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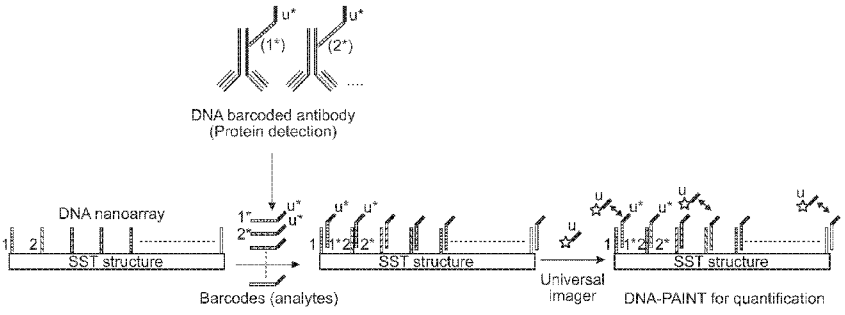
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(54) **Title**: TARGET DETECTION USING BARCODE CONJUGATES AND NUCLEIC ACID NANOARRAYS

FIG. 1A



(57) **Abstract**: Embodiments of the present disclosure relate molecular (e.g., proteomic and genomic) profiling of one or more cells using quantitative-based nucleic acid detection methods.

TARGET DETECTION USING BARCODE CONJUGATES AND NUCLEIC ACID NANOARRAYS

RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/264,821, filed December 8, 2015, which is incorporated by reference herein in its entirety.

FEDERALLY SPONSORED RESEARCH

This invention was made with government support under OD007292 and EB018659 awarded by National Institutes of Health, under 1054898 awarded by National Science Foundation, under N00014-11-1-0914 and N00014-13-1-0593 awarded by the Department of Defense Office of Naval Research, and under W911NF-12-1-0238 awarded by U.S. Army Research Laboratory. The government has certain rights in the invention.

BACKGROUND OF INVENTION

Analyzing a proteomic signature of a single cell is increasingly important in the fields of biomedical research and clinical diagnostics. Available proteomic analysis platforms, however, are either limited by low-multiplexing capability or by a requirement for large cell numbers. Further, available platforms are often highly expensive or lack the sensitivity required for detection at a single-cell level.

SUMMARY OF INVENTION

Provided herein are methods and compositions that permit accurate, reliable and cost-effective analyses of molecular (*e.g.*, protein or nucleic acid) signatures, in some instances, at single-cell resolution, enabling an understanding of the role of cellular heterogeneity, for example, in disease progression and drug response. The present methods and compositions may be used, in some embodiments, for molecular profiling (*e.g.*, proteomic and genomic analysis) of rare cells (*e.g.*, circulating tumor cells) for early disease detection and for devising appropriate therapeutic strategies.

Some embodiments of the present disclosure provide methods for detecting one or more target biomolecule(s), the methods comprising (a) combining a sample containing a

target biomolecule with a barcoded conjugate, wherein the barcoded conjugate comprises a biomolecule binding partner that binds specifically to the target biomolecule and is linked to a nucleic acid barcode that comprises a unique nucleotide sequence and a universal nucleotide sequence, (b) detaching the barcode from the conjugate, (c) combining the barcode with a nucleic acid nanoarray that comprises a nucleic acid docking probe located at a prescribed position on the nanoarray, wherein the docking probe comprises a sequence that is complementary to the unique sequence of the barcode, thereby producing a nanoarray containing the docking probe bound to the barcode, and (d) combining the nanoarray with a labeled universal imager strand that comprises a sequence that is complementary to and capable of binding transiently to the universal sequence of the barcode.

In some embodiments, the biomolecule is a protein.

In some embodiments, the sample is a biological sample. For example, the biological sample may be blood or saliva.

In some embodiments, the biological sample is a plurality of cells or cell lysate. In some embodiments, the biological sample is a single cell or a single-cell lysate.

In some embodiments, the biomolecule binding partner is protein, such as an antibody.

In some embodiments, the labeled universal imager strand comprises a fluorescent label.

In some embodiments, the methods further comprise imaging the labeled universal imager strand.

In some embodiments, the methods further comprise determining, based on binding kinetics of the labeled universal imager strand to the universal sequence of the barcode, whether the barcode is bound to the docking probe. For example, in some embodiments a relatively short binding time (*e.g.*, transient binding time of imager strand to docking probe) indicates a barcode is bound to a docking probe, and a relatively long binding time indicates a barcode is not bound to a docking probe.

In some embodiments, the nucleic acid nanoarray comprises a plurality of docking probes, each docking probe located at a prescribed position on the nanoarray and each comprising a sequence that is unique to a protein.

In some embodiments, the nucleic acid nanoarray comprises at least two fiduciary marker sites, each site comprising at least one fiduciary marker. Each fiduciary marker site may comprise, for example, at least two fiduciary markers.

In some embodiments, the length of the nucleic acid barcode is 20 to 40 nucleotides. In some embodiments, the length of the unique nucleotide sequence of the nucleic acid barcode is 10 to 30 nucleotides. In some embodiments, the length of the unique nucleotide sequence of the nucleic acid barcode is 20 nucleotides. In some embodiments, the length of the universal sequence of the nucleic acid barcode is 5 to 15 nucleotides. In some embodiments, the length of the universal sequence of the nucleic acid barcode is 9 nucleotides.

In some embodiments, the length of the unique nucleotide sequence of the nucleic acid barcode is longer than (*e.g.*, at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 5%-50%, 10%-50%, or 10% to 100% longer than) the length of the universal sequence of the nucleic acid barcode.

In some embodiments, the nucleic acid barcode comprises a spacer sequence that separates the unique nucleotide sequence and the universal nucleotide sequence. The spacer sequence may have a length of 2 to 5 nucleotides, for example. In some embodiments, the spacer sequence is a polyT (*e.g.*, TT, TTT or TTTT), polyA (*e.g.*, AA, AAA or AAAA), polyC (*e.g.*, CC, CCC or CCCC) or polyG sequence (*e.g.*, GG, GGG or GGG).

Some embodiments of the present disclosure provide methods for detecting one or more target nucleic acid analyte(s), the methods comprising (a) combining a nucleic acid nanoarray with a sample that contains a plurality of target nucleic acid analytes, wherein the nanoarray comprises at prescribed locations on the array nucleic acid docking probes, each docking probe comprising (i) a sequence that is complementary to a target analyte of the plurality and that uniquely identifies the analyte and (ii) a universal sequence, and (b) combining the nanoarray with labeled nucleic acid imager strands that comprise a sequence complementary to and capable of binding transiently to the universal sequences of the docking probes, wherein the length of time that an imager strand binds to a docking probe is indicative of whether the docking probe is bound to a target nucleic acid analyte. For example, in some embodiments, the length of time that an imager strand binds to a docking probe bound to a target nucleic acid analyte is less than the length of time that an imager strand binds to a docking probe not bound to a target nucleic acid analyte.

In some embodiments, the sample is a biological sample. For example, the sample may be blood or saliva.

In some embodiments, the biological sample is a plurality of cells or cell lysate.

In some embodiments, the biological sample is a single cell or a single-cell lysate.

In some embodiments, the target nucleic acid analytes of the plurality are deoxyribonucleic acid (DNA) analytes. In some embodiments, the target nucleic acid analytes of the plurality are ribonucleic acid (RNA) analytes. For example, the RNA analytes may be RNA interference molecules, such as short-interfering RNAs or micro RNAs.

In some embodiments, the labeled nucleic acid imager strands comprise a fluorescent label.

In some embodiments, the methods further comprise imaging the labeled nucleic acid imager strands.

In some embodiments, the methods further comprise determining, based on binding kinetics of the labeled nucleic acid imager strands to the universal sequences of the docking probes, whether one or more target nucleic acid analyte(s) of the sample is/are bound to the docking probe(s).

Some embodiments of the present disclosure provide methods for detecting one or more target nucleic acid analyte(s), the method comprising (a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a third sequence that is complementary to the first sequence of the analyte, (b) combining the nanoarray with a nucleic acid probe that comprises a fourth sequence and a universal fifth sequence, wherein the fourth sequence is complementary to the second sequence of the analyte, and (c) combining the nanoarray with a detectable nucleic acid imager strand that comprises a sixth sequence that is complementary to and capable of binding transiently to the universal fifth sequence of the probe.

In some embodiments, the docking probe is shorter than the target nucleic acid analyte. In some embodiments, the nucleic acid probe is shorter than the target nucleic acid analyte.

Some embodiments of the present disclosure provide methods for detecting one or more target nucleic acid analyte(s), the method comprising (a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a first nucleic

acid strand and a second nucleic acid strand, wherein (i) the first strand comprises a third sequence, a fourth sequence and a universal fifth sequence, wherein the third sequence is complementary to the first sequence and the fourth sequence is complementary to the second sequence, and (ii) the second strand comprises a sixth sequence and a universal seventh sequence, wherein the sixth sequence is complementary to and bound to the fourth sequence, and (b) combining the nanoarray with a labeled nucleic acid imager strand that comprises an eighth sequence and a ninth sequence, wherein the eighth sequence is complementary to and capable of binding transiently to the universal fifth sequence and the ninth sequence is complementary to and capable of binding transiently to the universal seventh sequence. In some embodiments, the second nucleic acid strand is shorter than the first nucleic acid strand.

Some embodiments of the present disclosure provide methods for detecting one or more target nucleic acid analyte(s), the method comprising (a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a third sequence, a fourth sequence, a universal fifth sequence, and a sixth sequence, wherein the third sequence is complementary to the first sequence, the fourth sequence is complementary to the second sequence, and the sixth sequence is complementary to and hybridized to the fourth sequence, and (b) combining the nanoarray with a labeled nucleic acid imager strand that comprises a seventh sequence that is complementary to and capable of binding transiently to the universal fifth sequence.

Some embodiments of the present disclosure provide methods for detecting one or more target nucleic acid analyte(s), the method comprising (a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein the analyte comprises a first sequence that uniquely identifies the analyte, and the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a second sequence flanked by a universal third sequence and a universal fourth sequence, wherein the second sequence is complementary to the first sequence, and (b) combining the nanoarray with a labeled nucleic acid imager strand that comprises a fifth sequence and a sixth sequence, wherein the fifth sequence is complementary to and capable of binding transiently to the universal third sequence, and the sixth sequence is complementary to and capable of binding transiently to the universal fourth sequence.

In some embodiments, regions of the second sequence bind to each other to form a hairpin loop.

Some embodiments of the present disclosure provide methods for detecting one or more target nucleic acid analyte(s), the method comprising (a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a first nucleic acid strand and a second nucleic acid strand, wherein (i) the first strand comprises a third sequence, a fourth sequence, a universal fifth sequence, and a universal sixth sequence, wherein the third sequence is complementary to the first sequence and the fourth sequence is complementary to the second sequence, and (ii) the second strand comprises a seventh sequence and an eighth sequence, wherein the seventh sequence is complementary to and hybridized to the fourth sequence and the eighth sequence is complementary to and hybridized to the universal fifth sequence, and (b) combining the nanoarray with a first labeled nucleic acid imager strand that comprises a ninth sequence and a tenth sequence, wherein the ninth sequence is complementary to and capable of binding transiently to the universal fifth sequence, and the tenth sequence is complementary to and capable of binding transiently to the universal sixth sequence. In some embodiments, the second nucleic acid strand is shorter than the first nucleic acid strand.

In some embodiments, the first nucleic acid strand further comprises a universal eleventh sequence.

In some embodiments, the methods further comprise combining the nanoarray with a second labeled imager strand that comprises a twelfth sequence and a thirteenth sequence, wherein the twelfth sequence is complementary to the universal sixth sequence and the thirteenth sequence is complementary to the universal eleventh sequence.

In some embodiments, the target nucleic acid analyte is a component of a biological sample. In some embodiments, the biological sample is a plurality of cells or cell lysate. In some embodiments, the biological sample is a single cell or a single-cell lysate.

In some embodiments, the nucleic acid analyte is a deoxyribonucleic acid (DNA) analyte. In some embodiments, the nucleic acid analyte is a ribonucleic acid (RNA) analyte. For example, the RNA analyte may be an RNA interference molecule, such as a short-interfering RNA or a micro RNA.

In some embodiments, the labeled nucleic acid imager strand comprises a fluorescent label.

In some embodiments, the methods further comprise imaging the labeled nucleic acid imager strand.

In some embodiments, the methods further comprise determining, based on binding kinetics of the labeled nucleic acid imager strand to the universal sequence of the docking probe, whether a target nucleic acid analyte is bound to the docking probe.

In some embodiments, the nucleic acid nanoarray comprises a plurality of docking probes, each probe located at a prescribed position on the nanoarray and each comprising a sequence that is unique to a target nucleic acid analyte.

In some embodiments, the nucleic acid nanoarray is a two-dimensional or three-dimensional nucleic acid nanostructure. In some embodiments, the nucleic acid nanoarray is a single-stranded nucleic acid tile array.

Provided herein are docking probes comprising a sequence of any one of SEQ ID NO: 1-39. In some embodiments, docking probes comprise a barcode binding site of a sequence of any one of SEQ ID NO: 1-39.

Also provided herein are nanoarrays comprising docking probes comprising a sequence of any one of SEQ ID NO: 1-39.

Also provided herein are nucleic acid barcodes comprising a sequence of any one of SEQ ID NO: 40-78.

Some embodiments provide biomolecule binding partners linked to nucleic acid barcodes comprising a sequence of any one of SEQ ID NO: 40-78.

Some embodiments provide nanoarrays comprising docking probes bounds to nucleic acid barcodes comprising a sequence of any one of SEQ ID NO: 40-78.

Also provided herein are fiduciary markers comprising a sequence of any one of SEQ ID NO: 79-93.

Some embodiments provide nanoarrays comprising fiduciary markers comprising a sequence of any one of SEQ ID NO: 79-93.

Also provided herein are kits comprising any two or more of the following components selected from the group consisting of barcoded conjugates, biomolecule binding partners, linkers, nucleic acid barcodes, nucleic acid nanoarrays, docking probes and universal imager strands.

These and other embodiments of the present disclosure are described in more detail herein.

The invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Each of the above embodiments may be linked to any other embodiment.

BRIEF DESCRIPTION OF DRAWINGS

The accompanying drawings are not intended to be drawn to scale. For purposes of clarity, not every component may be labeled in every drawing.

Fig. 1A shows a schematic of an example of a single-cell proteomic analysis of the present disclosure using DNA-antibody conjugates, a single-stranded tile (SST) nanoarray containing docking probes, and detection/quantification using labeled nucleic acid imager strands. FIG. 1B shows a schematic of an example of a SST nanoarray design.

Fig. 2 shows a schematic of an example of a nucleic acid analyte analysis of the present disclosure using a SST nanoarray containing docking probes, and detection/quantification using labeled nucleic acid imager strands. In this example, an intermediate nucleic acid probe is used for detection.

Figs. 3A-3E show schematics of examples of different strategies for nanoarray design and imager-strand-based detection methods of the present disclosure. Fig. 3A: Upon hybridization with targets, a specific "blinking" signature is observed based on the sequence presented. Fig. 3B: In this design, a hairpin based docking site is used on the origami structure. Upon hybridization with a target, an imager strand docking site opens up for detection. Figs. 3C-3E: Upon hybridization of docking probes with target analytes, a change in "blinking" (*e.g.*, binding = "ON"; not binding = "OFF") signature (*e.g.*, transient binding of labeled imager strand to docking probe) confirms the presence or absence of the targets.

Fig. 4 depicts an example of one embodiment of the present disclosure using a barcoded conjugate with photocleavable linkers to attach barcodes to antibodies.

Fig. 5 shows a schematic of an example nanoarray.

Fig. 6 shows docking probes (shorter strands), barcodes (longer strands) and dots, which indicate fiduciary marker sites. Each dot corresponds to three fiduciary markers (3 nucleic acid strands).

Fig. 7 shows different combinations of barcodes incubated with a nanoarray. The schematic and the image in Fig. 8 indicate the correspondence between the expected image and the obtained image. Spots corresponding to the incubated barcodes were observed. The images are overlays of two PAINT super-resolution images taken with imagers P1 and P2 with Cy3b dye. The colors are false colors, with dark dray (small dots) denoting P1 and light gray (large dots) denoting P2.

Fig. 8 shows a wide field view of the nanoarrays of Fig. 7, circled in white. The images are overlays of two PAINT super-resolution images taken with imagers P1 and P2 with Cy3b dye. The colors are false colors, with dark dray (small dots) denoting P1 and light gray (large dots) denoting P2.

DETAILED DESCRIPTION OF INVENTION

Provided herein are methods and compositions that enable molecular analysis at a single-cell level. The methods and compositions of the present disclosure may be used to detect, for example, biomolecules, such as proteins and/or nucleic acids. An example of a proteomic analysis is shown in Fig. 1A. First, a barcoded conjugate as provided herein is applied to a sample (*e.g.*, a single cell) containing a target protein. In this example, the barcoded conjugate contains (a) an antibody that binds specifically to a target protein, and (b) a nucleic acid barcode having a nucleotide sequence that uniquely identifies the target (Fig. 1A: sequence "1*" uniquely identifies (*e.g.*, is representative of) one target protein, and sequence "2*" uniquely identifies another target protein). In this example, the barcode also contains a universal sequence (Fig. 1A, "u*"), which is not unique to the barcode or target protein and is used to later detect the presence or absence of binding of the barcode to a docking probe on a nucleic acid nanoarray. Next, unbound barcoded conjugates are removed from the sample. Barcodes from conjugates remaining in the sample (presumably those with the antibody bound to the target protein) are then detached (*e.g.*, cleaved) from the antibody and applied to a nucleic acid nanoarray containing docking probes.

An example of a tile-like nucleic acid nanoarray architecture, as provided herein, is depicted in Fig. 1B. With reference again to the example depicted in Fig. 1A, nucleic acid

docking probes are positioned at prescribed (*e.g.*, pre-determined, known) locations on the nanoarray, each docking probe containing a nucleotide sequence that is complementary to, and thus binds to, the unique sequence of the barcode (Fig. 1A: sequence "1" of the first docking probe is complementary to sequence "1*" of the first barcode, and sequence "2" of the second docking probe is complementary to sequence "2*" of the second barcode). Thus, barcodes and docking probes with complementary sequences bind stably to each other on the nanoarray. Any unbound barcode is removed, and the detection phase is implemented. A single docking probe is designed to bind to only one barcode, or one species of barcode (*e.g.*, a barcode representative of one species of target biomolecule).

In the example shown in Fig. 1A, the barcodes have a universal sequence (that is, a sequence that is not unique to each barcode). This universal sequence is used to detect the presence and location of individual barcodes that are bound to docking probes. Detection is achieved, for example, by applying to the nanoarray labeled imager strands, each containing a nucleotide sequence (Fig. 1A, "u") complementary to and capable of binding transiently to the universal sequence (Fig. 1A, "u*") of the barcode, referred to herein as "universal imager strands." When the nanoarray is imaged, locations containing the prescribed docking probes bound by barcodes appear to "blink" on and off as the universal imager strands bind ("ON") and unbind ("OFF") the barcode, indicating the presence of a barcode bound to the docking probe, representative of the presence of the protein of interest in the original sample. This detection scheme can also be used to quantify the number of target proteins in a given sample. In some configurations, as described below, the universal sequence (or more than one universal sequence) may be on the docking probe instead of the barcode. The location of the universal sequence depends, in part, on the configuration of the docking probe (*e.g.*, selected from those depicted in Figs. 1A, 2, and 3A-3E).

In addition to offering tools for a proteomic analysis (*e.g.*, single-cell proteomic analysis), methods and compositions provided herein can be used for genomic analysis (*e.g.*, single-cell proteomic analysis) to detect and quantify nucleic acids, including deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and microRNA, as shown in the examples depicted in Figs. 2 and 3A-3E.

Barcoded Conjugates

Some embodiments of the present disclosure use barcoded conjugates to detect biomolecules (*e.g.*, proteins) in a sample. A "barcoded conjugate" herein refers to a biomolecule binding partner linked to a nucleic acid barcode.

A biomolecule binding partner may be any biomolecule (*e.g.*, protein) that has an affinity for (*e.g.*, binds to) a target biomolecule (*e.g.*, protein) of interest. Typically, a biomolecule binding partner "binds specifically" to a target biomolecule of interest. A biomolecule binding partner is considered to bind specifically to a target biomolecule of interest if the biomolecule binding partner binds to the target biomolecule and does not bind to a non-target biomolecule. As an example, a biomolecule binding partner is considered to bind specifically to a target biomolecule if the identity of the target biomolecule can be distinguished based on binding of the biomolecule binding partner to the target biomolecule, as provided herein. Examples of biomolecules for use as biomolecule binding partners in accordance with the present disclosure include, without limitation, proteins, such as antibodies (*e.g.*, monoclonal antibodies, polyclonal antibodies), antigen-binding antibody fragments (*e.g.*, Fab fragments, or other antigen-binding fragment/portion), receptors, peptides and protein/peptide aptamers; saccharides (*e.g.*, polysaccharides); lipids; nucleic acids (*e.g.*, DNA, RNA, microRNA); and small molecules (*e.g.*, low molecular weight (<900 Daltons) organic or inorganic molecules). Other biomolecule binding partners may be used as provided herein.

In some embodiments, a biomolecule binding partner is a protein.

In some embodiments, a protein binding partner is an antibody. Antibodies of the present disclosure include full-length antibodies and any antigen binding fragment (*e.g.*, "antigen-binding portion") or single chain thereof. The term "antibody" includes, without limitation, a glycoprotein comprising at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds, or an antigen binding portion thereof. Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof (*e.g.*, humanized, chimeric).

An "antigen-binding portion" of an antibody herein refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. The antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_H, V_L, C_L and C_{H1}

domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_H and V_L domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, *Nature* 341:544 546, 1989), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR) or (vii) a combination of two or more isolated CDRs, which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_H and V_L, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_H and V_L regions pair to form monovalent molecules (known as single chain Fv (scFv); *see, e.g.*, Bird *et al. Science* 242:423 426, 1988; and Huston *et al. Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988). Such single chain antibodies are also encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

In some embodiments, a biomolecule binding partner is a receptor. "Receptors" herein refer to cellular-derived molecules (*e.g.*, proteins) that bind to ligands such as, for example, other proteins, peptides or small molecules.

As used herein, "peptide aptamer" refers to a molecule with a variable peptide sequence inserted into a constant scaffold protein (*see, e.g.*, Baines IC, *et al. Drug Discov. Today* 11:334–341, 2006).

A "nucleic acid barcode," also referred to more simply as a "barcode," herein refers to a single-stranded, or partially single-stranded (*e.g.*, containing a double-stranded region and a single-stranded region), nucleic acid species that comprises a unique nucleotide sequence (a "unique sequence") and a universal nucleotide sequence (a "universal" sequence). Typically, but not always, a barcode is single-stranded. In some embodiments, a barcode has a length of 5 nucleotides to 500 nucleotides (*e.g.*, 5 to 500, 5 to 400, 5 to 300, 5 to 200, or 5 to 100 nucleotides). In some embodiments, a barcode has a length of 5 nucleotides to 100 nucleotides. For example, the length of a nucleic acid barcode may be 5 to 100, 5 to 90, 5 to 80, 5 to 70, 5 to 60, 5 to 50, 5 to 40, 5 to 30, 5 to 20, 5 to 10, 10 to 100, 10 to 90, 10 to 80, 10 to 70, 10 to 60, 10 to 50, 10 to 40, 10 to 30, 10 to 20, 15 to 100, 15 to 90, 15 to 80, 15 to 70, 15 to 60, 15 to 50, 15 to 40, 15 to 30, 15 to 20, 20 to 100, 20 to 90, 20 to 80, 20 to 70, 20 to

60, 20 to 50, or 20 to 40 nucleotides. In some embodiments, a barcode has a length of 5 nucleotides to 50 nucleotides. In some embodiments, a barcode has a length of 4 nucleotides to 60 nucleotides, 6 nucleotides to 40 nucleotides, 7 nucleotides to 30 nucleotides, 8 nucleotides to 20 nucleotides, or 9 nucleotides to 15 nucleotides. In some embodiments, a barcode has a length of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more, nucleotides. In some embodiments, a barcode has a length of 25 to 35 nucleotides.

