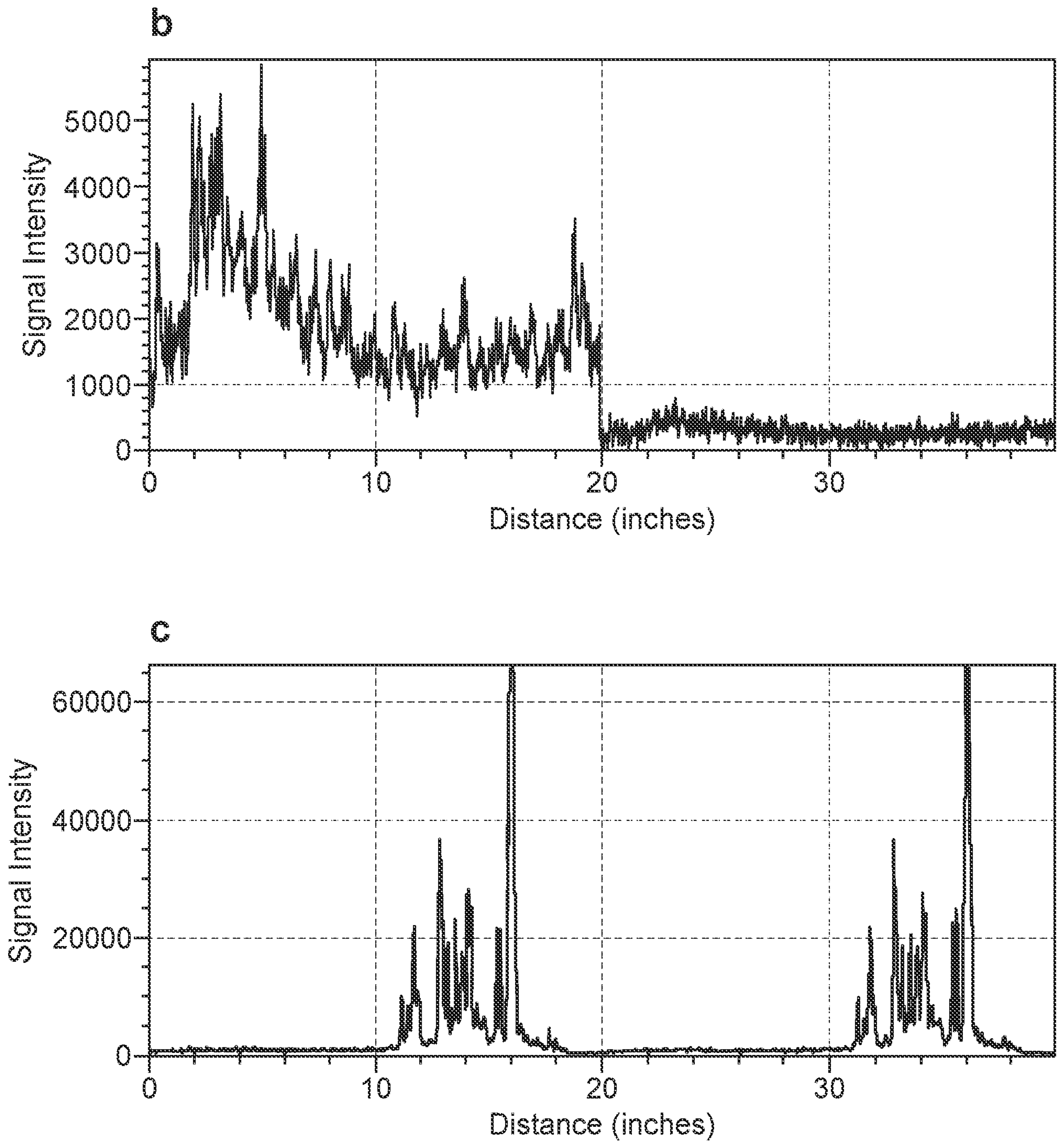


FIG. 10 (Cont.)