A "unique sequence" of a barcode is defined relative to a species of target biomolecule. A single barcode species (and, thus, a unique sequence of a single barcode species) is representative of a single target biomolecule species and is representative of only that biomolecule species. For example, if a sample contains a plurality of target protein species—protein 1, protein 2, protein 3, *etc.*—and the target protein species is protein 1, a barcoded conjugate of the present disclosure may contain an antibody that binds specifically to protein 1 and is linked to a barcode that comprises a unique nucleotide sequence specific to (*i.e.*, that uniquely identifies) protein 1. Thus, a plurality of barcoded conjugates used to detect protein 1 may comprise a plurality of the same species of antibody, each linked to a single barcode, each barcode containing the same unique sequence that uniquely identifies protein 1. As another example, if a sample contains a plurality of target protein species, and two particular species of target proteins are of interest—*e.g.*, protein 1 and protein 2—a plurality of barcoded conjugates of the present disclosure may contain a subset of conjugates that comprise (1) an antibody that binds specifically to protein 1 and is linked to a barcode that comprises a nucleotide sequence specific to (*i.e.*, that uniquely identifies) protein 1 and a subset of conjugates that comprise (2) an antibody that binds specifically to protein 2 and is linked to a barcode that comprises a nucleotide sequence specific to (*i.e.*, that uniquely identifies) protein 2. Thus, a plurality of barcoded conjugates used to detect protein 1 and protein 2 may comprise (1) one subset of antibodies of the same species, each antibody linked to a single barcode, each barcode containing the same unique sequence that uniquely identifies protein 1 and/or (2) another subset of antibodies of the same species, each antibody linked to a single barcode, each barcode containing the same unique sequence that uniquely

identifies protein 2. A unique sequence typically has a length of 5 nucleotides to 50 nucleotides. In some embodiments, a unique sequence has a length of 4 nucleotides to 60 nucleotides, 6 nucleotides to 40 nucleotides, 7 nucleotides to 30 nucleotides, 8 nucleotides to 20 nucleotides, or 9 nucleotides to 15 nucleotides. In some embodiments, a unique sequence has a length of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more, nucleotides. It should be understood that the length of a barcode is equal to or greater than the unique sequence of the barcode.

A "universal sequence" is defined relative to a labeled universal nucleic acid imager strand (an "imager strand"), defined in more detail below. A labeled imager strand comprises a detectable label linked to a single-stranded, or partially single stranded, nucleic acid that contains a nucleotide sequence complementary to the universal sequence of a barcode or docking probe such that the imager strand is capable of binding transiently to the respectively barcode or docking probe through the universal sequence. A universal sequence typically has a length of 5 consecutive nucleotides to 50 nucleotides. In some embodiments, a universal sequence has a length of 4 nucleotides to 60 nucleotides, 6 nucleotides to 40 nucleotides, 7 nucleotides to 30 nucleotides, 8 to 20 nucleotides, or 9 nucleotides to 15 nucleotides. In some embodiments, a universal sequence has a length of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more, nucleotides. A "universal sequence" may be present on a barcode or it may be present on a docking probe, as described below. The location of a universal sequence may depend, in part, on the target biomolecule and the choice of docking probe configuration.

A universal sequence, in some embodiments, is located on an end of a barcode or docking probe. For example, as depicted in Fig. 1A, a barcode may be linked to a biomolecule binding partner at one end (*e.g.*, the 5' end) and contain a universal sequence ("u*") at its other end (*e.g.*, the 3" end). As another example, as depicted in Fig. 3A, a docking probe may be linked to a nanoarray at one end and contain a universal sequence

("u") at its other end. In some embodiments, a universal sequence is located at the 3' end of a barcode or docking probe. In some embodiments, a universal sequence is located at the 5' end of a barcode or docking probe.

A unique nucleotide sequence may be longer than or shorter than a universal nucleotide sequence. Thus, in some embodiments, a unique nucleotide sequence is longer than a universal nucleotide sequence. For example, a unique nucleotide sequence may be at least 5, at least 10, at least 15, or at least 20 nucleotides longer than a universal nucleotide sequence. In some embodiments, a unique nucleotide sequence is 5 to 20, 5 to 15, or 5 to 10 nucleotides longer than a universal nucleotide sequence. In other embodiments, a universal nucleotide sequence is longer than a unique nucleotide sequence.

In some embodiments, a barcode may comprise a "spacer sequence" separating, for example, a unique sequence and a universal sequence. This spacer sequence minimizes steric hindrance. In some embodiments, a spacer sequence is a homopolymer of 2 to 10 nucleotides (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides). A spacer sequence may be a polyT, polyA, polyC or polyG sequence. In some embodiments, a spacer sequence includes (*e.g.*, consists of) 2 thymine nucleotides.

A biomolecule binding partner may be linked covalently or non-covalently to a nucleic acid barcode. In some embodiments, a biomolecule binding partner is directly linked to a nucleic acid barcode, without an intermediate linker molecule. In other embodiments, a biomolecule binding partner is indirectly linked to a nucleic acid barcode, through an intermediate linker molecule. In some embodiments, an intermediate linker is a photocleavable linker (*see, e.g.*, Agasti S, *et al. J Am Chem Soc.* 134(45): 18499–18502, 2012, incorporated by reference herein). In some embodiments, an intermediate linker includes an N-hydroxysuccinimide (NHS) linker. Other intermediate linkers may comprise biotin and/or streptavidin. For example, in some embodiments, biomolecule binding partner and a nucleic acid barcode may each be biotinylated (*i.e.*, linked to at least one biotin molecule) and linked to each other through biotin binding to an intermediate streptavidin molecule. Intermediate linkers provided herein may be used to link barcodes to biomolecule binding partners, to link docking probes to nucleic acid nanoarrays, or to link nucleic acid arrays to substrates (*e.g.*, glass).

Examples of target biomolecules include, without limitation, proteins, saccharides (*e.g.*, polysaccharides), lipids, nucleic acids (*e.g.*, DNA, RNA, microRNAs), and small

molecules. In some embodiments, a target biomolecule is a nucleic acid. A target nucleic acid may be referred to herein as a target "nucleic acid analyte." Target nucleic acid analytes may be DNA or RNA. In some embodiments, target nucleic acid analytes are RNA interference molecules, such as short-interfering RNAs (siRNAs) or micro RNAs (microRNAs). In some embodiments, target nucleic acids are antisense molecules, such as DNA antisense synthetic oligonucleotides (ASOs). Other target biomolecules (including target nucleic acid analytes) are contemplated.

In some embodiments, a barcode is considered a nucleic acid analyte. For example, after a barcode is detached (*e.g.*, cleaved) from a conjugate, it may be referred to as a nucleic acid analyte that is applied to a nucleic acid nanoarray as provided herein.

In some embodiments, a "complex" is formed upon binding of a barcoded conjugate to a target biomolecule—specifically, the biomolecule binding partner binding to the target biomolecule.

Pluralities of barcoded conjugates are provided herein. A plurality may be a population of the same species or distinct species. A plurality of barcoded conjugates of the same species may comprise conjugates that all bind to the same target (*e.g.*, biomolecule) (*e.g.*, the same epitope or region/domain). Conversely, a plurality of barcoded conjugates of distinct species may comprise conjugates, or subsets of conjugates, each conjugate or subset of conjugates binding to a distinct epitope on the same target or to a distinct target. The number of distinct species in a given plurality of barcoded conjugates is limited by the number of available biomolecule binding partners (*e.g.*, antibodies). In some embodiments, a plurality of barcoded conjugates (*e.g.*, protein-nucleic acid conjugates) comprises at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4, 50000, 10^5, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} barcoded conjugates. In some embodiments, a plurality may contain 1 to about 200 or more distinct species of barcoded conjugates. For example, a plurality may contain at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200 or more distinct species. In some embodiments, a plurality may contain less than about 5 to about 200 distinct species of barcoded conjugates. For example, a plurality may contain less than 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175 or 200 distinct species.

After a conjugate binds to a target biomolecule, the barcode is removed (*e.g.*, cleaved) from the conjugate and applied to a nucleic acid nanoarray for detection.

Nucleic Acid Nanoarrays

The present disclosure provides nucleic acid nanoarrays as substrates for detecting and quantifying biomolecules. A "nucleic acid nanoarray," or more simply a "nanoarray," herein refers to a nucleic acid nanostructures comprising one or more docking probe(s) and/or fiduciary marker(s) located at prescribed and identifiable positions on the nanoarray. Typically, a nanoarray comprises a plurality of different species of docking probes, as described below.

Nucleic Acid Nanostructures

A "nucleic acid nanostructure" herein refers to a rationally-designed, artificial (*e.g.*, non-naturally occurring) structure self-assembled from individual nucleic acids. "Self-assembly" refers to the ability of nucleic acids (and, in some instances, nucleic acid nanostructures) to anneal to each other, in a sequence-specific manner, in a predicted manner and without external control. In some embodiments, nucleic acid nanostructure self-assembly methods include combining nucleic acids (*e.g.*, single-stranded nucleic acids) in a single vessel and allowing the nucleic acids to anneal to each other, based on sequence complementarity. In some embodiments, this annealing process involves placing the nucleic acids at an elevated temperature and then reducing the temperature gradually in order to favor sequence-specific binding. Various nucleic acid nanostructures or self-assembly methods are known and described herein.

Nucleic acid nanostructures are typically nanometer-scale structures (*e.g.*, having length scale of 1 to 1000 nanometers), although, in some instances, the term "nucleic acid nanostructure" herein may refer to micrometer-scale structures (*e.g.*, assembled from more than one nanometer-scale or micrometer-scale structure). In some embodiments, a nucleic acid nanostructure has a length scale of 1 to 1000 nm, 1 to 900 nm, 1 to 800 nm, 1 to 700 nm, 1 to 600 nm, 1 to 500 nm, 1 to 400 nm, 1 to 300 nm, 1 to 200 nm, 1 to 100 nm or 1 to 50 nm. In some embodiments, a nucleic acid nanostructure has a length scale of greater than 1000 nm. In some embodiments, a nucleic acid nanostructure has a length scale of 1 micrometer to 2 micrometers, or more.

In some embodiments, a nucleic acid nanostructure self-assembles from a plurality of different nucleic acids (*e.g.*, single-stranded nucleic acids). For example, a nucleic acid

nanostructure may assemble from at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 nucleic acids. In some embodiments, a nucleic acid nanostructure assembles from at least 100, at least 200, at least 300, at least 400, at least 500, or more, nucleic acids. The term "nucleic acid" encompasses "oligonucleotides," which are short, single-stranded nucleic acids (*e.g.*, DNA) having a length of 10 nucleotides to 100 nucleotides. In some embodiments, an oligonucleotide has a length of 10 to 20 nucleotides, 10 to 30 nucleotides, 10 to 40 nucleotides, 10 to 50 nucleotides, 10 to 60 nucleotides, 10 to 70 nucleotides, 10 to 80 nucleotides or 10 to 90 nucleotides. In some embodiments, an oligonucleotide has a length of 20 to 50, 20 to 75 or 20 to 100 nucleotides. In some embodiments, an oligonucleotide has a length of 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides.

In some embodiments, a nucleic acid nanostructure is assembled from single-stranded nucleic acids, double-stranded nucleic acids, or a combination of single-stranded and double-stranded nucleic acids.

Nucleic acid nanostructures may assemble, in some embodiments, from a plurality of heterogeneous nucleic acids. "Heterogeneous" nucleic acids may differ from each other with respect to nucleotide sequence length and/or composition. For example, in a heterogeneous plurality that includes nucleic acids A, B and C, the nucleotide sequence of nucleic acid A differs from the nucleotide sequence of nucleic acid B, which differs from the nucleotide sequence of nucleic acid C. Heterogeneous nucleic acids may also differ with respect to length and chemical composition (*e.g.*, isolated v. synthetic, G/C content).

The fundamental principle for designing self-assembled nucleic acid nanostructures is that sequence complementarity in nucleic acid strands is encoded such that, by pairing up complementary segments, the nucleic acid strands self-organize into a predefined nanostructure under appropriate physical conditions. From this basic principle (*see*, *e.g.*, Seeman N.C. *J. Theor. Biol.* 99: 237, 1982, incorporated by reference herein), researchers have created diverse synthetic nucleic acid nanostructures (*see*, *e.g.*, Seeman N.C. *Nature* 421: 427, 2003; Shih W.M. *et al. Curr. Opin. Struct. Biol.* 20: 276, 2010, each of which is incorporated by reference herein). Examples of nucleic acid (*e.g.*, DNA) nanostructures, and methods of producing such structures, that may be used in accordance with the present disclosure are known and include, without limitation, lattices (*see*, *e.g.*, Winfree E. *et al. Nature* 394: 539, 1998; Yan H. *et al. Science* 301: 1882, 2003; Yan H. *et al. Proc. Natl.*

Acad. of Sci. USA 100; 8103, 2003; Liu D. *et al. J. Am. Chem. Soc.* 126: 2324, 2004; Rothemund P.W.K. *et al. PLoS Biology* 2: 2041, 2004, each of which is incorporated by reference herein), ribbons (*see, e.g.*, Park S.H. *et al. Nano Lett.* 5: 729, 2005; Yin P. *et al. Science* 321: 824, 2008, each of which is incorporated by reference herein), tubes (*see, e.g.*, Yan H. *Science*, 2003; P. Yin, 2008, each of which is incorporated by reference herein), finite two-dimensional and three dimensional objects with defined shapes (*see, e.g.*, Chen J. *et al. Nature* 350: 631, 1991; Rothemund P. W. K., *Nature*, 2006; He Y. *et al. Nature* 452: 198, 2008; Ke Y. *et al. Nano. Lett.* 9: 2445, 2009; Douglas S. M. *et al. Nature* 459: 414, 2009; Dietz H. *et al. Science* 325: 725, 2009; Andersen E. S. *et al. Nature* 459: 73, 2009; Liedl T. *et al. Nature Nanotech.* 5: 520, 2010; Han D. *et al. Science* 332: 342, 2011, each of which is incorporated by reference herein), and macroscopic crystals (*see, e.g.*, Meng J. P. *et al. Nature* 461: 74, 2009, incorporated by reference herein).

Nucleic acid nanostructures of the present disclosure may be two-dimensional or three-dimensional.

In some embodiments, a nucleic acid nanostructure is assembled from single-stranded tiles (SSTs) (*see, e.g.*, Wei B. *et al. Nature* 485: 626, 2012 and International Publication Number WO 2014/074597, published 15 May 2014, each incorporated by reference herein) or nucleic acid "bricks" (*see, e.g.*, Ke Y. *et al. Science* 388:1177, 2012; International Publication Number WO 2014/018675 A1, published 30 January 2014, each incorporated by reference herein). For example, single-stranded 2- or 4-domain oligonucleotides self-assemble, through sequence-specific annealing, into two- and/or three-dimensional nanostructures in a predetermined (*e.g.*, predicted) manner. As a result, the position of each oligonucleotide in the nanostructure is known. In this way, a nucleic acid nanostructure may be modified, for example, by adding, removing or replacing oligonucleotides at particular positions. The nanostructure may also be modified, for example, by attachment of moieties, at particular positions. This may be accomplished by using a modified oligonucleotide as a starting material or by modifying a particular oligonucleotide after the nanostructure is formed. Therefore, knowing the position of each of the starting oligonucleotides in the resultant nanostructure provides addressability to the nanostructure.

In some embodiments, a nucleic acid nanostructure is assembled using a nucleic acid (*e.g.*, DNA) origami approach. With a DNA origami approach, for example, a long "scaffold" nucleic acid strand is folded to a predesigned shape through interactions with relatively

shorter "staple" strands. Thus, in some embodiments, a single-stranded nucleic acid for assembly of a nucleic acid nanostructure has a length of at least 500 base pairs, at least 1 kilobase, at least 2 kilobases, at least 3 kilobases, at least 4 kilobases, at least 5 kilobases, at least 6 kilobases, at least 7 kilobases, at least 8 kilobases, at least 9 kilobases, or at least 10 kilobases. In some embodiments, a single-stranded nucleic acid for assembly of a nucleic acid nanostructure has a length of 500 base pairs to 10 kilobases, or more. In some embodiments, a single-stranded nucleic acid for assembly of a nucleic acid nanostructure has a length of 7 to 8 kilobases. In some embodiments, a single-stranded nucleic acid for assembly of a nucleic acid nanostructure comprises the M13 viral genome.

Nucleic acids of the present disclosure include DNA such as D-form DNA and L-form DNA and RNA, as well as various modifications thereof. Nucleic acid modifications include base modifications, sugar modifications, and backbone modifications. Non-limiting examples of such modifications are provided below.

Examples of modified DNA nucleic acids (*e.g.*, DNA variants) that may be used in accordance with the present disclosure include, without limitation, L-DNA (the backbone enantiomer of DNA, known in the literature), peptide nucleic acids (PNA) bisPNA clamp, a pseudocomplementary PNA, locked nucleic acid (LNA), and co-nucleic acids of the above such as DNA-LNA co-nucleic acids. Thus, the present disclosure contemplates nanostructures that comprise DNA, RNA, LNA, PNA or combinations thereof. It is to be understood that the nucleic acids used in methods and compositions of the present disclosure may be homogeneous or heterogeneous in nature. As an example, nucleic acids may be completely DNA in nature or they may be comprised of DNA and non-DNA (*e.g.*, LNA) monomers or sequences. Thus, any combination of nucleic acid elements may be used. The nucleic acid modification may render the nucleic acid more stable and/or less susceptible to degradation under certain conditions. For example, in some embodiments, nucleic acids are nuclease-resistant.

Nucleic acids of the present disclosure, in some embodiments, have a homogenous backbone (*e.g.*, entirely phosphodiester or entirely phosphorothioate) or a heterogeneous (or chimeric) backbone. Phosphorothioate backbone modifications may render an oligonucleotide less susceptible to nucleases and thus more stable (as compared to a native phosphodiester backbone nucleic acid) under certain conditions. Other linkages that may provide more stability to a nucleic acid of the present disclosure include, without limitation,

phosphorodithioate linkages, methylphosphonate linkages, methylphosphorothioate linkages, boranophosphonate linkages, peptide linkages, alkyl linkages and dephospho-type linkages. Thus, in some embodiments, nucleic acids have non-naturally occurring backbones.

Nucleic acids of the present disclosure, in some embodiments, additionally or alternatively comprise modifications in their sugars. For example, a β-ribose unit or a β-D-2'-deoxyribose unit can be replaced by a modified sugar unit, wherein the modified sugar unit is, for example, selected from β-D-ribose, α-D-2'-deoxyribose, L-2'-deoxyribose, 2'-F-2'-deoxyribose, arabinose, 2'-F-arabinose, 2'-O-(C_1-C_6)alkyl-ribose, preferably 2'-O-(C_1-C_6)alkyl-ribose is 2'-O-methylribose, 2'-O-(C_2-C_6)alkenyl-ribose, 2'-[O-(C_1-C_6)alkyl-O-(C_1-C_6)alkyl]-ribose, 2'-NH_2-2'-deoxyribose, β-D-xylo-furanose, α-arabinofuranose, 2,4-dideoxy-β-D-erythro-hexo-pyranose, and carbocyclic (*see*, *e.g.*, Froehler J. *Am. Chem. Soc.* 114:8320, 1992, incorporated by reference herein) and/or open-chain sugar analogs (*see*, *e.g.*, Vandendriessche *et al. Tetrahedron* 49:7223, 1993, incorporated by reference herein) and/or bicyclosugar analogs (*see*, *e.g.*, Tarkov M. *et al. Helv. Chim. Acta.* 76:481, 1993, incorporated by reference herein).

Nucleic acids of the present disclosure, in some embodiments, comprise modifications in their bases. Modified bases include, without limitation, modified cytosines (such as 5-substituted cytosines (*e.g.*, 5-methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (*e.g.*, N4-ethyl-cytosine), 5-aza-cytosine, 2-mercapto-cytosine, isocytosine, pseudo-isocytosine, cytosine analogs with condensed ring systems (*e.g.*, N,N'-propylene cytosine or phenoxazine), and uracil and its derivatives (*e.g.*, 5-fluoro-uracil, 5-bromo-uracil, 5-bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil), modified guanines such as 7-deazaguanine, 7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine), 7-deaza-8-substituted guanine, hypoxanthine, N2-substituted guanines (*e.g.* N2-methyl-guanine), 5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine, 2-aminopurine, purine, indole, adenine, substituted adenines (*e.g.* N6-methyl-adenine, 8-oxo-adenine) 8-substituted guanine (*e.g.* 8-hydroxyguanine and 8-bromoguanine), and 6-thioguanine. The nucleic acids may comprise universal bases (*e.g.* 3-nitropyrrole, P-base, 4-methyl-indole, 5-nitro-indole, and K-base) and/or aromatic ring systems (*e.g.* fluorobenzene, difluorobenzene, benzimidazole or

dichloro-benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid amide). A particular base pair that may be incorporated into the oligonucleotides of the invention is a dZ and dP non-standard nucleobase pair reported by Yang et al. NAR, 2006, 34(21):6095-6101. dZ, the pyrimidine analog, is 6-amino-5-nitro-3-(1'-β-D-2'-deoxyribofuranosyl)-2(1H)-pyridone, and its Watson-Crick complement dP, the purine analog, is 2-amino-8-(1'-β-D-1'-deoxyribofuranosyl)-imidazo[1,2-a]-1,3,5-triazin-4(8H)-one.

Nucleic acids of the present disclosure, in some embodiments, are synthesized *in vitro*. Thus, in some embodiments, nucleic acids are synthetic (*e.g.*, not naturally-occurring). Methods for synthesizing nucleic acids, including automated nucleic acid synthesis, are known. For example, nucleic acids having modified backbones, such as backbones comprising phosphorothioate linkages, and including those comprising chimeric modified backbones, may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries (*see*, *e.g.*, F. E. Eckstein, "Oligonucleotides and Analogues - A Practical Approach" *IRL Press, Oxford, UK*, 1991; and Matteucci M. D. *et al. Tetrahedron Lett.* 21: 719, 1980). Synthesis of nucleic acids with aryl- and alkyl-phosphonate linkages are also contemplated (*see*, *e.g.*, U.S. Patent No. 4,469,863). In some embodiments, nucleic acids with alkylphosphotriester linkages (in which the charged oxygen moiety is alkylated, *e.g.*, as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) are prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (*see*, *e.g.*, Uhlmann E. *et al. Chem. Rev.* 90:544, 1990; Goodchild J. *Bioconjugate Chem.* 1:165, 1990; Crooke S.T. *et al. Annu. Rev. Pharmacol. Toxicol.* 36:107, 1996; and Hunziker J. *et al. Mod Synth Methods* 7:331, 1995, each of which is incorporated by reference) and may be used in accordance with the present disclosure.

Some nucleic acid nanostructures are assembled using annealing processes. In some embodiments, nucleic acids are combined, in a single vessel such as, but not limited to, a tube, a well or a vial. The molar amounts of nucleic acids that are used may depend on the frequency of each nucleic acid in the nanostructure desired and the amount of nanostructure desired. In some embodiments, the nucleic acids may be present in equimolar concentrations. In some embodiments, each nucleic acid (*e.g.*, oligonucleotide) may be present at a concentration of about 200 nM. In some embodiments, the nucleic acids are placed in a solution. The solution may be buffered, although the annealing reaction can also occur in the

absence of buffer. The solution may further comprise divalent cations such as, but not limited, to Mg^{2+}. The cation or salt concentration may vary. An exemplary concentration is about 490 mM. The solution may also comprise EDTA or other nuclease inhibitors in order to prevent degradation of the nucleic acids.

An annealing reaction is carried out, in some embodiments, by heating the solution containing nucleic acids and then allowing the solution to slowly cool down (*e.g.*, heated and then placed in a room temperature environment). The temperature of the reaction should be sufficiently high to melt any undesirable secondary structure such as hairpin structures and to ensure that the nucleic acids are not bound incorrectly to other non-complementary nucleic acids. The temperature, therefore, may be initially raised to any temperature below or equal to 100 ºC. For example, the temperature may be initially raised to 100 ºC, 95 ºC, 90 ºC, 85 ºC, 80 ºC, 75 ºC, 70 ºC, 65 ºC or 60 ºC. The temperature may be raised by placing the vessel in a hot water bath, heating block or a device capable of temperature control, such as a thermal cycler (*e.g.*, polymerase chain reaction (PCR) machine). The vessel may be kept in that environment for seconds or minutes. In some embodiments, an incubation time of about 1-10 minutes is sufficient.

Once nucleic acid incubation at an elevated temperature is complete, the temperature may be dropped in a number of ways. The temperature may be dropped, for example, in an automated manner using a computer algorithm that drops the temperature by a certain amount and maintains that temperature for a certain period of time before dropping the temperature again. Such automated methods may involve dropping the temperature by a degree in each step or by a number of degrees at each step. The vessel may thus be heated and cooled in the same device. As another example, the heated solution may be placed at room temperature to cool. An exemplary process for dropping temperature is as follows. To effect a drop in temperature from about 80 ºC to about 24 ºC, the temperature is changed from 80 ºC to 61 ºC in one degree increments at a rate of 3 minutes per degree (*e.g.*, 80 ºC for 3 minutes, 79 ºC for 3 minutes, *etc.*). The temperature is then changed from 60 ºC to 24 ºC in one degree increments and at a rate of about 120 minutes per degree (*e.g.*, 60 ºC for 120 minutes, 59 ºC for 210 minutes, *etc.*). The total annealing time for this process is about 17 hours. In accordance with the present disclosure, under these conditions, nucleic acids self-assemble into a nanostructure of predetermined and desired shape and size.

An example of a specific annealing process uses one hundred different 200 nM oligonucleotides in solution (*e.g.*, 5 mM Tris-1 mM EDTA (TE), 40 mM $MgCl_2$) and the solution is heated to about 90 ºC and then cooled to about 24 ºC over a period of about 73 hours, as described above with a 3 minute per degree drop between 80 ºC and 61 ºC, and a 120 minute per degree drop between 60 ºC and 24 ºC. It should be understood that the foregoing annealing process is exemplary and that other annealing processes may be used in accordance with the present disclosure.

In some embodiments, nucleic acid nanoarrays are attached to a substrate. In some embodiments, the substrate is glass.

Fiduciary Markers

In some embodiments a nucleic acid nanoarray comprises a (at least one) fiduciary marker, for example, located at prescribed and identifiable positions on the nanoarray. Examples of fiduciary markers of the present disclosure are shown in Example 3.

A "fiduciary marker" herein refers to a single-stranded, or partially single-stranded, nucleic acid located on a nanoarray, comprises a nucleotide sequence that is complementary to a sequence on an imager strand, and does not bind to nucleic acid barcodes. Fiducial markers, generally, may be present on nanoarrays in the field of view of a system used to image the nanoarray. Fiducial markers typically are use as a point of reference or a measure and appear in an image produced (*e.g.*, an image of a nanoarray).

A fiduciary marker typically has a length of 5 nucleotides to 100 nucleotides. In some embodiments, a fiduciary marker has a length of 5 nucleotides to 50 nucleotides. The length of a fiduciary marker depends, in part, on the length of an imager strand (or a complementary sequence on the imager strand). In some embodiments, a fiduciary marker has a length of 4 nucleotides to 60 nucleotides, 6 nucleotides to 40 nucleotides, 7 nucleotides to 30 nucleotides, 8 to 20 nucleotides, or 9 nucleotides to 15 nucleotides. In some embodiments, a fiduciary marker has a length of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more, nucleotides.

Fiduciary markers may be linked covalently or non-covalently to a nucleic acid nanoarray. In some embodiments, a fiduciary marker is an extension of (*e.g.*, is contiguous with) a nucleic acid that forms a nanoarray. In some embodiments, a fiduciary marker is linked to a nanoarray through an intermediate linker (*e.g.*, biotin-streptavidin). Examples of intermediate linkers are described elsewhere herein. In some embodiments, a fiduciary marker contains a nucleotide sequence complementary to a sequence of an imager strand and also contains a nucleotide sequence complementary to a sequence of a nucleic acid nanoarray. For example, the a fiduciary markers in Example 3 contain a 42 nucleotide region that is complementary to a unique sequence of a barcode and also contain a 9 nucleotide region that is complementary to a sequence of an imager strand. Thus, in some embodiments, fiduciary markers comprise (1) a sequence having a length of 10 to 100 nucleotides (*e.g.*, 10 to 90, 10 to 80, 10 to 70, 10 to 60, 10 to 50, 10 to 40, 10 to 30, 10 to 20 nucleotides, or 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nucleotides) and is complementary to a sequence of a nucleic acid nanoarray and (2) a sequence having a length of 4 to 30 nucleotides (*e.g.*, 4 to 30, 4 to 20 nucleotides, or 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25, or 30 nucleotides) and is complementary to a sequence of an imager strand.

A "fiduciary marker site" is a discrete region of a nanoarray that contains one or more fiduciary markers. A single nanoarray may include 2 to 10, or more, fiduciary markers sites. In some embodiments, a nanoarray comprises 2, 3, 4, 5, 6, 7, 8, 9 or 10 fiduciary marker sites. Each fiduciary marker site may include 2 to 10, or more, fiduciary markers. In some embodiments, each fiduciary marker site comprises 2, 3, 4, 5, 6, 7, 8, 9 or 10 fiduciary markers. In some embodiments, a nanoarray contains 3 fiduciary marker sites, each site containing 3 fiduciary markers. In some embodiments, a nanoarray is configured as shown in Figure 5, having a fiduciary marker site located at each end (at one or more periphery or border/edge) of a nanoarray as well as a fiduciary marker site positioned between the end-located fiduciary marker sites.

In some embodiments, a fiducial marker may comprise a "spacer sequence" separating, for example, sequence complementary to a nanoarray and sequence complementary to an imager strand. This spacer sequence minimizes steric hindrance. In some embodiments, a spacer sequence is a homopolymer of 2 to 10 nucleotides (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides). A spacer sequence may be a polyT, polyA, polyC or polyG

sequence. In some embodiments, a spacer sequence includes (*e.g.*, consists of) 2 thymine nucleotides.

Docking Probes

Nucleic acid nanoarrays of the present disclosure comprise one or more docking probe(s). A "docking probe" herein refers to a single-stranded, or partially single-stranded, nucleic acid that comprises a nucleotide sequence that is complementary to a unique sequence of a barcode (*e.g.*, in embodiments where the target biomolecule is a protein) or a unique sequence of a target nucleic acid analyte such that the docking probe is capable of binding respectively to a barcode or target nucleic acid analyte. A nucleotide sequence that is complementary to a unique sequence of a barcode or target nucleic acid analyte is referred to herein as a "complementary unique sequence." A docking probe, like a barcode, is designed to be representative of a single species of biomolecule—a docking probe is designed to bind to only one species of barcode.

A docking probe typically has a length of 5 nucleotides to 100 nucleotides. In some embodiments, a docking probe has a length of 5 nucleotides to 50 nucleotides. The length of a docking probe, and the nucleotide sequence of a docking probe that is complementary to a "unique sequence," depends, in part, on the length of a barcode (or the unique sequence of the barcode) or nucleic acid analyte. In some embodiments, a docking probe, or the nucleotide sequence of a docking probe that is complementary to a unique sequence, has a length of 4 nucleotides to 60 nucleotides, 6 nucleotides to 40 nucleotides, 7 nucleotides to 30 nucleotides, 8 to 20 nucleotides, or 9 nucleotides to 15 nucleotides. In some embodiments, a docking probe, or the nucleotide sequence of a docking probe that is complementary to a unique sequence, has a length of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more, nucleotides. It should be understood that the length of a docking probe is equal to or greater than the length of the nucleotide sequence of a docking probe that is complementary to a unique sequence of a barcode or nucleic acid analyte.

A docking probe, in some embodiments, comprises one or more universal sequence(s). Whether a docking probe comprises a universal sequence and the number of

universal sequences depends, in part, on the selected configuration of the docking probe, which may depend on the type of target biomolecule (*e.g.*, protein v. nucleic acid). In some embodiments, a docking probe comprises two or more (*e.g.*, 2, 3 or 4) different universal sequences, each complementary to a sequence on a different imager strand. For example, a docking probe may contain a first universal sequence complementary to a sequence on a first imager strand, and the same docking probe may contain a second universal sequence complementary to a sequence on the same imager strand or on a second different imager strand (*see*, *e.g.*, Figs. 3A and 3E).

In some embodiments, a docking probe is single stranded. Examples of single-stranded docking probes are depicted in Figs. 1A, 2, and 3B-D. In some embodiments, a docking probe is partially single stranded. Examples of partially single-stranded docking probes are depicted in Figs. 3A and 3E. Partially single-stranded docking probes contain two nucleic strands arranged to form a double stranded region and one or more single-stranded region.

Docking probes may be linked covalently or non-covalently to a nucleic acid nanoarray. In some embodiments, a docking probe is an extension of (*e.g.*, is contiguous with) a nucleic acid that forms a nanoarray. In some embodiments, a docking probe is linked to a nanoarray through an intermediate linker (*e.g.*, biotin-streptavidin). Examples of intermediate linkers are described elsewhere herein. In some embodiments, a docking probe contains a nucleotide sequence complementary to a unique sequence of a barcode and also contains a nucleotide sequence complementary to a sequence of a nucleic acid nanoarray. For example, the docking probes in Example 3 contain a 42 nucleotide region that is complementary to a unique sequence of a barcode and also contain a 20 nucleotide region that is complementary to a unique sequence of a barcode. Thus, in some embodiments, docking probes (*e.g.*, single-stranded docking probes) comprise (1) a sequence having a length of 10 to 100 nucleotides (*e.g.*, 10 to 90, 10 to 80, 10 to 70, 10 to 60, 10 to 50, 10 to 40, 10 to 30, 10 to 20 nucleotides, or 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nucleotides) and is complementary to a sequence of a nucleic acid nanoarray and (2) a sequence having a length of 10 to 50 nucleotides (*e.g.*, 10 to 40, 10 to 30, 10 to 20 nucleotides, or 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides) and is complementary to a unique sequence of a barcode.

Docking probes are located at prescribed and identifiable positions on a nucleic acid nanoarray such that the presence of a target biomolecule in a sample can be identified based on the binding of a barcode or other nucleic acid analyte to the docking probes. A single nanoarray may have 2 to 200, or more, different (*e.g.*, each occurs as a single strand on the nanoarray) docking probes. In some embodiments, a nanoarray comprises 2 to 200, 2 to 150, 2 to 100, 2 to 50, 2 to 25, 5 to 200, 5 to 150, 5 to 100, 5 to 50, 5 to 25, 10 to 200, 10 to 150, 10 to 100, 10 to 50, 10 to 25, 15 to 200, 15 to 150, 15 to 100, 15 to 50, 15 to 25, 20 to 200, 20 to 150, 20 to 100, 20 to 50, 20 to 25, 30 to 200, 30 to 150, 30 to 100, 30 to 50, 40 to 200, 40 to 150, 40 to 100, 40 to 50, 50 to 200, 50 to 150, or 50 to 100 different docking probes.

Docking probes may be prescribed on a nanoarray in any number of ways to produce various nanoarray signature configurations, based on properties such as geometry and intensity, for example. For example, docking probes may be prescribed (relative to each other) beyond or within the spatial resolution of a particular imaging system. In some embodiments, docking probes are prescribed at a distance of 5, 10, 15, 20, 25, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295 or 300 nm relative to each other on a nanoarray. In some embodiments, docking probes are prescribed at a distance of 20 nm relative to each other on a nanoarray. In some embodiments docking probes are prescribed at a distance of 5 to 20, 5 to 30, or 5 to 40 nm relative to each other on a nanoarray. In some embodiments docking probes are prescribed at a distance of 20 to 200, 20 to 250, 20 to 300 nm relative to each other on a nanoarray. In some embodiments docking probes are prescribed at a distance of 200 nm to 250 nm, or 200 nm to 300 nm relative to each other on a nanoarray. (*e.g.*, greater than 250 nm for diffraction-limited and greater than 20–40 nm for super-resolution systems).

In some embodiments, the system used to image a nanoarray is a diffraction-limited imaging system. Thus, in some embodiments, for example, where visible green light is used as the wavelength of light, docking probes are prescribed at a distance of 200 ± 25 nm relative to each other on the nanoarray.

In some embodiments, the system used to image a nanoarray is a super-resolution imaging system. Thus, in some embodiments, for example, docking probes are prescribed at a distance of 5 to 20 nm, 5 to 30 nm, or 5 to 40 nm relative to each other on the nanoarray.

Labeled Imager Strands

A "labeled imager strand" herein refers to a single-stranded nucleic acid (*e.g.*, DNA) having a length of, for example, 4 to 30 nucleotides, comprises a nucleotide sequence that is complementary to a universal sequence of a barcode or docking probe, and is linked to a detectable label (*e.g.*, fluorescent label). In some embodiments, a labeled imager strand is 5 to 18 nucleotides, 6 to 15 nucleotides, 7 to 12 nucleotides, or 8 to 10 nucleotides in length. In some embodiments, a labeled imager strand is 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30, or more, nucleotides in length. In some embodiments, the length of an imager strand is equal to or greater than the length of the nucleotide sequence that is complementary to a universal sequence of a barcode or target nucleic acid analyte.

An imager strand of the present disclosure comprises a nucleotide sequence that is complementary to and transiently binds to a docking probe or a barcode, depending on the selected docking probe configuration. Two nucleic acids or two nucleic acid domains are "complementary" to one another if they base-pair, or bind, to each other to form a double-stranded nucleic acid molecule via Watson-Crick interactions (also referred to as hybridization). As used herein, "binding" refers to an association between at least two molecules due to, for example, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions. An imager strand is considered to "transiently bind" to a barcode or a docking probe (or other nucleic acid probe) if it binds to a complementary region of a respective barcode or docking probe (or other nucleic acid probe) and then disassociates (unbinds) from the respective barcode probe or docking probe (or other nucleic acid probe) within a short period of time, for example, at room temperature. In some embodiments, an imager strand remains bound to a barcode probe or docking probe for about 0.1 to about 10, or about 0.1 to about 5 seconds. For example, an imager strand may remain bound to a docking probe for about 0.1, about 1, about 5 or about 10 seconds.

Imager strands of the present disclosure may be labeled with a detectable label (*e.g.*, a fluorescent label, and thus are considered "fluorescently labeled"). For example, in some embodiments, an imager strand may comprise at least one (*i.e.*, one or more) fluorophore. Examples of fluorophores for use in accordance with the present disclosure include, without limitation, xanthene derivatives (*e.g.*, fluorescein, rhodamine, Oregon green, eosin and Texas

red), cyanine and cyanine derivatives (*e.g.*, cyanine (*e.g.*, Cy2, Cy3, Cy3b, Cy5), indocarbocyanine, oxacarbocyanine, thiacarbocyanine and merocyanine), naphthalene derivatives (*e.g.*, dansyl and prodan derivatives), coumarin derivatives, oxadiazole derivatives (*e.g.*, pyridyloxazole, nitrobenzoxadiazole and benzoxadiazole), pyrene derivatives (*e.g.*, cascade blue), oxazine derivatives (*e.g.*, Nile red, Nile blue, cresyl violet and oxazine 170), acridine derivatives (*e.g.*, proflavin, acridine orange and acridine yellow), arylmethine derivatives (*e.g.*, auramine, crystal violet and malachite green), and tetrapyrrole derivatives (*e.g.*, porphin, phthalocyanine and bilirubin). Other detectable labels may be used in accordance with the present disclosure, such us, for example, gold nanoparticles or other detectable particles or moieties.

As used herein, "spectrally distinct" molecules of the present disclosure (*e.g.*, conjugates and/or imager strands) refer to molecules with labels (*e.g.*, fluorophores) of different spectral signal or wavelength. For example, an imager strand labeled with a Cy2 fluorophore emits a signal at a wavelength of light of about 510 nm, while an imager strand labeled with a Cy5 fluorophore emits a signal at a wavelength of light of about 670 nm. Thus, the Cy2-labeled imager strand is considered herein to be spectrally distinct from the Cy5-labeled imager strand. Conversely, "spectrally indistinct" molecules of the present disclosure herein refer to molecules with labels having the same spectral signal or wavelength – that is, the emission wavelength of the labels cannot be used to distinguish between two spectrally indistinct fluorescently labeled molecules (*e.g.*, because the wavelengths are the same or close together).

Typically, a plurality of imager strands used in accordance with the present disclosure comprise the same species of label (*e.g.*, a Cy2 fluorophore). In some embodiments, however, a plurality of imager strands used as provided herein comprises subsets of imager strands, each subset containing a different species of label (*e.g.*, some linked to Cy2 fluorophores and some linked to Cy3 fluorophores).

Pluralities of imager strands are provided herein. A plurality may be a population of the same species or distinct species. A plurality of imager strands of the same species may comprise imager strands with the same nucleotide sequence and the same fluorescent label (*e.g.*, Cy2, Cy3 or Cy4). Conversely, a plurality of imager strands of distinct species may comprise imager strands with distinct nucleotide sequences (*e.g.*, DNA sequences) and distinct fluorescent labels (*e.g.*, Cy2, Cy3 or Cy4) or with distinct nucleotide sequences and

the same fluorescent (*e.g.*, all Cy2). In some embodiments, a plurality of fluorescently-labeled imager strands comprises at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4, 50000, 10^5, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} fluorescently-labeled imager strands. In some embodiments, a plurality may contain 1 to about 200 or more distinct species of imager strands. For example, a plurality may contain at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200 or more distinct species. In some embodiments, a plurality may contain less than about 5 to about 200 distinct species of imager strands. For example, a plurality may contain less than 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175 or 200 distinct species.

Samples and Target Molecules

A "sample" may comprise cells (or a cell), tissue, or bodily fluid such as blood (serum and/or plasma), urine, semen, lymphatic fluid, cerebrospinal fluid or amniotic fluid. A sample may be obtained from (or derived from) any source including, without limitation, humans, animals, bacteria, viruses, microbes and plants. In some embodiments, a sample is a cell lysate or a tissue lysate. A sample may also contain mixtures of material from one source or different sources. A sample may be a spatial area or volume (*e.g.*, a grid on an array, or a well in a plate or dish).

In some embodiments, a sample is a single cell, such as a rare cell. Examples of a rare cells include, without limitation, circulating tumor cells, epithelial progenitor and stem cells, mesenchymal cells, and fetal cells, for example, circulating in the blood stream.

A "target" is any moiety that one wishes to observe or quantitate and for which a binding partner exists. Typically, a target is a biomolecule. As used herein, a "biomolecule" is any molecule that is produced by a living organism, including large macromolecules such as proteins, polysaccharides, lipids and nucleic acids (*e.g.*, DNA and RNA such as mRNA), as well as small molecules such as primary metabolites, secondary metabolites, and natural products. Examples of target biomolecules include, without limitation, DNA, RNA, cDNA, or the DNA product of RNA subjected to reverse transcription, A23187 (Calcimycin, Calcium Ionophore), Abamectine, Abietic acid, Acetic acid, Acetylcholine, Actin, Actinomycin D, Adenosine, Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Adenosine triphosphate (ATP), Adenylate cyclase, Adonitol, Adrenaline,

epinephrine, Adrenocorticotropic hormone (ACTH), Aequorin, Aflatoxin, Agar, Alamethicin, Alanine, Albumins, Aldosterone, Aleurone, Alpha-amanitin, Allantoin, Allethrin, α-Amanatin, Amino acid, Amylase, Anabolic steroid, Anethole, Angiotensinogen, Anisomycin, Antidiuretic hormone (ADH), Arabinose, Arginine, Ascomycin, Ascorbic acid (vitamin C), Asparagine, Aspartic acid, Asymmetric dimethylarginine, Atrial-natriuretic peptide (ANP), Auxin, Avidin, Azadirachtin A – C35H44O16, Bacteriocin, Beauvericin, Bicuculline, Bilirubin, Biopolymer, Biotin (Vitamin H), Brefeldin A, Brassinolide, Brucine, Cadaverine, Caffeine, Calciferol (Vitamin D), Calcitonin, Calmodulin, Calmodulin, Calreticulin, Camphor - (C10H16O), Cannabinol, Capsaicin, Carbohydrase, Carbohydrate, Carnitine, Carrageenan, Casein, Caspase, Cellulase, Cellulose - (C6H10O5), Cerulenin, Cetrimonium bromide (Cetrimide) - C19H42BrN, Chelerythrine, Chromomycin A3, Chaparonin, Chitin, α-Chloralose, Chlorophyll, Cholecystokinin (CCK), Cholesterol, Choline, Chondroitin sulfate, Cinnamaldehyde, Citral, Citric acid, Citrinin, Citronellal, Citronellol, Citrulline, Cobalamin (vitamin B12), Coenzyme, Coenzyme Q, Colchicine, Collagen, Coniine, Corticosteroid, Corticosterone, Corticotropin-releasing hormone (CRH), Cortisol, Creatine, Creatine kinase, Crystallin, α-Cyclodextrin, Cyclodextrin glycosyltransferase, Cyclopamine, Cyclopiazonic acid, Cysteine, Cystine, Cytidine, Cytochalasin, Cytochalasin E, Cytochrome, Cytochrome C, Cytochrome c oxidase, Cytochrome c peroxidase, Cytokine, Cytosine – C4H5N3O, Deoxycholic acid, DON (DeoxyNivalenol), Deoxyribofuranose, Deoxyribose, Deoxyribose nucleic acid (DNA), Dextran, Dextrin, DNA, Dopamine, Enzyme, Ephedrine, Epinephrine – C9H13NO3, Erucic acid – CH3(CH2)7CH=CH(CH2)11COOH, Erythritol, Erythropoietin (EPO), Estradiol, Eugenol, Fatty acid, Fibrin, Fibronectin, Folic acid (Vitamin M), Follicle stimulating hormone (FSH), Formaldehyde, Formic acid, Formnoci, Fructose, Fumonisin B1, Gamma globulin, Galactose, Gamma globulin, Gamma-aminobutyric acid, Gamma-butyrolactone, Gamma-hydroxybutyrate (GHB), Gastrin, Gelatin, Geraniol, Globulin, Glucagon, Glucosamine, Glucose – C6H12O6, Glucose oxidase, Gluten, Glutamic acid, Glutamine, Glutathione, Gluten, Glycerin (glycerol), Glycine, Glycogen, Glycolic acid, Glycoprotein, Gonadotropin-releasing hormone (GnRH), Granzyme, Green fluorescent protein, Growth hormone, Growth hormone-releasing hormone (GHRH), GTPase, Guanine, Guanosine, Guanosine triphosphate (+GTP), Haptoglobin, Hematoxylin, Heme, Hemerythrin, Hemocyanin, Hemoglobin, Hemoprotein, Heparan sulfate, High density lipoprotein, HDL, Histamine, Histidine, Histone, Histone methyltransferase, HLA antigen, Homocysteine,

Hormone, human chorionic gonadotropin (hCG), Human growth hormone, Hyaluronate, Hyaluronidase, Hydrogen peroxide, 5-Hydroxymethylcytosine, Hydroxyproline, 5-Hydroxytryptamine, Indigo dye, Indole, Inosine, Inositol, Insulin, Insulin-like growth factor, Integral membrane protein, Integrase, Integrin, Intein, Interferon, Inulin, Ionomycin, Ionone, Isoleucine, Iron-sulfur cluster, K252a, K252b, KT5720, KT5823, Keratin, Kinase, Lactase, Lactic acid, Lactose, Lanolin, Lauric acid, Leptin, Leptomycin B, Leucine, Lignin, Limonene, Linalool, Linoleic acid, Linolenic acid, Lipase, Lipid, Lipid anchored protein, Lipoamide, Lipoprotein, Low density lipoprotein, LDL, Luteinizing hormone (LH), Lycopene, Lysine, Lysozyme, Malic acid, Maltose, Melatonin, Membrane protein, Metalloprotein, Metallothionein, Methionine, Mimosine, Mithramycin A, Mitomycin C, Monomer, Mycophenolic acid, Myoglobin, Myosin, Natural phenols, Nucleic Acid, Ochratoxin A, Oestrogens, Oligopeptide, Oligomycin, Orcin, Orexin, Ornithine, Oxalic acid, Oxidase, Oxytocin, p53, PABA, Paclitaxel, Palmitic acid, Pantothenic acid (vitamin B5), parathyroid hormone (PTH), Paraprotein, Pardaxin, Parthenolide, Patulin, Paxilline, Penicillic acid, Penicillin, Penitrem A, Peptidase, Pepsin, Peptide, Perimycin, Peripheral membrane protein, Perosamine, Phenethylamine, Phenylalanine, Phosphagen, phosphatase, Phospholipid, Phenylalanine, Phytic acid, Plant hormones, Polypeptide, Polyphenols, Polysaccharides, Porphyrin, Prion, Progesterone, Prolactin (PRL), Proline, Propionic acid, Protamine, Protease, Protein, Proteinoid, Putrescine, Pyrethrin, Pyridoxine or pyridoxamine (Vitamin B6), Pyrrolysine, Pyruvic acid, Quinone, Radicicol, Raffinose, Renin, Retinene, Retinol (Vitamin A), Rhodopsin (visual purple), Riboflavin (vitamin B2), Ribofuranose, Ribose, Ribozyme, Ricin, RNA - Ribonucleic acid, RuBisCO, Safrole, Salicylaldehyde, Salicylic acid, Salvinorin-A – $C_{23}H_{28}O_8$, Saponin, Secretin, Selenocysteine, Selenomethionine, Selenoprotein, Serine, Serine kinase, Serotonin, Skatole, Signal recognition particle, Somatostatin, Sorbic acid, Squalene, Staurosporin, Stearic acid, Sterigmatocystin, Sterol, Strychnine, Sucrose (sugar), Sugars (in general), superoxide, T2 Toxin, Tannic acid, Tannin, Tartaric acid, Taurine, Tetrodotoxin, Thaumatin, Topoisomerase, Tyrosine kinase, Taurine, Testosterone, Tetrahydrocannabinol (THC), Tetrodotoxin, Thapsigargin, Thaumatin, Thiamine (vitamin B1) – $C_{12}H_{17}ClN_4OS \cdot HCl$, Threonine, Thrombopoietin, Thymidine, Thymine, Triacsin C, Thyroid-stimulating hormone (TSH), Thyrotropin-releasing hormone (TRH), Thyroxine (T4), Tocopherol (Vitamin E), Topoisomerase, Triiodothyronine (T3), Transmembrane receptor, Trichostatin A, Trophic

hormone, Trypsin, Tryptophan, Tubulin, Tunicamycin, Tyrosine, Ubiquitin, Uracil, Urea, Urease, Uric acid – C5H4N4O3, Uridine, Valine, Valinomycin, Vanabins, Vasopressin, Verruculogen, Vitamins (in general), Vitamin A (retinol), Vitamin B, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B3 (niacin or nicotinic acid), Vitamin B4 (adenine), Vitamin B5 (pantothenic acid), Vitamin B6 (pyridoxine or pyridoxamine), Vitamin B12 (cobalamin), Vitamin C (ascorbic acid), Vitamin D (calciferol), Vitamin E (tocopherol), Vitamin F, Vitamin H (biotin), Vitamin K (naphthoquinone), Vitamin M (folic acid), Wortmannin and Xylose.

In some embodiments, a target biomolecule is a protein target such as, for example, proteins of a cellular environment (*e.g.*, intracellular or membrane proteins). Examples of proteins include, without limitation, fibrous proteins such as cytoskeletal proteins (*e.g.*, actin, arp2/3, coronin, dystrophin, FtsZ, keratin, myosin, nebulin, spectrin, tau, titin, tropomyosin, tubulin and collagen) and extracellular matrix proteins (*e.g.*, collagen, elastin, f-spondin, pikachurin, and fibronectin); globular proteins such as plasma proteins (*e.g.*, serum amyloid P component and serum albumin), coagulation factors (*e.g.*, complement proteins,C1-inhibitor and C3-convertase, Factor VIII, Factor XIII, fibrin, Protein C, Protein S, Protein Z, Protein Z-related protease inhibitor, thrombin, Von Willebrand Factor) and acute phase proteins such as C-reactive protein; hemoproteins; cell adhesion proteins (*e.g.*, cadherin, ependymin, integrin, Ncam and selectin); transmembrane transport proteins (*e.g.*, CFTR, glycophorin D and scramblase) such as ion channels (*e.g.*, ligand-gated ion channels such nicotinic acetylcholine receptors and GABAa receptors, and voltage-gated ion channels such as potassium, calcium and sodium channels), synport/antiport proteins (*e.g.*, glucose transporter); hormones and growth factors (*e.g.*, epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), peptide hormones such as insulin, insulin-like growth factor and oxytocin, and steroid hormones such as androgens, estrogens and progesterones); receptors such as transmembrane receptors (*e.g.*, G-protein-coupled receptor, rhodopsin) and intracellular receptors (*e.g.*, estrogen receptor); DNA-binding proteins (*e.g.*, histones, protamines, CI protein); transcription regulators (*e.g.*, c-myc, FOXP2, FOXP3, MyoD and P53); immune system proteins (*e.g.*, immunoglobulins, major histocompatibility antigens and T cell receptors); nutrient storage/transport proteins (*e.g.*, ferritin); chaperone proteins; and enzymes.

In some embodiments, a target biomolecule is a nucleic acid target such as, for example, nucleic acids of a cellular environment (*e.g.*, genomic nucleic acids). As used herein with respect to targets, barcodes, docking probes, and imager strands, a "nucleic acid" refers to a polymeric form of nucleotides of any length, such as deoxyribonucleotides or ribonucleotides, or analogs thereof. For example, a nucleic acid may be a DNA, RNA or the DNA product of RNA subjected to reverse transcription. Non-limiting examples of nucleic acids include coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. Other examples of nucleic acids include, without limitation, cDNA, aptamers, peptide nucleic acids ("PNA"), 2'-5' DNA (a synthetic material with a shortened backbone that has a base-spacing that matches the A conformation of DNA; 2'-5' DNA will not normally hybridize with DNA in the B form, but it will hybridize readily with RNA), locked nucleic acids ("LNA"), and nucleic acids with modified backbones (*e.g.*, base- or sugar-modified forms of naturally-occurring nucleic acids). A nucleic acid may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs ("analogous" forms of purines and pyrimidines are well known in the art). If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. A nucleic acid may be a single-stranded, double-stranded, partially single-stranded, or partially double-stranded DNA or RNA.

In some embodiments, a nucleic acid (*e.g.*, a nucleic acid target) is naturally-occurring. As used herein, a "naturally occurring" refers to a nucleic acid that is present in organisms or viruses that exist in nature in the absence of human intervention. In some embodiments, a nucleic acid naturally occurs in an organism or virus. In some embodiments, a nucleic acid is genomic DNA, messenger RNA, ribosomal RNA, micro-RNA, pre-micro-RNA, pro-micro-RNA, viral DNA, viral RNA or piwi-RNA. In some embodiments, a nucleic acid target is not a synthetic DNA nanostructure (*e.g.*, two-dimensional (2-D) or three-dimensional (3-D) DNA nanostructure that comprises two or more nucleic acids hybridized to each other by Watson-Crick interactions to form the 2-D or 3-D nanostructure).

The nucleic acid barcodes, docking probes and imager strands described herein can be any one of the nucleic acids described above (*e.g.*, DNA, RNA, modified nucleic acids, nucleic acid analogues, naturally-occurring nucleic acids, synthetic nucleic acids).

Nanoarray/Docking Probe Configurations and Detection Methods

Barcodes and target nucleic acid analytes, or simply "analytes," are applied to a nucleic acid nanoarrays for detection and/or quantification. The present disclosure provides various detection methods, which depend on the choice of docking probe configuration.

Docking probes are, in some embodiments, linked to a nucleic acid nanoarray through an intermediate linker, such as a biotin-streptavidin linker. Docking probes may be linked to a nucleic acid nanoarray via the 5' end or the 3' end of the probe.

In some embodiments, a docking probe is a substantially linear single-stranded nucleic acid comprising a sequence complementary to a unique sequence of a barcode or nucleic acid analyte. Examples of such docking probes are depicted in Figs. 1A and 2.

In some embodiments, a docking probe comprises a first strand and a second strand arranged to form a double-stranded region. In some embodiments, with reference to Fig. 3A, the first strand (the longer of the two strands) comprises two contiguous sequences "1a*" + "1b*", which are complementary to unique sequences "1a" + "1b" of a barcode or target nucleic acid analyte. The first strand also comprises a universal sequence "u". The second strand comprises a sequence "1b" complementary to sequence "1b*. The second strand also comprises a universal sequence "v" that is different from the universal sequence "u" of the first strand. Sequence "1b*" of the first strand forms a double-stranded region with "1b" of the second strand. In the presence of a barcode or target nucleic acid analyte comprising sequences "1a" and "1b", the second strand of the docking probe is displaced, and the analyte binds to regions "1a*" and "1b*" of the first strand of the docking probe. Unbound analyte is removed (*e.g.*, washed away), and then labeled imager strands comprising a sequence "u*", complementary to universal sequence "u", and sequence "v*", complementary to sequence "v", are applied to the array. Labeled imager strands transiently binding to a docking probe that is bound to a target analyte "blink" for a shorter period of time relative to labeled imager strands transiently binding to a docking probe that is not bound to a target analyte. In Fig. 3A, "S" indicates a short blinking time, and "L" indicates a longer blinking time, relative to the short blinking time. "Blinking" refers to binding ("ON") and unbinding ("OFF") of a labeled

imager strand to a barcode or docking probe. When imaged by, for example, time-lapse fluorescent microscopy, prescribed locations on the nanoarray appear to "blink" depending on the binding/unbinding interaction of a labeled imager strand with a barcode or docking probe. In the configuration depicted in Fig. 3A, a shorter, or faster, blinking rate is indicative of target analyte binding to a docking probe, and a longer, or slower, blinking rate is indicative that no target analyte is bound to the docking probe.

In some embodiments, a docking probe comprises a single strand that forms a hairpin loop. In some embodiments, with reference to Fig. 3B, the single-stranded docking probe comprises two contiguous sequences "1a*" + "1b*", each complementary to unique sequences "1a" + "1b" of a barcode or target nucleic acid analyte. The single-stranded docking probe also comprises a universal sequence "u", and a sequence "1b", which is complementary to and thus binds to sequence "1b*". In the presence of a barcode or target nucleic acid analyte comprising sequences "1a" and "1b", the "1b" region of the docking probe is displaced, and the analyte binds to regions "1a*" and "1b*" docking probe. Unbound analyte is removed (*e.g.*, washed away), and then labeled imager strands comprising a sequence "u*", complementary to universal sequence "u", applied to the array. Labeled imager strands transiently binding to a docking probe that is bound to a target analyte "blink." When imaged by, for example, time-lapse fluorescent microscopy, prescribed locations on the nanoarray appear to "blink" depending on the binding/unbinding interaction of a labeled imager strand with a barcode or docking probe.

In some embodiments, a docking probe comprises a substantially linear single strand. In some embodiments, with reference to Fig. 3C, the single-stranded docking probe comprises a sequence "1" that is complementary to a unique sequence "1*" of a barcode or target nucleic acid analyte. The single-stranded docking probe also comprises flanking universal sequences "u" and "v". In the presence of a barcode or target nucleic acid analyte comprising sequence "1*", the target binds to region "1" of the docking probe. Unbound analyte is removed (*e.g.*, washed away), and then labeled imager strands comprising a sequence "u*", complementary to universal sequence "u", and sequence "v*", complementary to sequence "v", are applied to the array. Labeled imager strands transiently binding to a docking probe that is bound to a target analyte "blink" for a shorter period of time relative to labeled imager strands transiently binding to a docking probe that is not bound to a target analyte. In the configuration depicted in Fig. 3C, a shorter, or faster,

blinking rate is indicative of target analyte binding to a docking probe, and a longer, or slower, blinking rate is indicative that no target analyte is bound to the docking probe.

In some embodiments, a docking probe comprises a single strand that forms a hairpin loop. In some embodiments, with reference to Fig. 3D, the single-stranded docking probe comprises a sequence "1" that is complementary to a unique sequence "1*" of a barcode or target nucleic acid analyte. The single-stranded docking probe also comprises flanking sequences that bind to each other to form a hairpin as well as flanking universal sequences "u" and "v". In the presence of a barcode or target nucleic acid analyte comprising sequence "1*", the target binds to region "1" of the docking probe, thereby linearizing the docking probe. Unbound analyte is removed (*e.g.*, washed away), and then labeled imager strands comprising a sequence "u*", complementary to universal sequence "u", and sequence "v*", complementary to sequence "v", are applied to the array. In the configuration depicted in Fig. 3D, a shorter, or faster, blinking rate is indicative of target analyte binding to a docking probe, and a longer, or slower, blinking rate is indicative that no target analyte is bound to the docking probe.

In some embodiments, a docking probe comprises a first strand and a second strand arranged to form a double-stranded region. In some embodiments, with reference to Fig. 3E, the first strand (the longer of the two strands) comprises two contiguous sequences "1a" + "1b", which are complementary to unique sequences "1a*" + "1b*" of a barcode or target nucleic acid analyte. The first strand also comprises universal sequences "u" and "v". The second strand comprises a sequence "1b*" complementary to sequence "1* and a sequence "u*" complementary to sequence "u". Sequences "1b" and "u" of the first strand form a double-stranded region with "1b*" and "u*" of the second strand. In some instances, the first strand further comprises a sequence "w" for confirming the presence of the docking strand. In the presence of a barcode or target nucleic acid analyte comprising sequences "1a*" and "1b*", the second strand of the docking probe is displaced, and the target binds to regions "1a*" and "1b*" of the first strand of the docking probe. Unbound analyte is removed (*e.g.*, washed away), and then labeled imager strands comprising a sequence "u*", complementary to universal sequence "u", and sequence "v*", complementary to sequence "v", are applied to the array. In the configuration depicted in Fig. 3E, a shorter, or faster, blinking rate is indicative of target analyte binding to a docking probe, and a longer, or slower, blinking rate is indicative that no target analyte is bound to the docking probe. To confirm the presence of

the docking strands, another labeled imager strand comprising sequences "v*" and "w*" ("w*" complementary to "w") may be applied to the nanoarray.

Methods provided herein are based, in part, on the programmability of barcode probes or docking probes and imager strands. That is, for example, barcode probes or docking probes and imager strands can be designed such that they bind to each other (*e.g.*, barcode binding to imager strand, or docking probe binding to imager strand) under certain conditions for a certain period of time. This programmability permits transient binding of imager strands to barcode probes or docking probes, as provided herein. Generally, the methods provided herein are directed to identifying one or more target(s) (*e.g.*, biomolecule(s) such as a protein or nucleic acid) in a particular sample (*e.g.*, biological sample). In some instances, whether or not one or more target(s) is present in sample is unknown. Thus, methods of the present disclosure may be used to determine the presence or absence of one or more target(s) in a sample suspected of containing the target(s). In any one of the embodiments and embodiments provided herein, a sample may contain or may be suspected of containing one or more target(s).

Methods as provided herein can also be used to identify the absolute quantity of a single target (*e.g.*, such as, for example, a particular protein), or the quantity of a single target relative to one or more other targets.

Further, as methods provided herein may be used to identify the location of a target within a sample or relative to other targets in the sample.

In some embodiments, methods comprise contacting a sample with (a) barcoded conjugates that comprises a biomolecule binding partner linked to a barcode probe having a unique sequence and a universal sequence under conditions sufficient for binding of the barcoded conjugate to a target biomolecule of interest, (b) removing unbound barcoded conjugates, (c) dissociating (*e.g.*, cleaving) barcodes from bound barcoded conjugates, (d) applying the dissociated barcodes to a nanoarray comprising at prescribed locations docking probes that comprise sequences complementary to the unique sequences of the barcodes under conditions sufficient for binding of the barcodes to the docking probes, (e) removing unbound barcodes, (f) applying to the nanoarray labeled imager strands that comprise a sequence complementary and bind transiently to the universal sequence of the barcodes, and (g) then determining whether the barcoded conjugates bind to the target biomolecule (such in the sample. In some embodiments, the determining step comprises imaging (*e.g.*, with time-

lapsed fluorescent microscopy techniques) transient binding of the labeled imager strands to the barcodes.

In some embodiments, methods comprise (a) applying nucleic acid analytes (*e.g.*, barcodes or genomic nucleic acids, such as microRNAs) to a nanoarray comprising at prescribed locations docking probes that comprise sequences complementary to unique sequences of the analytes under conditions sufficient for binding of the analytes to the docking probes, (b) removing unbound analytes, (c) applying to the nanoarray labeled imager strands that comprise a sequence complementary and bind transiently to the universal sequence of the barcodes, and (g) then determining whether the barcoded conjugates bind to the target biomolecule (such in the sample. In some embodiments, the determining step comprises imaging (*e.g.*, with time-lapsed fluorescent microscopy techniques) transient binding of the labeled imager strands to the barcodes.

Imaging

Imaging of the nanoarrays of the present disclosure rely, in some embodiments, relies on the kinetics of photoswitching of fluorescent signals. This stochastic super-resolution imaging uses transient binding of universal imager strands to its complementary universal sequence located on a barcode or docking probe. This method permits imaging in a diffraction-limited area. "Switching" molecules from a fluorescent OFF- to an ON-state is facilitated by single-molecule nucleic acid (*e.g.*, DNA) hybridization events, which are governed by a predictable kinetic model with a second order association rate k_{on} and a first order dissociation rate k_{off}:

$$S_{imager} + S_{docking} \xrightarrow{k_{on}} S_{imager}:S_{docking}$$

$$S_{imager}:S_{docking} \xrightarrow{k_{off}} S_{imager} + S_{docking}$$

The kinetic parameters k_{on} and k_{off} are directly linked to fluorescent ON- and OFF-times (τ_b and τ_d, respectively). The fluorescence ON-time τ_b is determined by the dissociation rate k_{off}: $\tau_b = 1/k_{off}$, and the fluorescence OFF-time τ_d is determined by the association rate k_{on}, the concentration of imager strands in solution c_{imager}, and the number of observed binding sites bs:

$$\tau_d = \frac{1}{k_{on} \cdot c_{imager} \cdot bs}$$

After calibrating $k_{on} \cdot c_{imager}$ using a sample with a known number of binding sites bs (which can be easily done using, *e.g.*, a DNA nanostructure), the number of binding sites for an unknown molecule or area can be obtained according to the equation:

$$bs = \frac{1}{k_{on} \cdot c_{imager} \cdot \tau_d}$$

Accordingly, the quantification of a fluorescence image may be done automatically using binding kinetics analysis software. In brief, a typical image is recorded in a time-lapsed fashion (*e.g.*, 15000 frames with a frame rate of 10 Hz). Fluorescence spot detection and fitting (*e.g.*, Gaussian fitting, Centroid fitting, or Bessel fitting) is performed on the diffraction-limited image, and thus a super-resolved image is obtained. In the next step, a fiduciary marker is selected. The software automatically calculates the fluorescence dark time τ_d by fitting the OFF-time distribution to a cumulative distribution function. Using the equations described above, the product of $k_{on} \cdot c_{imager}$ can be calculated. This product is used to calculate the number of docking sites, and thus targets in the imaged area.

In some embodiments, the selection of areas of interest in the resolved (*e.g.*, super-resolved) imaged can be performed automatically by applying a second spot detection step, *e.g.*, to calculate the number of targets in a cluster.

Thus, in some embodiments, the methods of the present disclosure comprise providing a nanoarray that comprises docking probes bound to complementary barcodes and fluorescently-labeled imager strands transiently bound directly or indirectly to the docking probes or barcodes, obtaining a time-lapsed diffraction-limited fluorescence image of the nanoarray, performing fluorescence spot detection and fitting (*e.g.*, Gaussian fitting, Centroid fitting, or Bessel fitting) on the diffraction-limited image to obtain a high-resolution image of the nanoarray, calibrating $k_{on} \cdot c_{imager}$ using a region of the nanoarry with a known number of targets, wherein k_{on} is a second order association constant, and c_{imager} is the concentration of fluorescently-labeled imager strands on the nanoarray, including unbound imager strands, determining variable τ_d by fitting the fluorescence OFF-time distribution to a cumulative distribution function, and determining the number of barcodes on the nanoarray based on the equation, $number\ of\ barcodes = (k_{on} \cdot c_{imager} \cdot \tau_d)^{-1}$.

Some aspects of the present disclosure relate to fitting functions. A "fitting function," as used herein, refers to a mathematical function used to fit the intensity profile of molecules. Examples of fitting functions for use as provided herein include, without limitation, Gaussian

fitting, Centroid fitting, and Bessel fitting. It should be understood that while many embodiments of the present disclosure refer to Gaussian fitting, other fitting functions may be used instead of, or in addition to, Gaussian fitting.

Kits

The present disclosure further provides kits comprising one or more components as provided herein. The kits may comprise, for example, barcoded conjugates having a target biomolecule linked to a nucleic acid barcode, docking probes, imager strands (*e.g.*, fluorescently-labeled imager strands), fiduciary markers, nanoarrays, or any combination of two or more of the foregoing. The kits may also comprise components for producing a barcoded conjugate or for labeling an imager strand. Thus, the kits may comprise target biomolecules (*e.g.*, antibodies), nucleic acid barcodes, docking strands, structural subunits of a nanoarray (*e.g.*, staple strands, scaffold strands, tiles, *etc.*) and intermediate linkers such as, for example, biotin and streptavidin molecules, imager strands, or any combination of two or more of the foregoing. The kits can be used for any purpose apparent to those of skill in the art, including, those described above.

The kits may include other reagents as well, for example, buffers for performing hybridization reactions and for assembling the nanoarrays. The kits may also include instructions for using the components of the kit and/or for producing various components (*e.g.*, barcoded conjugates, labeled imager strands, nanoarrays (*e.g.*, with or without docking probes)).

The present disclosure further provides the following numbered paragraphs:

1. A method for detecting one or more target biomolecule(s), the method comprising:

(a) combining a sample containing a target biomolecule with a barcoded conjugate, wherein the barcoded conjugate comprises a biomolecule binding partner that binds specifically to the target biomolecule and is linked to a nucleic acid barcode that comprises a unique nucleotide sequence and a universal nucleotide sequence;

(b) detaching the barcode from the conjugate;

(c) combining the barcode with a nucleic acid nanoarray that comprises a nucleic acid docking probe located at a prescribed position on the nanoarray, wherein the docking probe

comprises a sequence that is complementary to the unique sequence of the barcode, thereby producing a nanoarray containing the docking probe bound to the barcode; and

(d) combining the nanoarray with a labeled universal imager strand that comprises a sequence that is complementary to and capable of binding transiently to the universal sequence of the barcode.

2. The method of paragraph 1, wherein the biomolecule is a protein.

3. The method of paragraph 1 or 2, wherein the sample is a biological sample.

4. The method of paragraph 3, wherein the biological sample is blood or saliva.

5. The method of paragraph 3, wherein the biological sample is a plurality of cells or cell lysate.

6. The method of paragraph 3, wherein the biological sample is a single cell or a single-cell lysate.

7. The method of any one of paragraphs 1-6, wherein the biomolecule binding partner is an antibody.

8. The method of any one of paragraphs 1-7, wherein the labeled universal imager strand comprises a fluorescent label.

9. The method of any one of paragraphs 1-8, further comprising imaging the labeled universal imager strand.

10. The method of paragraph 9, further comprising determining, based on binding kinetics of the labeled universal imager strand to the universal sequence of the barcode, whether the barcode is bound to the docking probe.

11. The method of any one of paragraphs 1-10, wherein the nucleic acid nanoarray comprises a plurality of docking probes, each docking probe located at a prescribed position on the nanoarray and each comprising a sequence that is unique to a protein.

12. The method of any one of paragraphs 1-11, wherein the nucleic acid nanoarray comprises at least two fiduciary marker sites, each site comprising at least one fiduciary marker.

13. The method of paragraph 12, wherein each fiduciary marker site comprises at least two fiduciary markers.

14. The method of any one of paragraphs 1-13, wherein the length of the nucleic acid barcode is 20 to 40 nucleotides.

15. The method of any one of paragraphs 1-14, wherein the length of the unique nucleotide sequence of the nucleic acid barcode is 10 to 30 nucleotides.

16. The method of paragraph 15, wherein the length of the unique nucleotide sequence of the nucleic acid barcode is 20 nucleotides.

17. The method of any one of paragraphs 1-16, wherein the length of the universal sequence of the nucleic acid barcode is 5 to 15 nucleotides.

18. The method of paragraph 17, wherein the length of the universal sequence of the nucleic acid barcode is 9 nucleotides.

19. The method of any one of paragraphs 1-18, wherein the length of the unique nucleotide sequence of the nucleic acid barcode is longer than the length of the universal sequence of the nucleic acid barcode.

20. The method of any one of paragraphs 1-19, wherein the nucleic acid barcode comprises a spacer sequence that separates the unique nucleotide sequence and the universal nucleotide sequence.

21. The method of paragraph 20, wherein the spacer sequence has a length of 2 to 5 nucleotides.

22. The method of paragraph 20 or 21, wherein the spacer sequence is a polyT, polyA, polyC or polyG sequence.

23. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a sample that contains a plurality of target nucleic acid analytes, wherein the nanoarray comprises at prescribed locations on the array nucleic acid docking probes, each docking probe comprising (i) a sequence that is complementary to a target analyte of the plurality and that uniquely identifies the analyte and (ii) a universal sequence; and

(b) combining the nanoarray with labeled nucleic acid imager strands that comprise a sequence complementary to and capable of binding transiently to the universal sequences of the docking probes, wherein the length of time that an imager strand binds to a docking probe is indicative of whether the docking probe is bound to a target nucleic acid analyte.

24. The method of paragraph 23, wherein the sample is a biological sample.

25. The method of paragraph 24, wherein the biological sample is blood or saliva.

26. The method of paragraph 24, wherein the biological sample is a plurality of cells or cell lysate.

27. The method of paragraph 24, wherein the biological sample is a single cell or a single-cell lysate.

28. The method of any one of paragraphs 23-27, wherein the target nucleic acid analytes of the plurality are deoxyribonucleic acid (DNA) analytes.

29. The method of any one of paragraphs 23-27, wherein the target nucleic acid analytes of the plurality are ribonucleic acid (RNA) analytes.

30. The method of paragraph 29, wherein the RNA analytes are RNA interference molecules.

31. The method of paragraph 30, wherein the RNA interference molecules are short-interfering RNAs or micro RNAs.

32. The method of any one of paragraphs 23-31, wherein the labeled nucleic acid imager strands comprise a fluorescent label.

33. The method of any one of paragraphs 23-32, further comprising imaging the labeled nucleic acid imager strands.

34. The method of paragraph 44, further comprising determining, based on binding kinetics of the labeled nucleic acid imager strands to the universal sequences of the docking probes, whether one or more target nucleic acid analyte(s) of the sample is/are bound to the docking probe(s).

35. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a third sequence that is complementary to the first sequence of the analyte;

(b) combining the nanoarray with a nucleic acid probe that comprises a fourth sequence and a universal fifth sequence, wherein the fourth sequence is complementary to the second sequence of the analyte; and

(c) combining the nanoarray with a detectable nucleic acid imager strand that comprises a sixth sequence that is complementary to and capable of binding transiently to the universal fifth sequence of the probe.

36. The method of paragraph 35, wherein the docking probe is shorter than the target nucleic acid analyte.

37. The method of paragraph 35 or 36, wherein the nucleic acid probe is shorter than the target nucleic acid analyte.

38. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a first nucleic acid strand and a second nucleic acid strand, wherein:

(i) the first strand comprises a third sequence, a fourth sequence and a universal fifth sequence, wherein the third sequence is complementary to the first sequence and the fourth sequence is complementary to the second sequence, and

(ii) the second strand comprises a sixth sequence and a universal seventh sequence, wherein the sixth sequence is complementary to and bound to the fourth sequence; and

(b) combining the nanoarray with a labeled nucleic acid imager strand that comprises an eighth sequence and a ninth sequence, wherein the eighth sequence is complementary to and capable of binding transiently to the universal fifth sequence and the ninth sequence is complementary to and capable of binding transiently to the universal seventh sequence.

39. The method of paragraph 38, wherein the second nucleic acid strand is shorter than the first nucleic acid strand.

40. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a third sequence, a fourth sequence, a universal fifth sequence, and a sixth sequence, wherein the third sequence is complementary to the first sequence, the fourth sequence is complementary to the second sequence, and the sixth sequence is complementary to and hybridized to the fourth sequence; and

(b) combining the nanoarray with a labeled nucleic acid imager strand that comprises a seventh sequence that is complementary to and capable of binding transiently to the universal fifth sequence.

41. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence that uniquely identifies the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a second sequence flanked by a universal third sequence and a universal fourth sequence, wherein the second sequence is complementary to the first sequence; and

(b) combining the nanoarray with a labeled nucleic acid imager strand that comprises a fifth sequence and a sixth sequence, wherein the fifth sequence is complementary to and capable of binding transiently to the universal third sequence, and the sixth sequence is complementary to and capable of binding transiently to the universal fourth sequence.

42. The method of paragraph 41, wherein regions of the second sequence bind to each other to form a hairpin loop.

43. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a first nucleic acid strand and a second nucleic acid strand, wherein:

(i) the first strand comprises a third sequence, a fourth sequence, a universal fifth sequence, and a universal sixth sequence, wherein the third sequence is complementary to the first sequence and the fourth sequence is complementary to the second sequence, and

(ii) the second strand comprises a seventh sequence and an eighth sequence, wherein the seventh sequence is complementary to and hybridized to the fourth sequence and the eighth sequence is complementary to and hybridized to the universal fifth sequence; and

(b) combining the nanoarray with a first labeled nucleic acid imager strand that comprises a ninth sequence and a tenth sequence, wherein the ninth sequence is complementary to and capable of binding transiently to the universal fifth sequence, and the tenth sequence is complementary to and capable of binding transiently to the universal sixth sequence.

44. The method of paragraph 43, wherein the second nucleic acid strand is shorter than the first nucleic acid strand.

45. The method of paragraph 43 or 44, wherein the first nucleic acid strand further comprises a universal eleventh sequence.

46. The method of paragraph 45, further comprising combining the nanoarray with a second labeled imager strand that comprises a twelfth sequence and a thirteenth sequence, wherein the twelfth sequence is complementary to the universal sixth sequence and the thirteenth sequence is complementary to the universal eleventh sequence.

47. The method of any one of paragraphs 23-46, wherein the target nucleic acid analyte is a component of a biological sample.

48. The method of paragraph 47, wherein the biological sample is a plurality of cells or cell lysate.

49. The method of paragraph 47, wherein the biological sample is a single cell or a single-cell lysate.

50. The method of any one of paragraphs 23-49, wherein the nucleic acid analyte is a deoxyribonucleic acid (DNA) analyte.

51. The method of any one of paragraphs 23-49, wherein the nucleic acid analyte is a ribonucleic acid (RNA) analyte.

52. The method of paragraph 51, wherein the RNA analyte is an RNA interference molecule.

53. The method of paragraph 52, wherein the RNA interference molecule is a short-interfering RNA or a micro RNA.

54. The method of any one of paragraphs 23-53, wherein the labeled nucleic acid imager strand comprises a fluorescent label.

55. The method of any one of paragraphs 23-54, further comprising imaging the labeled nucleic acid imager strand.

56. The method of paragraph 55, further comprising determining, based on binding kinetics of the labeled nucleic acid imager strand to the universal sequence of the docking probe, whether a target nucleic acid analyte is bound to the docking probe.

57. The method of any one of paragraphs 23-56, wherein the nucleic acid nanoarray comprises a plurality of docking probes, each probe located at a prescribed position on the nanoarray and each comprising a sequence that is unique to a target nucleic acid analyte.

58. The method of any one of paragraphs 23-57, wherein the nucleic acid nanoarray is a two-dimensional or three-dimensional nucleic acid nanostructure.

59. The method of any one of paragraphs 23-58, wherein the nucleic acid nanoarray is a single-stranded nucleic acid tile array.

60. A docking probe comprising a sequence of any one of SEQ ID NO: 1-39.

61. A docking probe comprising a barcode binding site of a sequence of any one of SEQ ID NO: 1-39.

62. A nanoarray comprising a docking probe of paragraph 61.

63. A nucleic acid barcode comprising a sequence of any one of SEQ ID NO: 40-78.

64. A biomolecule binding partner linked to a nucleic acid barcode of paragraph 63.

65. A nanoarray comprising docking probes bounds to nucleic acid barcodes of paragraph 63.

66. A fiduciary marker comprising a sequence of any one of SEQ ID NO: 79-93.

67. A nanoarray comprising fiduciary markers of paragraph 66.

68. A kit comprising any two or more of the following components selected from the group consisting of barcoded conjugates, biomolecule binding partners, linkers, nucleic acid barcodes, nucleic acid nanoarrays, docking probes and universal imager strands.

The present disclosure is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teachings that are referenced herein.

EXAMPLES

Example 1

An example of a method for detecting a target biomolecule is present below and depicted, in part, in Fig. 4.

A method of the present disclosure comprises at least two components: one or more barcodes (obtained from barcoded conjugates) or target nucleic acid analyte(s) (*e.g.*, genomic nucleic acids such as microRNA) and a nucleic acid nanoarray. A nucleic acid nanoarray comprises at prescribed and identifiable locations docking probes for docking and detecting (and, in some embodiments, quantifying) barcodes or target nucleic acid analytes.

Barcodes or nucleic acid analytes are hybridized to the nucleic acid nanoarray under TBE-buffered conditions, supplemented with ~5 to 20 mM Mg^{2+} at room temperature to 37 ºC. The nanoarray is then immobilized on an appropriate glass surface using, for example, biotin-streptavidin binding. To detect and quantify barcodes, imaging is performed using labeled (*e.g.*, fluorescently-labeled) imager strands in TBE-buffer conditions (supplemented with ~5 to 20 mM Mg^{2+} at room temperature) using ~1 to10 nM concentration of imager strands. Images obtained (*e.g.*, by fluorescent microscopy) are then be analyzed using localization software to quantify the analyte.

Example 2

DNA Origami Nanoarray Protocol for Quantifying DNA Barcodes

This Examples describes an example of a protocol for quantitation of orthogonal DNA barcodes using a DNA nanostructure and a DNA PAINT technique (use of docking strands and labeled imager strands). The orthogonal DNA barcodes dock at a unique position

on the DNA nanostructure and are imaged using a universal DNA PAINT imager strand. Counting the number of spots imaged permits quantification of barcodes. The method provided herein can assay 39 distinct barcodes, for example. It is possible to modify the method to accommodate more barcodes, as required.

The DNA nanostructure used in this example contains a six helix bundle folded from a standard M13mp18 scaffold and staple strands (short, single-stranded oligonucleotides). The nanostructure has the following modifications:

a. Biotin Handles: These are eight staple strands with a common sequence extension that is complementary to a biotinylated strand. This is how the nanostructure is attached to a streptavidin coated surface.

b. Fiduciary Markers: There are three versions of the six-helix bundle, each can assay thirteen barcodes. The three versions are distinguished from each other by the use of Fiduciary Markers. These are three 'spots', two at the ends of the six-helix bundle, common to all three versions, and one in between the end spots (indicated as green spots in the figure above). The position of the center 'spot' permits identification of the version. Each spot is a set of three staples with PAINT handle extensions. The PAINT sequence P2 as used for the fiduciary markers.

c. Docking probes: These are three sets of thirteen staples each with extensions complementary to barcode sequences. Each set of thirteen staples corresponds to one of the three versions of the six-helix bundle.

The 39 barcodes each have two domains. One domain is the barcode ID, with a distinct orthogonal sequence. The other domain is a common PAINT handle sequence for the docking of a PAINT strand. The PAINT sequence P1 was used for the barcodes.

A. Folding six helix bundle DNA origami

There are four categories of staples that fold the six helix bundle DNA origami – core strands, biotin handles, fiduciary (version) markers and docking probes. The core strands make up the core of the structure. The biotin handles are used to hybridize a biotinylated strand. The fiduciary (version) markers help identify the orientation of the six-helix bundle on the surface and distinguish the version number. The docking probes are sites where the barcodes can hybridize.

DNA nanostructures are folded in 1X TE buffer with 10mM Mg++ at 5-10nM scaffold concentration with a 10X excess of staples and a 100X excess of the biotinylated strand. The annealing protocol is a slow cooling from 90 °C to 30 °C overnight. Store at 4 °C before purification (see below).

B. Purifying DNA Origami

DNA nanostructures are purified to remove excess staple and biotinylated strands. A standard native gel electrophoresis technique was used:

1. Pour a 50mL volume 1% Agarose Gel in 1X TAE with 10mM Mg++ buffer and SYBR safe stain.

-Weigh 0.5g agarose powder and add to 1X TAE buffer in a conical flask.

-Add 500uL of 1M MgCl2 solution.

-Microwave for about a minute or two till the powder is completely dissolved and you get a clear solution.

-Allow to cool a little (should feel warm when the flask is touched but not hot) and add 5uL of SYBR safe stain.

-Allow to set.

-Mix 1 part (by volume) origami with 5 parts 6X native gel loading buffer. Do not load all the annealed origami, save some for the later step of estimating the concentration of purified origami.

-Load on gel and run for 2-3hrs at 80V in an ice bath.

-Image on Safe Imager Blue Light Transilluminator and cut out the bright origami band.

-Transfer the cut band to a Freeze N Squeeze tube. Crush the gel with a pestle and spin for 4 mins at 2000 g at 4 °C in a temperature controlled centrifuge.

-Discard the crushed gel. Pipette the origami solution into 20 μL aliquots and store at -20 °C. Freeze-thawing purified origami multiple times is not recommended. Keep thawed nanostructure on ice.

C. Drift Markers

Rectangular DNA was used as drift markers with 84 sites extended to P1 docking sites and another 84 sites extended to P2 docking sites. The folding and purifying of drift markers is analogous to that for DNA nanostructures. Any other drift markers (*e.g.*, two color (P1/P2)) may be used and should give similar results.

D. Hybridization of Barcodes to Nanoarray

Prior to PAINT imaging, we must hybridize the barcodes to purified nanoarrays. If the barcode concentration is too low, use a vacuum centrifuge to concentrate. Mix 5uL of purified nanoarray to 5uL barcode solution. Leave at room temperature for 2 to 12 hours. We will refer to the nanoarrays with hybridized barcodes as hybridized nanoarrays.

E. PAINT Imaging

Buffer A: 10mM Tris (pH 8), 100mM NaCl

Buffer B: 5mM Tris (pH 8), 10mM $MgCl_2$, 1mM EDTA

Prior to imaging the hybridized nanoarray was diluted with buffer B to the appropriate concentration for imaging and drift markers were mix in. The target concentrations were: hybridized nanoarrays – 100pM, and drift markers – 10pM. This solution is referred to as "the nanostructure mix."

Using standard glass slide and coverslip:

1. For sample preparation, a piece of coverslip (No. 1.5, 18×18 mm2, ≈0.17 mm thick) and a glass slide (3×1 inch2, 1 mm thick) were sandwiched together by two strips of double-sided tape to form a flow chamber with inner volume of ≈20 µl.
2. First, 20 µl of biotin-labeled bovine albumin (1 mg/ml, dissolved in buffer A) was flown into the chamber and incubated for 2 min. The chamber was then washed twice using 40 µl of buffer A.
3. 20 µl of streptavidin (0.5 mg/ml, dissolved in buffer A) was then flown through the chamber and allowed to bind for 2 min.
4. Wash with 40 µl of buffer A and subsequently with 40 µl of buffer B.
5. Flow in 20 µl of the nanostructure mix and incubate for 2 min.
6. Wash using 40 µl of buffer B.

7. Flow in PAINT imager P2-Cy3b at 5nM concentration to image the fiduciary markers on the nanoarray.

8. Take a PAINT image (10000 frames at 100ms per frame. 10% laser power on the Nikon NSTORM)

9. Wash with 40uL of Buffer B.

10. Flow in PAINT imager P1-Cy3b at 5nM concentration to image the barcodes on the nanoarray.

F. Image analysis

Software was used to construct a super-resolution image. The drift marker was used to correct drift in the two channels (P1 and P2). Spot detection software was used to identify the ends, orientation and version of the nanoarrays. The presence/absence of the spots at the barcode hybridization positions were counted.

Example 3

Sequence Design: DNA Nanoarray

A. Docking probes (d_1^* to d_{39}^*) have the format:

5' <staple sequence> <barcode binding site> 3'

where <staple sequence> is a 42 base segment which is incorporated into the nanostructure (complementary to the scaffold) and <barcode binding site> is a 20 base segment for hybridizing the corresponding barcode sequences (b_1 to b_{39}).

Table 1

Docking Probe Sequences (from 5' to 3')	**ID**	**SEQ ID NO:**
ATCCTGATTGTTTGACAATAAACAACATTCATTACCGCGCCCACTATGGTG CGGAACAAGTT	d_1^*	1
AGCTACAATTTTATTCATATGGTTTACCATGGCTTTTGATGATATTAGAGAA ATAGTATTTT	d_2^*	2
AGGCGAATTATTCAGGAATCATAATTACAAATAAACAGCCATAAATTTGA TTAGAATTTGAT	d_3^*	3
CTGAGACTCCTCAAACGTGGCACAGACAATTACATTTAACAATTAGTAAA ATTGAATGAATT	d_4^*	4

Docking Probe Sequences (from 5' to 3')	ID	SEQ ID NO:
CCTGAACAAAGTCAAAAAGTAAGCAGATAGAGGGTTGATATAAAAGATAT AGTATGTTTAAT	d_5^*	5
AGCTTAGATTAAGAATCATAGGTCTGAGAAGCAACACTATCATATGTTTTT AGTATATGTAA	d_6^*	6
AGGAACCCATGTACCAAACTATCGGCCTTGTAGATGGGCGCAAATTTGTTA TTAGATTTTGT	d_7^*	7
TAAATATTCATTGACAGGACGTTGGGAACAGCCCTCATAGTTAAATAAGTG TAAATGAAATA	d_8^*	8
AGGCTGCGCAACTGAAGATTGTATAAGCTCAGGTCTTTACCCTAAAGAAA GTTTAATGTATT	d_9^*	9
TGCGAATAATAATTTAACGTGCTTTCCTGATGTGCTGCAAGGATAGAATAT AGTTAAGTATA	d_{10}^*	10
TCAGGATTAGAGAGGCTGGCTGACCTTCGGTTTATCAGCTTGTATAGTGTT ATAAGATATAT	d_{11}^*	11
ATGGTCATAGCTGTACAGTCAAATCACCATTGCTGAATATAATATTTAGAT AAGTTTAAGAA	d_{12}^*	12
CGGGATCGTCACCCAATCGGCAAAATCCATAAAGTGTAAAGCAAATGTAG TAATATGAAATT	d_{13}^*	13
ATCCTGATTGTTTGACAATAAACAACATTCATTACCGCGCCCTTTAGTTTG AAAAAGTATTT	d_{14}^*	14
AAATAAATCCTCATTATCAAACCCTCAACGTAAAACAGAAATATGTTATGT TAGCAGTGGTT	d_{15}^*	15
AGCTACAATTTTATTCATATGGTTTACCATGGCTTTTGATGAAAAGATTAA AGTATATAGAT	d_{16}^*	16
CTGAGACTCCTCAAACGTGGCACAGACAATTACATTTAACAAAATTTTTGA ATTAGATGAAT	d_{17}^*	17
CCTGAACAAAGTCAAAAAGTAAGCAGATAGAGGGTTGATATATTCAATGG GCGTTTTGGTAA	d_{18}^*	18
AGCTTAGATTAAGAATCATAGGTCTGAGAAGCAACACTATCATTGTAATG ATAAAAGATTTA	d_{19}^*	19
AGGAACCCATGTACCAAACTATCGGCCTTGTAGATGGGCGCAAAGATATG TATTAAGTTAAT	d_{20}^*	20
TAAATATTCATTGACAGGACGTTGGGAACAGCCCTCATAGTTTTTGATTTG TTTTGTAAATT	d_{21}^*	21
AGGCTGCGCAACTGAAGATTGTATAAGCTCAGGTCTTTACCCATAAGTTGT TTTTGATTATT	d_{22}^*	22
TGCGAATAATAATTTAACGTGCTTTCCTGATGTGCTGCAAGGATGATTTAA	d_{23}^*	23

Docking Probe Sequences (from 5' to 3')	ID	SEQ ID NO:
AGTTATAGAAT		
TCAGGATTAGAGAGGCTGGCTGACCTTCGGTTTATCAGCTTGAATTAGTATTTAAGGAAAAA	d_{24}^*	24
ATGGTCATAGCTGTACAGTCAAATCACCATTGCTGAATATAATAAATGATATGTTTTAAGAA	d_{25}^*	25
CGGGATCGTCACCCAATCGGCAAAATCCATAAAGTGTAAAGCAATTTGTAGAAAAAGTAATA	d_{26}^*	26
ATCCTGATTGTTTGACAATAAACAACATTCATTACCGCGCCCAATTTGTAGAAAAAGTAATA	d_{27}^*	27
AAATAAATCCTCATTATCAAACCCTCAACGTAAAACAGAAATATTGTAAAGTAAGAAATTAA	d_{28}^*	28
AGCTACAATTTTATTCATATGGTTTACCATGGCTTTTGATGAATTAAGAAAAGTAAAAAGAT	d_{29}^*	29
AGGCGAATTATTCAGGAATCATAATTACAAATAAACAGCCATTAGAAAATATGATTGATATA	d_{30}^*	30
CTGAGACTCCTCAAACGTGGCACAGACAATTACATTTAACAATAGTTTATTAGAAAATGATT	d_{31}^*	31
AGCTTAGATTAAGAATCATAGGTCTGAGAAGCAACACTATCATAATATTGGATAATGTATAT	d_{32}^*	32
AGGAACCCATGTACCAAACTATCGGCCTTGTAGATGGGCGCAGTATTAGTTATTTGTAATAA	d_{33}^*	33
TAAATATTCATTGACAGGACGTTGGGAACAGCCCTCATAGTTTATAGATGTTAAGATAAAAA	d_{34}^*	34
AGGCTGCGCAACTGAAGATTGTATAAGCTCAGGTCTTTACCCAATAAGTTTTTAGTTTAGTT	d_{35}^*	35
TGCGAATAATAATTTAACGTGCTTTCCTGATGTGCTGCAAGGAAATTGATAATAGAAGAATA	d_{36}^*	36
TCAGGATTAGAGAGGCTGGCTGACCTTCGGTTTATCAGCTTGTTAATAGAGTAAATTGTATA	d_{37}^*	37
ATGGTCATAGCTGTACAGTCAAATCACCATTGCTGAATATAATTTTGTTTATATGTTATTGA	d_{38}^*	38
CGGGATCGTCACCCAATCGGCAAAATCCATAAAGTGTAAAGCTTGTTATATGAATATTGATA	d_{39}^*	39

B. Barcodes (b_1 to b_{39}) have the format:

5' <barcode> <TT> <P1 PAINT docking sequence> 3'

where <barcode> is a 20 base segment which is complementary to the corresponding barcode docking sites, <TT> is a two base spacer sequence to minimize steric hindrance and <P1 PAINT docking sequence> is a 9 base segment for docking the P1 PAINT imager sequence (through which the super-resolution image is obtained). A "PAINT docking sequence" is an example of a "universal sequence," which is a sequence to which a universal imager strand binds.

Table 2

Barcode Sequences (from 5' to 3')	**ID**	**SEQ ID NO:**
AACTTGTTCCGCACCATAGTTTATACATCTA	b_1	40
AAAATACTATTTCTCTAATATTATACATCTA	b_2	41
ATCAAATTCTAATCAAATTTTTATACATCTA	b_3	42
AATTCATTCAATTTTACTAATTATACATCTA	b_4	43
ATTAAACATACTATATCTTTTTATACATCTA	b_5	44
TTACATATACTAAAAACATATTATACATCTA	b_6	45
ACAAAATCTAATAACAAATTTTATACATCTA	b_7	46
TATTTCATTTACACTTATTTTTATACATCTA	b_8	47
AATACATTAAACTTTCTTTATTATACATCTA	b_9	48
TATACTTAACTATATTCTATTTATACATCTA	b_{10}	49
ATATATCTTATAACACTATATTATACATCTA	b_{11}	50
TTCTTAAACTTATCTAAATATTATACATCTA	b_{12}	51
AATTTCATATTACTACATTTTTATACATCTA	b_{13}	52
AAATACTTTTTCAAACTAAATTATACATCTA	b_{14}	53
AACCACTGCTAACATAACATTTATACATCTA	b_{15}	54
ATCTATATACTTTAATCTTTTTATACATCTA	b_{16}	55
ATTCATCTAATTCAAAAATTTTATACATCTA	b_{17}	56
ATTATTCCTAATATCATAAATTATACATCTA	b_{18}	57
TTACCAAAACGCCCATTGAATTATACATCTA	b_{19}	58
TAAATCTTTTATCATTACAATTATACATCTA	b_{20}	59
ATTAACTTAATACATATCTTTTATACATCTA	b_{21}	60
AATTTACAAAACAAATCAAATTATACATCTA	b_{22}	61
AATAATCAAAAACAACTTATTTATACATCTA	b_{23}	62
ATTCTATAACTTTAAATCATTTATACATCTA	b_{24}	63
TTTTTCCTTAAATACTAATTTTATACATCTA	b_{25}	64
TTCTTAAAACATATCATTTATTATACATCTA	b_{26}	65
TATTACTTTTTCTACAAATTTTATACATCTA	b_{27}	66

Barcode Sequences (from 5' to 3')	ID	SEQ ID NO:
TTAATTTCTTACTTTACAATTTATACATCTA	b_{28}	67
ATCTTTTTACTTTTCTTAATTTATACATCTA	b_{29}	68
TATATCAATCATATTTTCTATTATACATCTA	b_{30}	69
AATCATTTTCTAATAAACTATTATACATCTA	b_{31}	70
ATATACATTATCCAATATTATTATACATCTA	b_{32}	71
TTATTACAAATAACTAATACTTATACATCTA	b_{33}	72
TTTTTATCTTAACATCTATATTATACATCTA	b_{34}	73
AACTAAACTAAAAACTTATTTTATACATCTA	b_{35}	74
TATTCTTCTATTATCAATTTTTATACATCTA	b_{36}	75
TATACAATTTACTCTATTAATTATACATCTA	b_{37}	76
TCAATAACATATAAACAAAATTATACATCTA	b_{38}	77
TATCAATATTCATATAACAATTATACATCTA	b_{39}	78

C. Fiduciary markers have the format:

5' <staple sequence> <TT> <P2 PAINT docking sequence> 3'

where <staple sequence> is a 42 base segment which is incorporated into the nanostructure (complementary to the scaffold), <TT> is a two base spacer sequence to minimize steric hindrance and <P2 PAINT docking sequence> is a 9 base segment for docking the P2 PAINT imager sequence (through which the super-resolution image in channel P2 is obtained). There are three fiduciary marker sites on each of the three versions of the six helix bundle DNA nanoastructure (V2.1, V2.2 and V2.3). Each fiduciary marker site consists of three fiduciary markers. The two fiduciary marker sites (and the corresponding six fiduciary marker sequences) at the ends of the six helix bundle are common to all three versions (V2.1, V2.2 and V2.3). The site in between these two sites varies and has distinct sequences.

Table 3

Fiduciary Marker Sequences (from 5' to 3')	Note	SEQ ID NO:
ATTTTGCGGAACAATCCTAATTTACGAGTCCTTATCATTCCAT TGATCTACAT	End marker (commmon)	79
AGAACGGGTATTAACCGTAATCAGTAGCCCCTCAGAGCCGCC TTGATCTACAT	End marker (commmon)	80
CATCGATAGCAGCAACCAAGTACCGCACAGTCCTGAACAAGA TTGATCTACAT	End marker (commmon)	81
AAATGGTCAATAACAAAACACTCATCTTGACTAAAGACTTTT	End marker	82

Fiduciary Marker Sequences (from 5' to 3')	Note	SEQ ID NO:
TTGATCTACAT	(commmon)	
CAAAAGAATACACTCTGTTTAGCTATATGGTTGTACCAAAAA TTGATCTACAT	End marker (commmon)	83
CTGATTGCCCTTCACCATTAAACGGGTATAAAACGAAAGAGG TTGATCTACAT	End marker (commmon)	84
AAGAACGCGAGGCGATTAAAGGTGAATTTGATATTCACAAAC TTGATCTACAT	In-between marker (V2.1)	85
AAATAAATCCTCATTATCAAACCCTCAACGTAAAACAGAAAT TTGATCTACAT	In-between marker (V2.1)	86
GACGGAAATTATTCTTTTAGCGAACCTCGCATTTTCGAGCCAT TGATCTACAT	In-between marker (V2.1)	87
TTGAGTAACAGTGCGAACGAACCACCAGACAAAATCGCGCA GTTGATCTACAT	In-between marker (V2.2)	88
AGGCGAATTATTCAGGAATCATAATTACAAATAAACAGCCAT TTGATCTACAT	In-between marker (V2.2)	89
CCATTAAAAATACCCCGTATAAACAGTTGGTGGCAACATATA TTGATCTACAT	In-between marker (V2.2)	90
CAATATATGTGAGTCGCAAGACAAAGAAAATTAACTGAACAC TTGATCTACAT	In-between marker (V2.3)	91
CCTGAACAAAGTCAAAAAGTAAGCAGATAGAGGGTTGATAT ATTGATCTACAT	In-between marker (V2.3)	92
GATGCAAATCCAATGAATAACCTTGCTTCACGACCAGTAATA TTGATCTACAT	In-between marker (V2.3)	93

D. Biotin handles have the format:

5' <biotin strand docking site> <staple sequence>

where <biotin strand docking site> is a 21 base segment which is complementary to the biotinylated strand and <staple sequence> is a 42 base segment (complementary to the scaffold) which is incorporated into the nanostructure.

Table 4

Biotin Handle Sequences (from 5' to 3')
CGGTTGTACTGTGACCGATTCGAACGCGCCTGTTTTATCAGATGATGGCATAGATTAGAGCCG (SEQ ID NO: 94)
CGGTTGTACTGTGACCGATTCGTTTTGAAGCCTTAACCGATTGAGGGAGAAAGCGCAGTCTCT (SEQ ID NO: 95)
CGGTTGTACTGTGACCGATTCATTTCGGAACCTATTGCGCGAACTGATAGCAAAAGAAGATGA (SEQ ID NO: 96)
CGGTTGTACTGTGACCGATTCTATTAATTAATTTTTTGGGTTATATAACGCTAATATCAGAGA (SEQ ID NO: 97)
CGGTTGTACTGTGACCGATTCGACTCTAGAGGATCGATAAATTAATGCCCCTTTTGATAAGAG (SEQ ID NO: 98)
CGGTTGTACTGTGACCGATTCAGTTTCAGCGGAGTGAGGCCGATTAAAGCGGTGCGGGCCTCT (SEQ ID NO: 99)
CGGTTGTACTGTGACCGATTCAAATGTTTAGACTGACGGAACAACATTATCGTCACCAGTACA (SEQ ID NO: 100)
CGGTTGTACTGTGACCGATTCTGCGAACGAGTAGAAACAAAGTACAACGCAGCATCGGAACGA (SEQ ID NO: 101)

E. Core strands of a nucleic acid nanoarray have the format:

5' <staple sequence> 3'

where <staple sequence> is a 42 base segment (complementary to the scaffold) which is incorporated into the nanostructure.

Table 5

Core strand sequences (from 5' to 3')	**Notes**	**SEQ ID NO:**
GGAACCAGAGCCACAACTCGTATTAAATTGAGTAA CATTATC	Common for V2.1 V2.2 and V2.3	102
TGCCTTTAGCGTCATAATCGGCTGTCTTCATGTAGA AACCAA	Common for V2.1 V2.2 and V2.3	103
ACAAACAATTCGACCACCGGAACCGCCTGACAGAA TCAAGTT	Common for V2.1 V2.2 and V2.3	104
AAAATAATATCCCAAGAAACCACCAGAAAAGTATT AGACTTT	Common for V2.1 V2.2 and V2.3	105
ACCCTCAGAACCGCATTTGAGGATTTAGGGAGCGG AATTATC	Common for V2.1 V2.2 and V2.3	106
ATCATATTCCTGATATCAACAATAGATATCATCGAG	Common for V2.1	107

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
AACAAG	V2.2 and V2.3	
TCAATAGATAATACCACCCTCAGAGCCAGTCACCA ATGAAAC	Common for V2.1 V2.2 and V2.3	108
CAAGCCGTTTTTATGCAAGGCCGGAAACCCACCCTC AGAGCC	Common for V2.1 V2.2 and V2.3	109
GCCACCAGAACCACAGCACTAACAACTAAATTCAT CAATATA	Common for V2.1 V2.2 and V2.3	110
CACCATTACCATTATTTCATCGTAGGAAGTTCAGCT AATGCA	Common for V2.1 V2.2 and V2.3	111
AAAATATCTTTAGGCACCAGAGCCGCCGAAAATCA CCAGTAG	Common for V2.1 V2.2 and V2.3	112
AATAGCAAGCAAATGAATTAGAGCCAGCCCAGCAT TGACAGG	Common for V2.1 V2.2 and V2.3	113
CTGTCCAGACGACGGATTATACTTCTGAGAGGAAG GTTATCT	Common for V2.1 V2.2 and V2.3	114
AGGTTGAGGCAGGTAGTTGAAAGGAATTATAATGG AAGGGTT	Common for V2.1 V2.2 and V2.3	115
CTTGAGCCATTTGGCAGATATAGAAGGCAAGGTAA AGTAATT	Common for V2.1 V2.2 and V2.3	116
AGAACCTACCATATTAAAGTACCGACAATTATCCGG TATTCT	Common for V2.1 V2.2 and V2.3	117
GTTGGCAAATCAACCAGACGATTGGCCTATCACCGT CACCGA	Common for V2.1 V2.2 and V2.3	118
GTAATAAGAGAATACAAAATTATTTGCATCAATATC TGGTCA	Common for V2.1 V2.2 and V2.3	119
AAAGAAATTGCGTATAATTTAGGCAGAGCCGACTT GCGGGAG	Common for V2.1 V2.2 and V2.3	120
TGCTGAACCTCAAATAAAGCCAGAATGGGGAAGGT AAATATT	Common for V2.1 V2.2 and V2.3	121
ACAACGCCAACATGGATTTTCAGGTTTACTAAAGCA TCACCT	Common for V2.1 V2.2 and V2.3	122
GAATTTACCGTTCCAGCAAATGAAAAATACGTCAG ATGAATA	Common for V2.1 V2.2 and V2.3	123
AAGGGCGACATTCAAATCAAGATTAGTTAATCGCC ATATTTA	Common for V2.1 V2.2 and V2.3	124
TACAGTAACAGTACAGGGCTTAATTGAGGCTATTTT GCACCC	Common for V2.1 V2.2 and V2.3	125

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
CGCTGAGAGCCAGCAGTAAGCGTCATACAGCGCCA AAGACAA	Common for V2.1 V2.2 and V2.3	126
CAACGCTCAACAGTCTTTTACATCGGGACTGCAACA GTGCCA	Common for V2.1 V2.2 and V2.3	127
TACAGGAGTGTACTAGTATTAACACCGCGAAACAA TAACGGA	Common for V2.1 V2.2 and V2.3	128
AATCAATAGAAAATCCTGAATCTTACCATACCAGTA TAAAGC	Common for V2.1 V2.2 and V2.3	129
TTCGCCTGATTGCTGTTATACAAATTCTACGCTAAC GAGCGT	Common for V2.1 V2.2 and V2.3	130
GAGGTGAGGCGGTCGGTAATAAGTTTTAGTTTATTT TGTCAC	Common for V2.1 V2.2 and V2.3	131
CTTTCCAGAGCCTAACACCACGGAATAAACGGGGT CAGTGCC	Common for V2.1 V2.2 and V2.3	132
TTAGTATCATATGCTTGAATACCAAGTTCAGAAGAT AAAACA	Common for V2.1 V2.2 and V2.3	133
AAAGAAACGCAAAGATTTGCCAGTTACATAGAAAA AGCCTGT	Common for V2.1 V2.2 and V2.3	134
ATTATTTATCCCAAAATACATACATAAAAATGCCCC CTGCCT	Common for V2.1 V2.2 and V2.3	135
TAAGAATAAACACCTTTCAATTACCTGAGCCCTAAA ACATCG	Common for V2.1 V2.2 and V2.3	136
TAGCAAACGTAGAATCCAAATAAGAAACAATAAGG CGTTAAA	Common for V2.1 V2.2 and V2.3	137
TGAAACAAACATCACCGACCGTGTGATAGATTTTTT GTTTAA	Common for V2.1 V2.2 and V2.3	138
CTATTAGTCTTTAATATTCTGAAACATGATTACGCA GTATGT	Common for V2.1 V2.2 and V2.3	139
CGTCAAAAATGAAAGATTAAGACTCCTTAAAGTATT AAGAGG	Common for V2.1 V2.2 and V2.3	140
AATGGTTTGAAATAAGAAAACAAAATTAATATTTTT GAATGG	Common for V2.1 V2.2 and V2.3	141
AAAAGAACTGGCATATAGCAGCCTTTACTCTGACCT AAATTT	Common for V2.1 V2.2 and V2.3	142
TTTCATTTGAATTAAGTTAATTTCATCTAGAGAGAA TAACAT	Common for V2.1 V2.2 and V2.3	143
GAAAGCGTAAGAATGAGAAGGATTAGGAATAACGG	Common for V2.1	144

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
AATACCC	V2.2 and V2.3	
AAAAACAGGGAAGCGAGGAAACGCAATATTAGCG GGGTTTTG	Common for V2.1 V2.2 and V2.3	145
TTCAAATATATTTTCCTTTTTTAATGGAAACCCTTCT GACCT	Common for V2.1 V2.2 and V2.3	146
CTCAGTACCAGGCGGCCAACAGAGATAGAACAGTA CATAAAT	Common for V2.1 V2.2 and V2.3	147
ACCAGAAGGAAACCGCATTAGACGGGAGCGCGAGA AAACTTT	Common for V2.1 V2.2 and V2.3	148
AAAGGGACATTCTGGATAAGTGCCGTCGAGCCGAA CAAAGTT	Common for V2.1 V2.2 and V2.3	149
AGTATAGCCCGGAAAGATTCACCAGTCACTGTAAA TCGTCGC	Common for V2.1 V2.2 and V2.3	150
AAGCCCTTTTTAAGGAGGGTAATTGAGCTATATGTA AATGCT	Common for V2.1 V2.2 and V2.3	151
TTATTTACATTGGCTAGGTGTATCACCGTAGCTATC TTACCG	Common for V2.1 V2.2 and V2.3	152
GATAACCCACAAGACAATGAAATAGCAATACTCAG GAGGTTT	Common for V2.1 V2.2 and V2.3	153
ACCTCCGGCTTAGGCCCTTAGAATCCTTTCGTCTGA AATGGA	Common for V2.1 V2.2 and V2.3	154
AGTACCGCCACCCTATTTTGACGCTCAAGAAAACAT AGCGAT	Common for V2.1 V2.2 and V2.3	155
ATAAGAGCAAGAAAATTGAGTTAAGCCCAGACTAC CTTTTTA	Common for V2.1 V2.2 and V2.3	156
ATGGAAATACCTACCAGAACCGCCACCCGGCATAG TAAGATA	Common for V2.1 V2.2 and V2.3	157
TAACCCTCGTTTACAAAAGGAATTACGATCAGAACC GCCACC	Common for V2.1 V2.2 and V2.3	158
GTGAATTTATCAAACGCTGAGAAGAGTCCAGGAAA AACGCTC	Common for V2.1 V2.2 and V2.3	159
CTCAGAGCCACCACGCCAGCCATTGCAAAAACAAA CGGCGGA	Common for V2.1 V2.2 and V2.3	160
AGATACATAACGCCCAGACGACGATAAATTCTCCG TGGGATA	Common for V2.1 V2.2 and V2.3	161
TTGACCGTAATGGGAACAACCCGTCGGAAACCAAA ATAGCGA	Common for V2.1 V2.2 and V2.3	162

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
AGAACAATATTACCCCTCATTTTCAGGGCATTCAAC TAATGC	Common for V2.1 V2.2 and V2.3	163
GAGGCTTTTGCAAAATTTAGGAATACCAATAGCAA GCCCAAT	Common for V2.1 V2.2 and V2.3	164
AAATGTGAGCGAGTATAGGTCACGTTGGTGCTGGT AATATCC	Common for V2.1 V2.2 and V2.3	165
ATTCATCAGTTGAGAGAAGTTTTGCCAGCTTTCATC AACATT	Common for V2.1 V2.2 and V2.3	166
TCGTAACCGTGCATCTTCCTGTAGCCAGAGGGGGTA ATAGTA	Common for V2.1 V2.2 and V2.3	167
TGAGTAGAAGAACTCGTAACACTGAGTTTTACAGGT AGAAAG	Common for V2.1 V2.2 and V2.3	168
AATTCGCGTCTGGCCTGCCAGTTTGAGGTAACATCA CTTGCC	Common for V2.1 V2.2 and V2.3	169
AACTACAACGCCTGTCTTTGATTAGTAAGGACGACG ACAGTA	Common for V2.1 V2.2 and V2.3	170
AATAAAACGAACTAGATAGCGTCCAATAACGCCAT CAAAAAT	Common for V2.1 V2.2 and V2.3	171
TCGGCCTCAGGAAGTTTAACCAATAGGACTGCGGA ATCGTCA	Common for V2.1 V2.2 and V2.3	172
GTTGTAGCAATACTTAGCATTCCACAGAGAAAAATC TACGTT	Common for V2.1 V2.2 and V2.3	173
AAATCAGCTCATTTATCGCACTCCAGCCCACGCAAA TTAACC	Common for V2.1 V2.2 and V2.3	174
AGCGTAACGATCTAAAGAGTCTGTCCATAGCTTTCC GGCACC	Common for V2.1 V2.2 and V2.3	175
CTCATTATACCAGTATCCCCCTCAAATGATTAAATT TTTGTT	Common for V2.1 V2.2 and V2.3	176
GCTTCTGGTGCCGGTTGTTAAAATTCGCCTTTAAAC AGTTCA	Common for V2.1 V2.2 and V2.3	177
AGGCCACCGAGTAAAAGTTTTGTCGTCTATTTTAAG AACTGG	Common for V2.1 V2.2 and V2.3	178
GAAAACGAGAATGAAATTACCTTATGCGTTCCAGA CGTTAGT	Common for V2.1 V2.2 and V2.3	179
TAAACGTTAATATTAAACCAGGCAAAGCTTTTATAA TCAGTG	Common for V2.1 V2.2 and V2.3	180
AAATGAATTTTCTGATCCTGAGAAGTGTGCCATTCG	Common for V2.1	181

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
CCATTC	V2.2 and V2.3	
CTTTAATCATTGTGCCATAAATCAAAAAAAATATTTAAATTG	Common for V2.1 V2.2 and V2.3	182
AACGGTACGCCAGATATGGGATTTTGCTTGGTTTAATTTCAA	Common for V2.1 V2.2 and V2.3	183
TGACTATTATAGTCAATTGGGCTTGAGAAAACAACTTTCAAC	Common for V2.1 V2.2 and V2.3	184
GCCCCAAAAACAGGTTGGGAAGGGCGATGGATTTTAGACAGG	Common for V2.1 V2.2 and V2.3	185
CAGAACGAGTAGTAAGAAGCAAAGCGGATGATAATCAGAAAA	Common for V2.1 V2.2 and V2.3	186
TCGCTATTACGCCAATATGTACCCCGGTTTGCATCAAAAAGA	Common for V2.1 V2.2 and V2.3	187
CGGGAGCTAAACAGGAGAATAGAAAGGACTGACGAGAAACAC	Common for V2.1 V2.2 and V2.3	188
TTAAGAGGAAGCCCGAATAAGGCTTGCCACAACTAAAGGAAT	Common for V2.1 V2.2 and V2.3	189
CTAGCATGTCAATCGCTGGCGAAAGGGGCGTTAGAATCAGAG	Common for V2.1 V2.2 and V2.3	190
GCTGCTCATTCAGTGAAAGACTTCAAATCGGTAATCGTAAAA	Common for V2.1 V2.2 and V2.3	191
CGATTAAGTTGGGTAGAGAATCGATGAAATCGCGTTTTAATT	Common for V2.1 V2.2 and V2.3	192
TTGACGAGCACGTATTTTCACGTTGAAAATCAACGTAACAAA	Common for V2.1 V2.2 and V2.3	193
CGAGCTTCAAAGCGTATTCATTACCCAAATCTCCAAAAAAAA	Common for V2.1 V2.2 and V2.3	194
GTCTGGAGCAAACAAACGCCAGGGTTTTGTACTATGGTTGCT	Common for V2.1 V2.2 and V2.3	195
GGCTCCAAAAGGAGCCGCTACAGGGCGCCCCAGTCACGACGT	Common for V2.1 V2.2 and V2.3	196
TGACAAGAACCGGAAACCAGACCGGAAGGTCATTGCCTGAGA	Common for V2.1 V2.2 and V2.3	197
TGTAAAACGACGGCACAAAGGCTATCAGCAAACTCCAACAGG	Common for V2.1 V2.2 and V2.3	198
CCGCGCTTAATGCGCCTTTAATTGTATCATCAAGAGTAATCT	Common for V2.1 V2.2 and V2.3	199

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
TTTTTGAGAGATCTCAGTGCCAAGCTTGAACCACCA CACCCG	Common for V2.1 V2.2 and V2.3	200
CTTTCGAGGTGAATTCAACGCTGCGCGTCATGCCTG CAGGTC	Common for V2.1 V2.2 and V2.3	201
GACCAGGCGCATAGTACCTTTAATTGCTGGAGAGG GTAGCTA	Common for V2.1 V2.2 and V2.3	202
CGAAAAACCGTCTATTCTTAAACAGCTTATGAACGG TGTACA	Common for V2.1 V2.2 and V2.3	203
GTCATTTTTGCGGATTGAAAGAGGACAGGATACCG ATAGTTG	Common for V2.1 V2.2 and V2.3	204
CAACCGTTCTAGCTCCCGGGTACCGAGCCCAACGTC AAAGGG	Common for V2.1 V2.2 and V2.3	205
CGCCGACAATGACAAAAGAACGTGGACTTCGAATT CGTAATC	Common for V2.1 V2.2 and V2.3	206
CGAACTGACCAACTTGGCTTAGAGCTTAATCAATAT GATATT	Common for V2.1 V2.2 and V2.3	207
AAGAGTCCACTATTACAACCATCGCCCAAATCATAA GGGAAC	Common for V2.1 V2.2 and V2.3	208
TGCTGTAGCTCAACAGGCGCAGACGGTCCGCATAA CCGATAT	Common for V2.1 V2.2 and V2.3	209
GAGAAAGGCCGGAGTTCCTGTGTGAAATTTCCAGTT TGGAAC	Common for V2.1 V2.2 and V2.3	210
ATTCGGTCGCTGAGAGGGTTGAGTGTTGTGTTATCC GCTCAC	Common for V2.1 V2.2 and V2.3	211
ACTTAGCCGGAACGATGTTTTAAATATGAGATTCAA AAGGGT	Common for V2.1 V2.2 and V2.3	212
AATTCCACACAACATAATGTGTAGGTAACAACTAA AGTACGG	Common for V2.1 V2.2 and V2.3	213
GAATAGCCCGAGATGCTTGCAGGGAGTTACCTGCTC CATGTT	Common for V2.1 V2.2 and V2.3	214
TGTCTGGAAGTTTCTGTCGAAATCCGCGAAAGGCCG CTTTTG	Common for V2.1 V2.2 and V2.3	215
ATGCAATGCCTGAGTACGAGCCGGAAGCCTTATAA ATCAAAA	Common for V2.1 V2.2 and V2.3	216
CGCCTGATAAATTGATTCCATATAACAGCTCATATA TTTTAA	Common for V2.1 V2.2 and V2.3	217
CTGGGGTGCCTAATAAATTTTTAGAACCTTGATTCC	Common for V2.1	218

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
CAATTC	V2.2 and V2.3	
GATGGTGGTTCCGATCAGCAGCGAAAGAGAGATTTGTATCAT	Common for V2.1 V2.2 and V2.3	219
CAACGCAAGGATAAGAGTGAGCTAACTCCGAAAATCCTGTTT	Common for V2.1 V2.2 and V2.3	220
GGGTAGCAACGGCTTTTGCCCCAGCAGGACATTAATTGCGTT	Common for V2.1 V2.2 and V2.3	221
TATACCAAGCGCGATTTAGTTTGACCATAGAAGCCTTTATTT	Common for V2.1 V2.2 and V2.3	222
GCGCTCACTGCCCGAATACTTTTGCGGGTAGATACATTTCGC	Common for V2.1 V2.2 and V2.3	223
GCGGTCCACGCTGGACAGAGGCTTTGAGTGACCCCCAGCGAT	Common for V2.1 V2.2 and V2.3	224
CATTATGACCCTGTCTTTCCAGTCGGGAGAGAGTTGCAGCAA	Common for V2.1 V2.2 and V2.3	225
TCATGAGGAAGTTTCCGCCTGGCCCTGAAACCTGTCGTGCCA	Common for V2.1 V2.2 and V2.3	226
GCTGCATTAATGAACATAAAGCTAAATCTTTCATTTGGGGCG	Common for V2.1 V2.2 and V2.3	227
CGAGCTGAAAAGGTCGAAGGCACCAACCAAATACGTAATGCC	Common for V2.1 V2.2 and V2.3	228
ATAAAGCCTCAGAGTCGGCCAACGCGCGGAGACGGGCAACAG	Common for V2.1 V2.2 and V2.3	229
TTGAGTAACAGTGCGAACGAACCACCAGACAAAATCGCGCAG	Only for V2.1	230
CCATTAAAAATACCCCGTATAAACAGTTGGTGGCAACATATA	Only for V2.1	231
CAATATATGTGAGTCGCAAGACAAAGAAAATTAACTGAACAC	Only for V2.1	232
CCTGAACAAAGTCAAAAAGTAAGCAGATAGAGGGTTGATATA	Only for V2.1	233
AAGAACGCGAGGCGATTAAAGGTGAATTTGATATTCACAAAC	Only for V2.2	234
GACGGAAATTATTCTTTTAGCGAACCTCGCATTTTCGAGCCA	Only for V2.2	235
CAATATATGTGAGTCGCAAGACAAAGAAAATTAACTGAACAC	Only for V2.2	236

Core strand sequences (from 5' to 3')	Notes	SEQ ID NO:
CCTGAACAAAGTCAAAAAGTAAGCAGATAGAGGGTTGATATA	Only for V2.2	237
TTGAGTAACAGTGCGAACGAACCACCAGACAAAATCGCGCAG	Only for V2.3	238
CCATTAAAAATACCCCGTATAAACAGTTGGTGGCAACATATA	Only for V2.3	239
AAGAACGCGAGGCGATTAAAGGTGAATTTGATATTCACAAAC	Only for V2.3	240
GACGGAAATTATTCTTTTAGCGAACCTCGCATTTTCGAGCCA	Only for V2.3	241

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, *i.e.*, "one or more" of the elements so conjoined.

As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

CLAIMS

What is claimed is:

1. A method for detecting one or more target biomolecule(s), the method comprising:

(a) combining a sample containing a target biomolecule with a barcoded conjugate, wherein the barcoded conjugate comprises a biomolecule binding partner that binds specifically to the target biomolecule and is linked to a nucleic acid barcode that comprises a unique nucleotide sequence and a universal nucleotide sequence;

(b) detaching the barcode from the conjugate;

(c) combining the barcode with a nucleic acid nanoarray that comprises a nucleic acid docking probe located at a prescribed position on the nanoarray, wherein the docking probe comprises a sequence that is complementary to the unique sequence of the barcode, thereby producing a nanoarray containing the docking probe bound to the barcode; and

(d) combining the nanoarray with a labeled universal imager strand that comprises a sequence that is complementary to and capable of binding transiently to the universal sequence of the barcode.

2. The method of claim 1, wherein the biomolecule is a protein.

3. The method of claim 1, wherein the sample is a biological sample.

4. The method of claim 3, wherein the biological sample is blood or saliva.

5. The method of claim 3, wherein the biological sample is a plurality of cells or cell lysate.

6. The method of claim 3, wherein the biological sample is a single cell or a single-cell lysate.

7. The method of claim 1, wherein the biomolecule binding partner is an antibody.

8. The method of claim 1, wherein the labeled universal imager strand comprises a fluorescent label.

9. The method of claim 1, further comprising imaging the labeled universal imager strand.

10. The method of claim 9, further comprising determining, based on binding kinetics of the labeled universal imager strand to the universal sequence of the barcode, whether the barcode is bound to the docking probe.

11. The method of claim 1, wherein the nucleic acid nanoarray comprises a plurality of docking probes, each docking probe located at a prescribed position on the nanoarray and each comprising a sequence that is unique to a protein.

12. The method of claim 1, wherein the nucleic acid nanoarray comprises at least two fiduciary marker sites, each site comprising at least one fiduciary marker.

13. The method of claim 12, wherein each fiduciary marker site comprises at least two fiduciary markers.

14. The method of claim 1, wherein the length of the nucleic acid barcode is 20 to 40 nucleotides.

15. The method of claim 1, wherein the length of the unique nucleotide sequence of the nucleic acid barcode is 10 to 30 nucleotides.

16. The method of claim 15, wherein the length of the unique nucleotide sequence of the nucleic acid barcode is 20 nucleotides.

17. The method of claim 1, wherein the length of the universal sequence of the nucleic acid barcode is 5 to 15 nucleotides.

18. The method of claim 17, wherein the length of the universal sequence of the nucleic acid barcode is 9 nucleotides.

19. The method of claim 1, wherein the length of the unique nucleotide sequence of the nucleic acid barcode is longer than the length of the universal sequence of the nucleic acid barcode.

20. The method of claim 1, wherein the nucleic acid barcode comprises a spacer sequence that separates the unique nucleotide sequence and the universal nucleotide sequence.

21. The method of claim 20, wherein the spacer sequence has a length of 2 to 5 nucleotides.

22. The method of claim 20, wherein the spacer sequence is a polyT, polyA, polyC or polyG sequence.

23. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a sample that contains a plurality of target nucleic acid analytes, wherein the nanoarray comprises at prescribed locations on the array nucleic acid docking probes, each docking probe comprising (i) a sequence that is complementary to a target analyte of the plurality and that uniquely identifies the analyte and (ii) a universal sequence; and

(b) combining the nanoarray with labeled nucleic acid imager strands that comprise a sequence complementary to and capable of binding transiently to the universal sequences of the docking probes, wherein the length of time that an imager strand binds to a docking probe is indicative of whether the docking probe is bound to a target nucleic acid analyte.

24. The method of claim 23, wherein the sample is a biological sample.

25. The method of claim 24, wherein the biological sample is blood or saliva.

26. The method of claim 24, wherein the biological sample is a plurality of cells or cell lysate.

27. The method of claim 24, wherein the biological sample is a single cell or a single-cell lysate.

28. The method of claim 23, wherein the target nucleic acid analytes of the plurality are deoxyribonucleic acid (DNA) analytes.

29. The method of claim 23, wherein the target nucleic acid analytes of the plurality are ribonucleic acid (RNA) analytes.

30. The method of claim 29, wherein the RNA analytes are RNA interference molecules.

31. The method of claim 30, wherein the RNA interference molecules are short-interfering RNAs or micro RNAs.

32. The method of claim 23, wherein the labeled nucleic acid imager strands comprise a fluorescent label.

33. The method of claim 23, further comprising imaging the labeled nucleic acid imager strands.

34. The method of claim 44, further comprising determining, based on binding kinetics of the labeled nucleic acid imager strands to the universal sequences of the docking probes, whether one or more target nucleic acid analyte(s) of the sample is/are bound to the docking probe(s).

35. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a third sequence that is complementary to the first sequence of the analyte;

(b) combining the nanoarray with a nucleic acid probe that comprises a fourth sequence and a universal fifth sequence, wherein the fourth sequence is complementary to the second sequence of the analyte; and

(c) combining the nanoarray with a detectable nucleic acid imager strand that comprises a sixth sequence that is complementary to and capable of binding transiently to the universal fifth sequence of the probe.

36. The method of claim 35, wherein the docking probe is shorter than the target nucleic acid analyte.

37. The method of claim 35, wherein the nucleic acid probe is shorter than the target nucleic acid analyte.

38. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a first nucleic acid strand and a second nucleic acid strand, wherein:

(i) the first strand comprises a third sequence, a fourth sequence and a universal fifth sequence, wherein the third sequence is complementary to the first sequence and the fourth sequence is complementary to the second sequence, and

(ii) the second strand comprises a sixth sequence and a universal seventh sequence, wherein the sixth sequence is complementary to and bound to the fourth sequence; and

(b) combining the nanoarray with a labeled nucleic acid imager strand that comprises an eighth sequence and a ninth sequence, wherein the eighth sequence is complementary to and capable of binding transiently to the universal fifth sequence and the ninth sequence is complementary to and capable of binding transiently to the universal seventh sequence.

39. The method of claim 38, wherein the second nucleic acid strand is shorter than the first nucleic acid strand.

40. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a third sequence, a fourth sequence, a universal fifth sequence, and a sixth sequence, wherein the third sequence is complementary to the first sequence, the fourth sequence is complementary to the second sequence, and the sixth sequence is complementary to and hybridized to the fourth sequence; and

(b) combining the nanoarray with a labeled nucleic acid imager strand that comprises a seventh sequence that is complementary to and capable of binding transiently to the universal fifth sequence.

41. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence that uniquely identifies the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a second sequence flanked by a universal third sequence

and a universal fourth sequence, wherein the second sequence is complementary to the first sequence; and

(b) combining the nanoarray with a labeled nucleic acid imager strand that comprises a fifth sequence and a sixth sequence, wherein the fifth sequence is complementary to and capable of binding transiently to the universal third sequence, and the sixth sequence is complementary to and capable of binding transiently to the universal fourth sequence.

42. The method of claim 41, wherein regions of the second sequence bind to each other to form a hairpin loop.

43. A method for detecting one or more target nucleic acid analyte(s), the method comprising:

(a) combining a nucleic acid nanoarray with a target nucleic acid analyte, wherein

the analyte comprises a first sequence and a second sequence that uniquely identify the analyte, and

the nanoarray comprises at a prescribed location on the array a nucleic acid docking probe that comprises a first nucleic acid strand and a second nucleic acid strand, wherein:

(i) the first strand comprises a third sequence, a fourth sequence, a universal fifth sequence, and a universal sixth sequence, wherein the third sequence is complementary to the first sequence and the fourth sequence is complementary to the second sequence, and

(ii) the second strand comprises a seventh sequence and an eighth sequence, wherein the seventh sequence is complementary to and hybridized to the fourth sequence and the eighth sequence is complementary to and hybridized to the universal fifth sequence; and

(b) combining the nanoarray with a first labeled nucleic acid imager strand that comprises a ninth sequence and a tenth sequence, wherein the ninth sequence is complementary to and capable of binding transiently to the universal fifth sequence, and the tenth sequence is complementary to and capable of binding transiently to the universal sixth sequence.

44. The method of claim 43, wherein the second nucleic acid strand is shorter than the first nucleic acid strand.

45. The method of claim 43, wherein the first nucleic acid strand further comprises a universal eleventh sequence.

46. The method of claim 45, further comprising combining the nanoarray with a second labeled imager strand that comprises a twelfth sequence and a thirteenth sequence, wherein the twelfth sequence is complementary to the universal sixth sequence and the thirteenth sequence is complementary to the universal eleventh sequence.

47. The method of claim 23, wherein the target nucleic acid analyte is a component of a biological sample.

48. The method of claim 47, wherein the biological sample is a plurality of cells or cell lysate.

49. The method of claim 47, wherein the biological sample is a single cell or a single-cell lysate.

50. The method of claim 23, wherein the nucleic acid analyte is a deoxyribonucleic acid (DNA) analyte.

51. The method of claim 23, wherein the nucleic acid analyte is a ribonucleic acid (RNA) analyte.

52. The method of claim 51, wherein the RNA analyte is an RNA interference molecule.

53. The method of claim 52, wherein the RNA interference molecule is a short-interfering RNA or a micro RNA.

54. The method of claim 23, wherein the labeled nucleic acid imager strand comprises a fluorescent label.

55. The method of claim 23, further comprising imaging the labeled nucleic acid imager strand.

56. The method of claim 55, further comprising determining, based on binding kinetics of the labeled nucleic acid imager strand to the universal sequence of the docking probe, whether a target nucleic acid analyte is bound to the docking probe.

57. The method of claim 23, wherein the nucleic acid nanoarray comprises a plurality of docking probes, each probe located at a prescribed position on the nanoarray and each comprising a sequence that is unique to a target nucleic acid analyte.

58. The method of any one of claims 23-57, wherein the nucleic acid nanoarray is a two-dimensional or three-dimensional nucleic acid nanostructure.

59. The method of claim 23, wherein the nucleic acid nanoarray is a single-stranded nucleic acid tile array.

60. A docking probe comprising a sequence of any one of SEQ ID NO: 1-39.

61. A docking probe comprising a barcode binding site of a sequence of any one of SEQ ID NO: 1-39.

62. A nanoarray comprising a docking probe of claim 61.

63. A nucleic acid barcode comprising a sequence of any one of SEQ ID NO: 40-78.

64. A biomolecule binding partner linked to a nucleic acid barcode of claim 63.

65. A nanoarray comprising docking probes bounds to nucleic acid barcodes of claim 63.

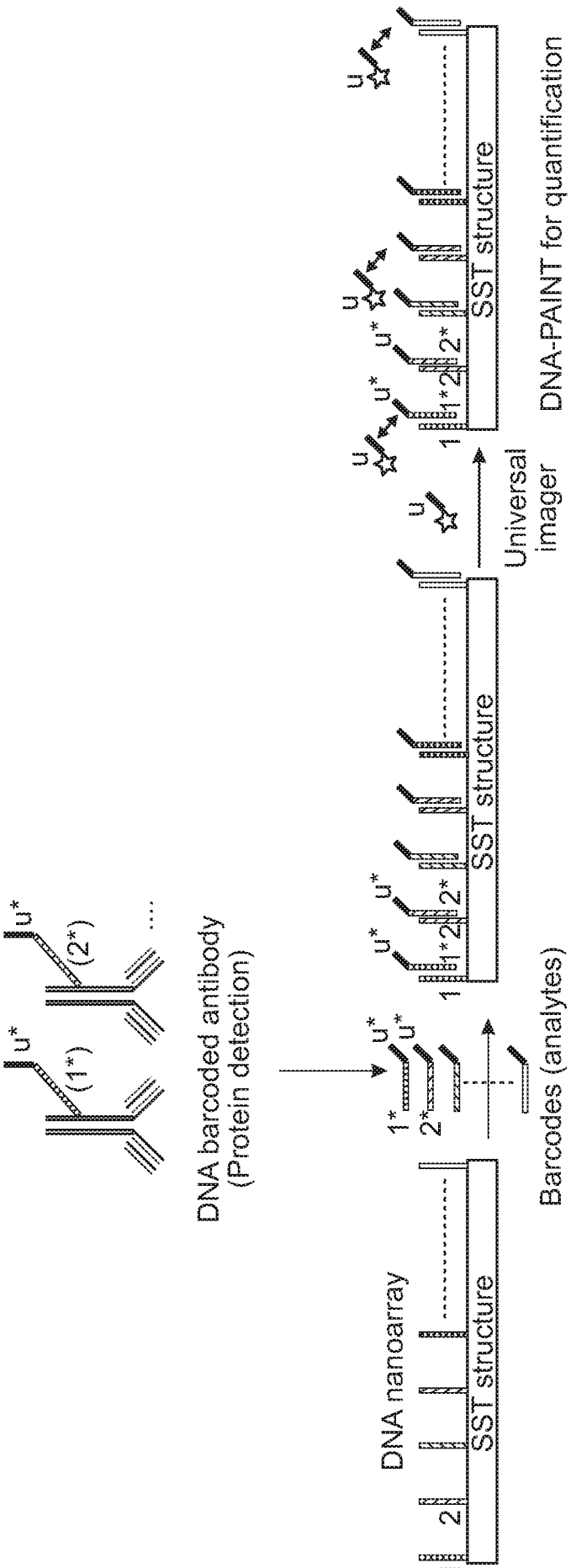
66. A fiduciary marker comprising a sequence of any one of SEQ ID NO: 79-93.

67. A nanoarray comprising fiduciary markers of claim 66.

5

68. A kit comprising any two or more of the following components selected from the group consisting of barcoded conjugates, biomolecule binding partners, linkers, nucleic acid barcodes, nucleic acid nanoarrays, docking probes and universal imager strands.

FIG. 1A


DNA barcoded antibody
(Protein detection)
DNA nanoarray
SST structure
Barcodes (analytes)
Universal
imager
DNA-PAINT for quantification

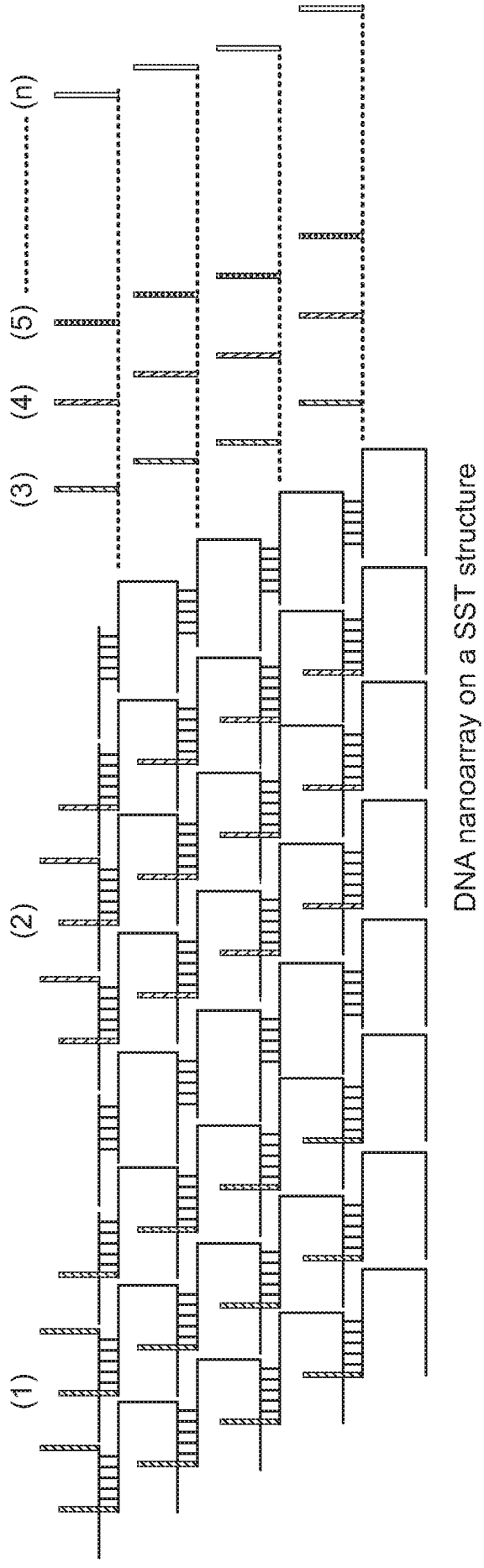


FIG. 1B

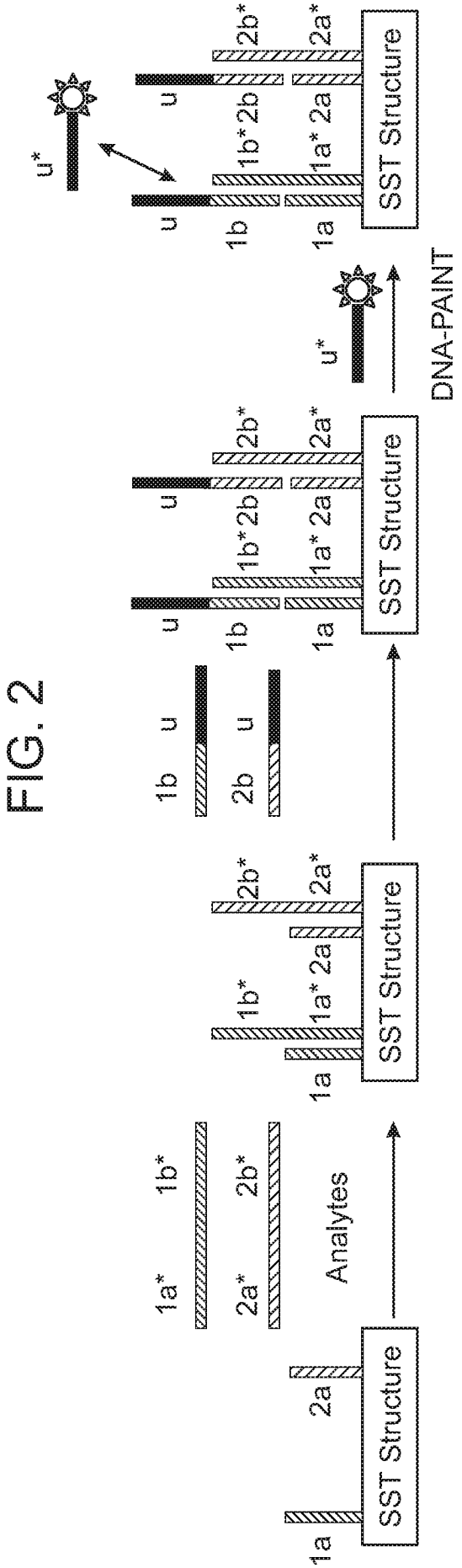


FIG. 2

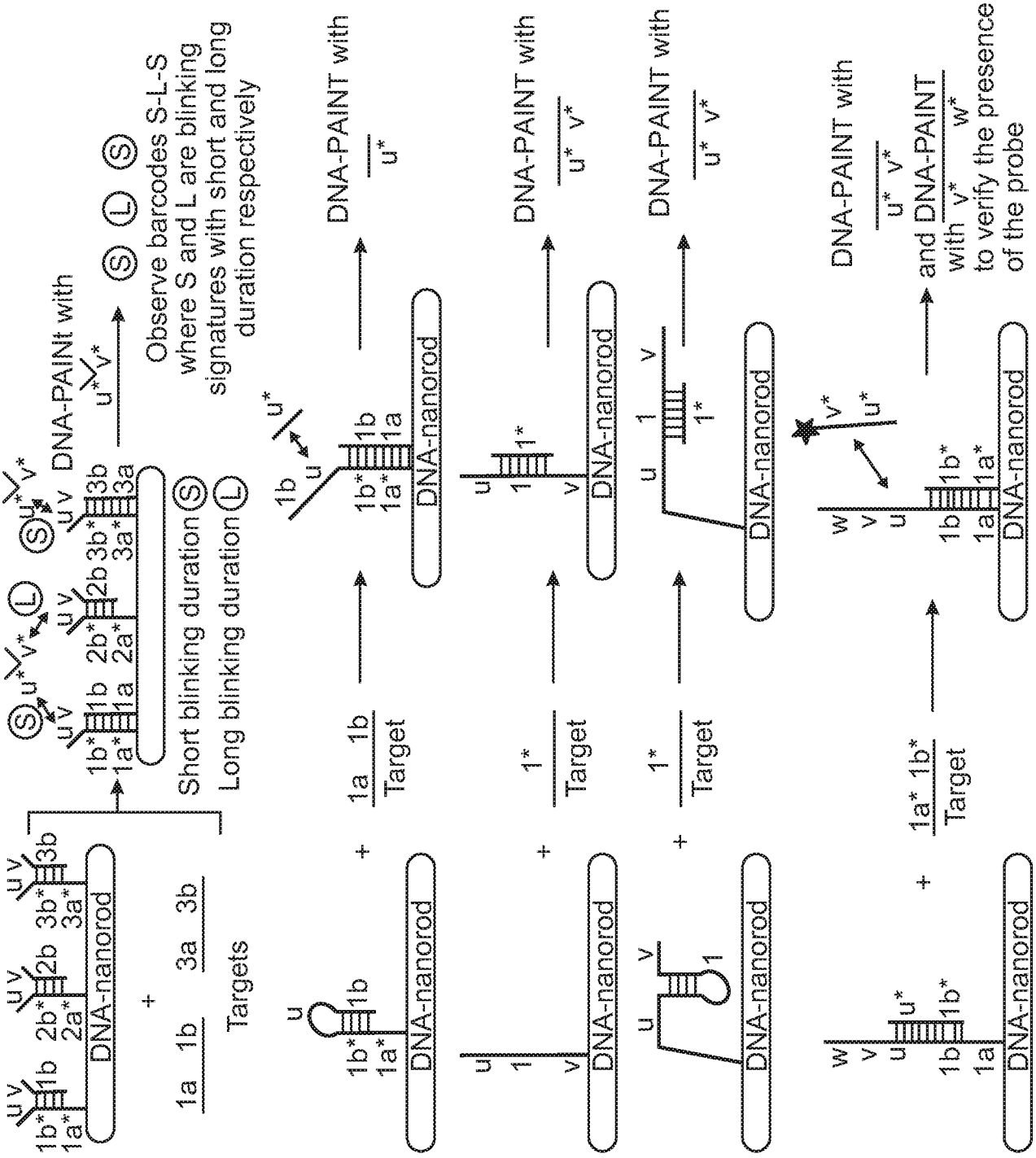


FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

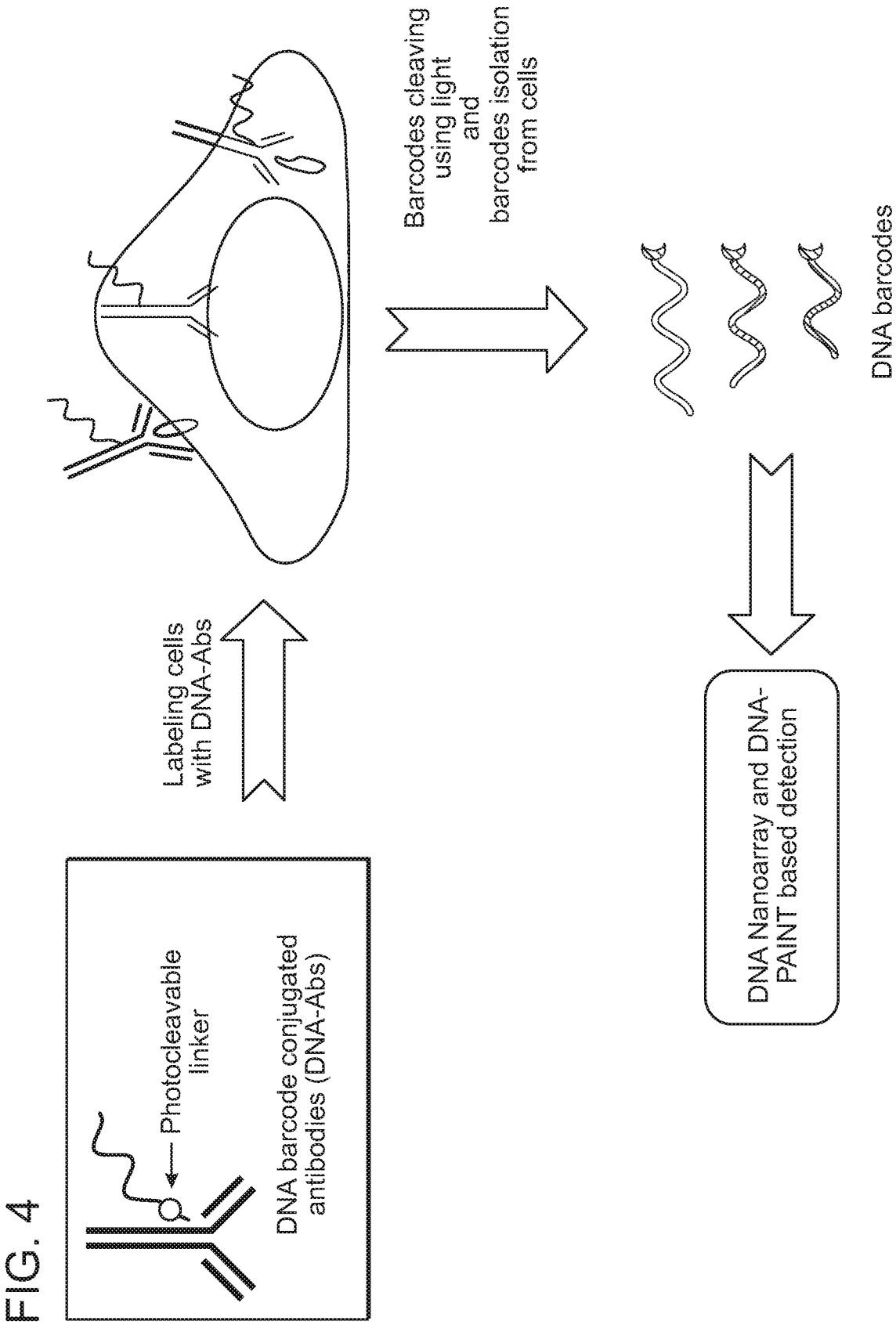

Photocleavable linker
DNA barcode conjugated antibodies (DNA-Abs)
Labeling cells with DNA-Abs
Barcodes cleaving using light and barcodes isolation from cells
DNA barcodes
DNA Nanoarray and DNA-PAINT based detection

FIG. 4

FIG. 5

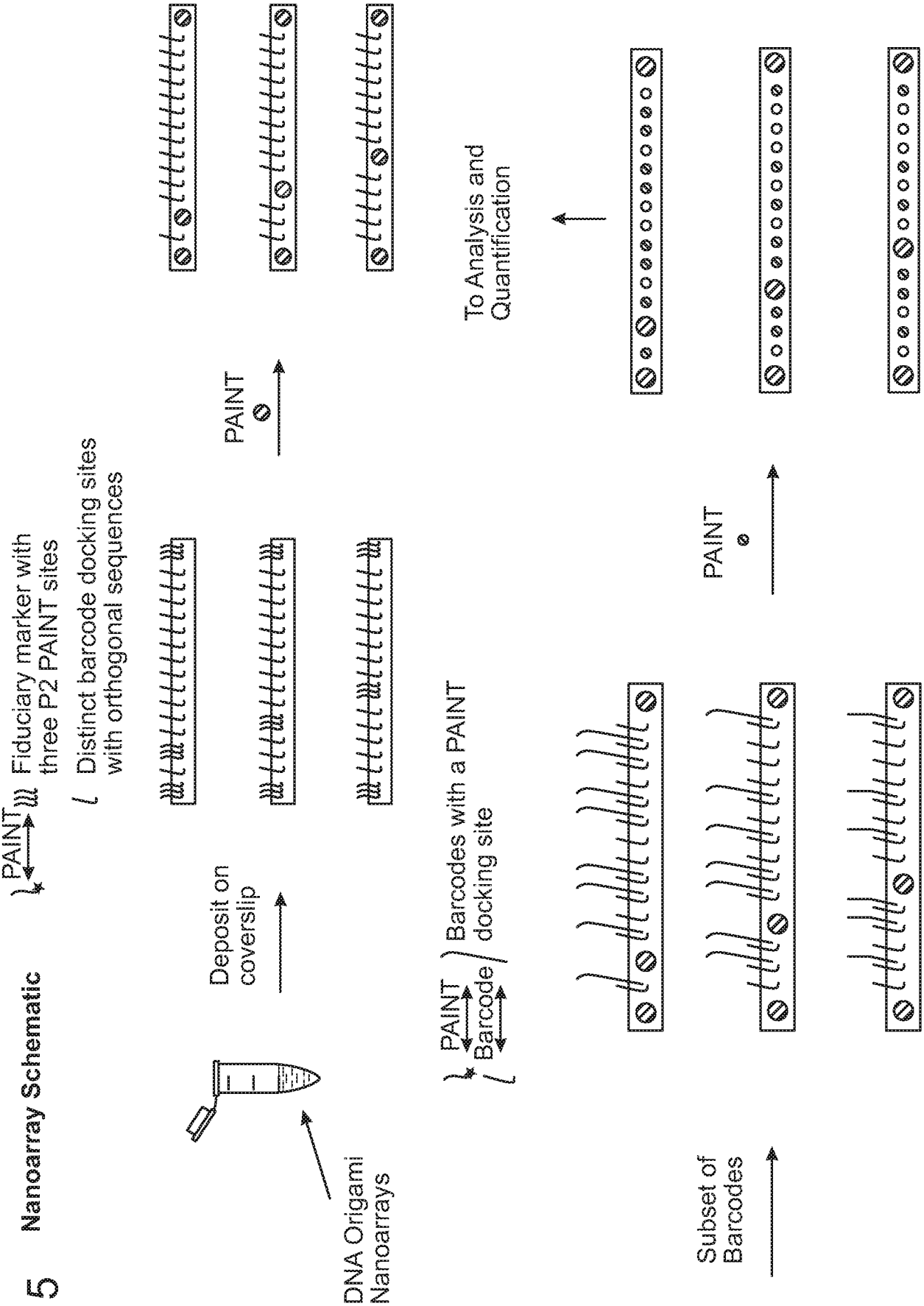
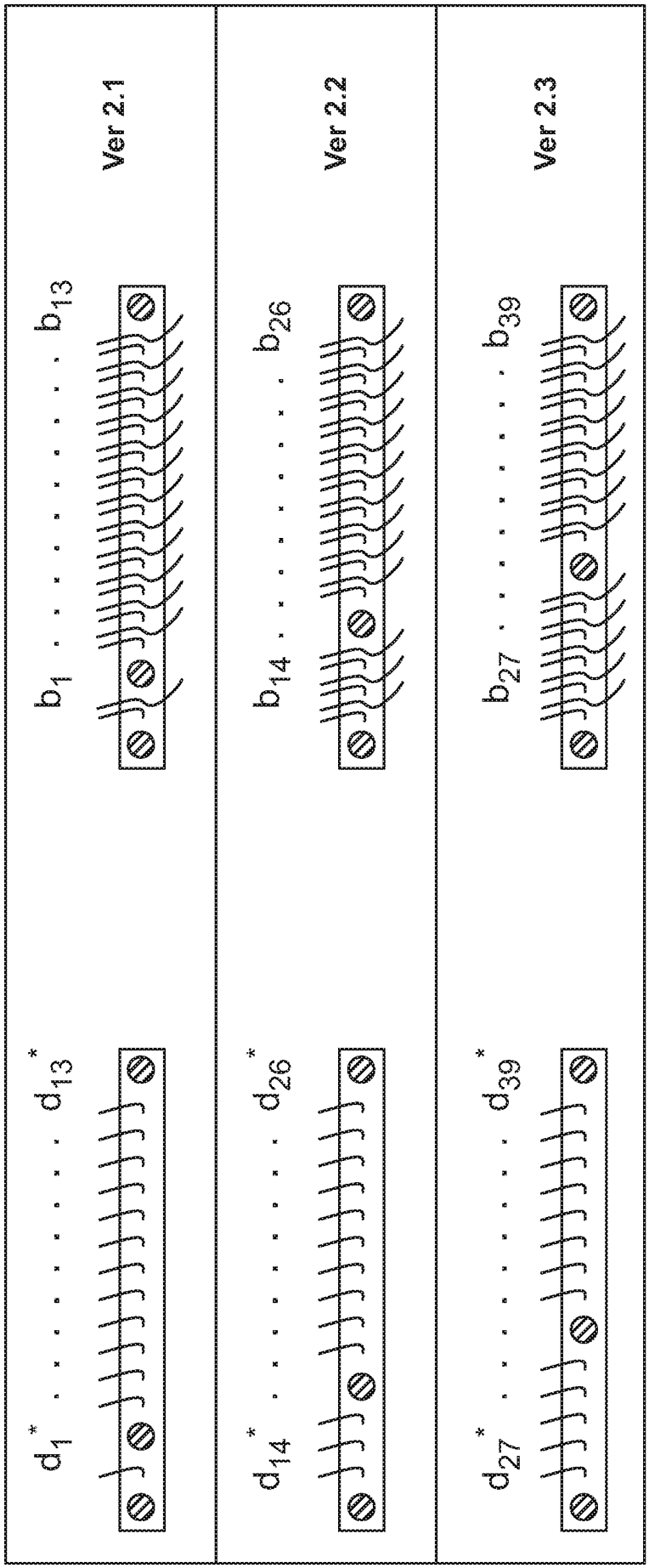

Nanoarray Schematic
PAINT
Fiduciary marker with three P2 PAINT sites
Distinct barcode docking sites with orthogonal sequences
Deposit on coverslip
DNA Origami Nanoarrays
PAINT
Barcode
Barcodes with a PAINT docking site
PAINT
To Analysis and Quantification
PAINT
Subset of Barcodes

FIG. 6


Barcodes
Docking Probes
Ver 2.1
Ver 2.2
Ver 2.3
b_1 . . . . . b_{13}
b_{14} . . . . . b_{26}
b_{27} . . . . . b_{39}
d_1^* . . . . . d_{13}^*
d_{14}^* . . . . . d_{26}^*
d_{27}^* . . . . . d_{39}^*

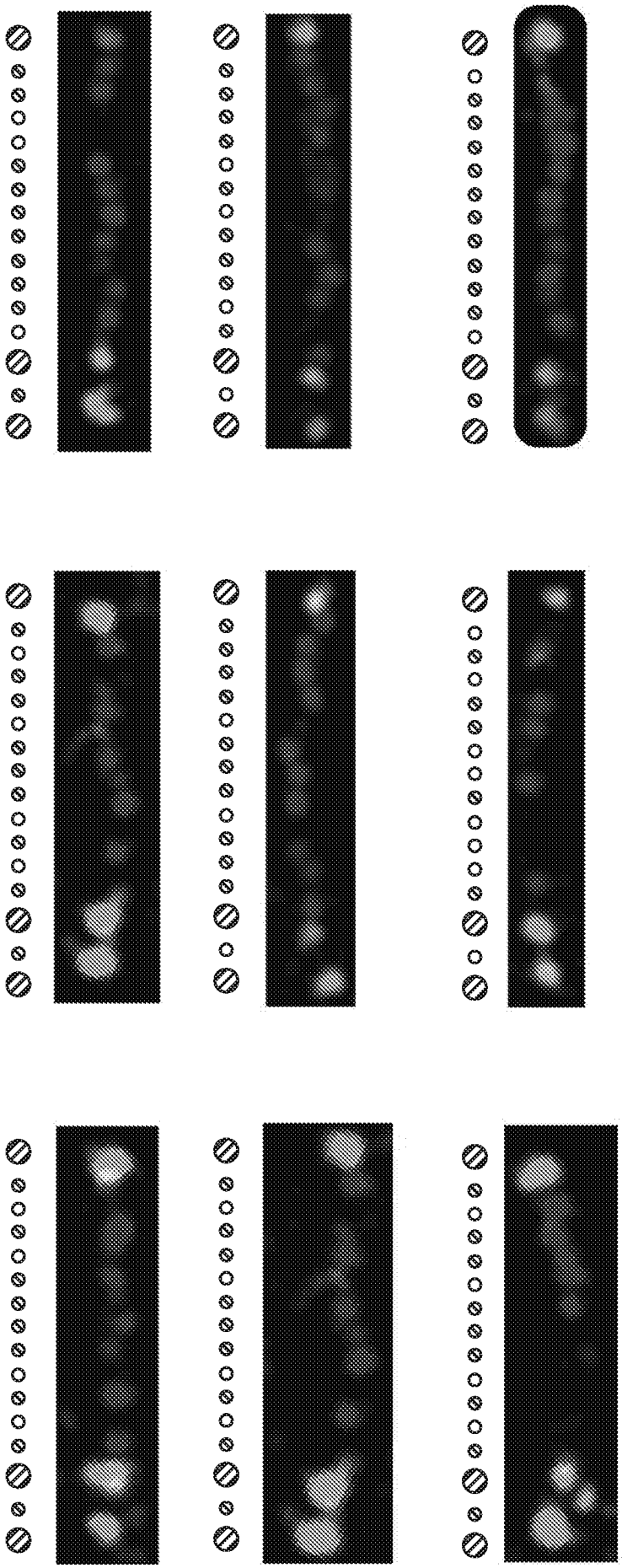
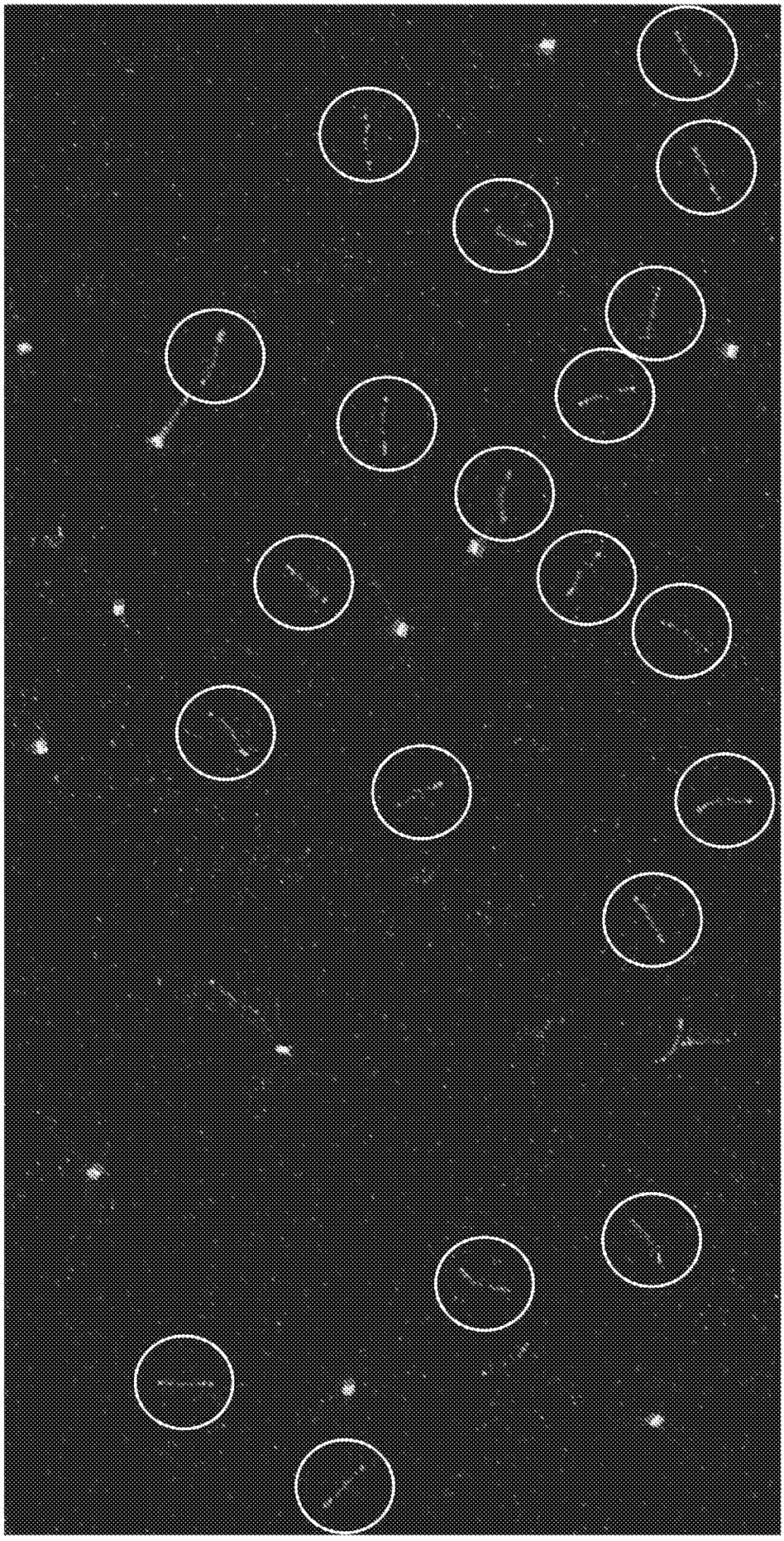


FIG. 7

FIG. 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/65270

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/533, G01N 33/58, C12N 15/115, C12Q 1/68 (2017.01)
CPC - G01N 33/533, G01N 33/58, C12N 15/115, C12Q 1/68, C12Q 1/6811, C12Q 1/682, C12Q 1/6837

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/0081134 A1 (Mirkin et al.) 01 April 2010 (01.04.2010) abstract, para [0125], [0357], [0360], [0366], Fig. 6, 9,	1-22
Y	WO 2015/089506 A2 (President And Fellows Of Harvard College) 18 June 2015 (18.06.2015) pg 6, ln 15-28, pg 7, ln 11-23, FIG. 2A	1-22
Y	US 6,942,968 B1 (Dickinson et al.) 13 September 2005 (13.09.2005) abstract, col 23, ln 25-52	12, 13
T	"DNA-barcoded labeling probes for highly multiplexed Exchange-PAINT imaging" (Agasti et al.) Chem. Sci., 2017, 8, 3080?3091, entire doc.	1-22

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 April 2017	28 APR 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/65270

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
- Please see extra sheet for Observations where unity of invention is lacking -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/65270

Continuation of: Box NO III. Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-59, drawn to a method of detecting one or more target molecule(s) comprising 1) a target analyte(s), 2) a nanoarray comprising specifically addressable docking probe(s) and 3) a labeled universal imaging strand.

The docking probe comprises the following configurations:
[claim 1] the docking probe comprises a sequence that is complementary to the target nucleic acid (barcode) (see instant Specification, Fig. 1);
[claim 23] the docking probe comprises a sequence that is complementary to the target nucleic acid and a universal sequence;
[claim 35] the docking probe comprises a third sequence (1a), a fourth sequence (1b), a universal fifth sequence (U) (see instant Specification, Fig. 2, 1a, 1b, U as indicated in the figure);
[claim 38] the docking probe comprises a first nucleic acid strand and a second nucleic acid strand, wherein: (i) the first strand comprises a third sequence (1a*), a fourth sequence (1b*) and a universal fifth sequence (U), (ii) the second strand comprises a sixth sequence (1b) and a universal seventh sequence (U) (see instant Specification, Fig. 3A, 1a*, 1b*, 1b, U as indicated in the figure);
[claim 40] the docking probe comprises a third sequence (1a*), a fourth sequence (1b*), a universal fifth sequence (U), and a sixth sequence (1b) (see instant Specification, Fig. 3B, 1a*, 1b*, 1b, U as indicated in the figure);
[claim 41] docking probe that comprises a second sequence (I) flanked by a universal third sequence (U) and a universal fourth sequence (U) (see instant Specification, Fig. 3C, 1, U as indicated in the figure);
[claim 43] docking probe comprises a first nucleic acid strand and a second nucleic acid strand, wherein: (i) the first strand comprises a third sequence (1a), a fourth sequence (1b), a universal fifth sequence (U), and a universal sixth sequence (V), and (ii) the second strand comprises a seventh sequence (1b*) and an eighth sequence (U*) (see instant Specification, Fig. 3E, 1a, 1b, U, V, 1b*, U* as indicated in the figure);

The method of detecting one or more target molecules will be searched to the extent that the docking probe encompasses the first named configuration (claim 1). It is believed that claims 1-22 read on this first named invention and thus these claims will be searched without fee to the extent that they comprise first named docking probe configuration. Additional docking probe configurations will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected docking pprobe configurations. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: a docking probe comprising the second cconfiguration (claims 23-34, 47-57, 58 (in part), 59).

Group II+: Claims 60-62, drawn to a composition comprising a docking probe sequence. Group II+ will be searched upon payment of additional fee(s). The composition may be searched, for example, to the extent that the docking probe comprises SEQ ID NO: 1 for an additional fee and election as such. It is believed that claims 60-62 read on this exemplary invention. Additional docking probes will be searched upon the payment of additional fees. Applicants must indicate, if applicable, which claims read on this named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the '+' group(s) will result in only the first named invention to be searched/examined. An exemplary election would be the docking probe comprises SEQ ID NO: 2 (claims 60-62).

Group III+: Claims 63-65, drawn to a nucleic acid barcode. Group III+ will be searched upon payment of additional fee(s). The composition may be searched, for example, to the extent that the nucleic acid barcode comprises SEQ ID NO: 40, for an additional fee and election as such. It is believed that claims 63-65 read on this exemplary invention. Additional nucleic acid barcodes will be searched upon the payment of additional fees. Applicants must indicate, if applicable, which claims read on this named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the '+' group(s) will result in only the first named invention to be searched/examined. An exemplary election would be the barcode comprises SEQ ID NO: 78 (claims 63-65).

Group IV+: Claims 66-67, drawn to a fiduciary marker. Group IV+ will be searched upon payment of additional fee(s). The composition may be searched, for example, to the extent that the fiduciary marker comprises SEQ ID NO: 79, for an additional fee and election as such. It is believed that claims 66-67 read on this exemplary invention. Additional fiduciary markers will be searched upon the payment of additional fees. Applicants must indicate, if applicable, which claims read on this named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the '+' group(s) will result in only the first named invention to be searched/examined. An exemplary election would be the fiduciary marker comprises SEQ ID NO: 85 (claims 66-67).

Group V: Claim 68, drawn to a kit comprising two or more components.

The inventions listed as Groups I+, II+, III+, IV+ and V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I+ has the special technical feature of a method detecting a target biomolecule(s), not required by Groups II+, III+, IV+ or V.

Group II+ has the special has the special technical feature of a docking probe composition, not required by Groups I+, III+, IV+ or V.

Group III+ has the special has the special technical feature of a nucleic acid barcode composition, not required by Groups I+, II+, IV+ or V.

- Please see extra sheet for continuation -

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/65270

Continuation of:
Box NO III. Observations where unity of invention is lacking

Group IV+ has the special technical feature of a fiduciary marker composition, not required by Groups I+, II+, III+ or V.

Group V has the special technical feature of a kit composition, not required by Groups I+, II+, III+, or IV+.

Among the inventions listed as Groups I+ are the specific docking probe configurations, recited therein. Each of the inventions of Group I+ requires a unique docking probe configuration, not required by the other inventions,

Among the inventions listed as Groups II+, III+ and IV+ are the specific (docking probe, nucleic acid barcode or fiduciary marker) sequences, recited therein. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among sequences.

Common Technical Feature:

1. Group I+ inventions share the common technical features of target analytes comprising unique sequences (e.g., barcode, optionally conjugated to a biomolecule target binding moiety), nanoarrays comprising addressable docking probes [in part complementary to a target analyte unique sequence], and universal labeled imaging strands.

2. Groups I+, II+ and V share the common technical feature of docking probes.

3. Groups I+, III+ and V share the common technical feature of barcodes.

4. Groups I+, V share the common technical feature of nanoarrays and universal imager strands.

However, said common technical features do not represent a contribution over the prior art, and is obvious over WO 2015/138653 A1 to President and Fellows of Harvard College (hereinafter "Harvard") [published 17 September 2015], in view of US 2013/0261019 A1 to LIN et al. (hereinafter "Lin")

As to common technical features #1 (in part), #2, and #4, Harvard teaches addressable docking probes on nanoarrays and universal imager strands (claim 1; "1. A method comprising
(1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
(2) optionally removing unbound target-specific binding partners,
(3) contacting the sample with labeled imager strands having a nucleotide sequence that is complementary to a docking strand,
(4) optionally removing unbound labeled imager strands,
(5) imaging the sample to detect location and number of bound labeled imager strands"; pg 22 ln 10-" a spatial area or volume (e.g., a grid on an array)"). Harvard does not specifically teach that the imager strands are universal. However, it would have been obvious to have included a bipartite docking strand/probe that had a sequence complementary to a specific target analyte and also had a sequence complementary to a universal labeled imager strand.

As to common technical features #1 (in part) and #3, Lin teaches a barcode (optionally) conjugated to a target binding moiety (para [0019]; "In some embodiments, the barcode probe further comprises a target binding moiety. In some embodiments, the target binding moiety is a single-stranded nucleic acid complementary to a nucleic acid target. In some embodiments, the target binding moiety is an antibody. In some embodiments, the target binding moiety is a protein or peptide").

As the common technical features were known in the art at the time of the invention, they cannot be considered common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+, II+, III+, IV+ and V lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.