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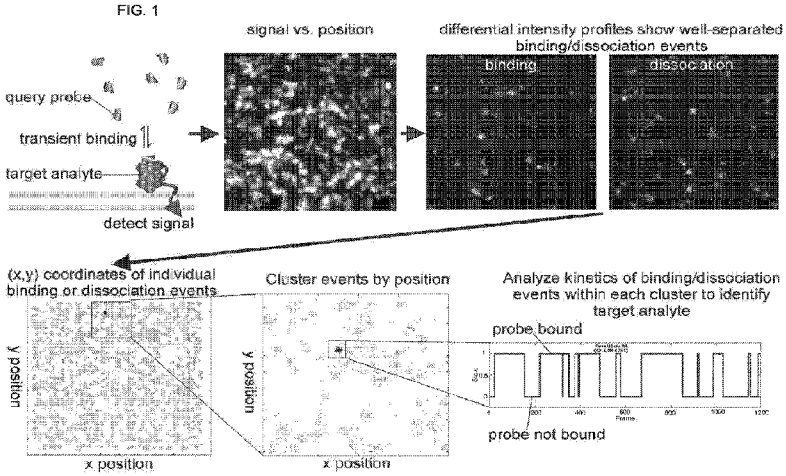
(54) Title: ANALYTE DETECTION

FIG. 1



(57) Abstract: Provided herein is technology relating to the detection of analytes and particularly, but not exclusively, to methods, systems, compositions, and kits for detecting analytes such as nucleic acids, proteins, small molecules, and other molecules using a technology based on the transient binding of detection probes.

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WO 2018/165309 A1

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ANALYTE DETECTION

This application claims priority to United States provisional patent application serial number 62/468,578, filed March 8, 2017, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under grant GM062357 awarded by the U.S. National Institutes of Health, and under grant W911NF-12-1-0420 awarded by the U.S. Navy, Office of Naval Research. The government has certain rights in the invention.

FIELD

Provided herein is technology relating to the detection of analytes and particularly, but not exclusively, to methods, systems, compositions, and kits for detecting analytes such as nucleic acids, proteins, small molecules, and other molecules using a technology based on the transient binding of detection probes.

BACKGROUND

Detecting and quantifying low-concentration analytes in complex mixtures has numerous applications in biological research and clinical diagnostics. Many important biological analytes are biomarkers of disease and other biological states. For example, the detection of a small fraction of circulating nucleic acids bearing oncogenic mutations in blood, urine, saliva, and other body fluids has been correlated to the incidence of certain types of cancer. In addition, protein analytes such as prostate-specific antigen (PSA) and interleukins also have current or potential clinical and research significance. The levels of small molecule metabolites provide information about health and drug processing in a biological system. Antibodies, aptamers, nucleic acids, and other affinity reagents are widely used for these assays in molecular diagnostics and research.

A challenge in the detection of low-abundance molecular analytes is the trade-off between sensitivity and specificity. That is, as the sensitivity of an assay is increased, the potential for false positives is increased, which reduces specificity. For instance, false positives in common ELISA (enzyme-linked immunosorbent assay) methods result from nonspecific binding of primary or secondary antibodies to the assay surface, thus producing a signal when the intended target analyte is not present. In such cases,

improving detector sensitivity or signal amplification efficiency increases both true positive and false positive detection events, resulting in lower specificity. While strategies have been developed to reduce false positive events, including incubation of surfaces with blocking solutions, stringent washing protocols, and use of split probes that must jointly bind a target analyte to generate a signal, current approaches nonetheless suffer from false positives because the false positive signal cannot be completely eliminated in practice. As a result, detecting target analytes at the single-molecule level with high specificity remains elusive for many classes of biomarkers.

SUMMARY

Previously, a strategy for detecting nucleic acids using kinetic modeling and analysis of intensity vs. time data for transient binding of a query probe has been described (see Johnson-Buck et al. (2015) Nat. Biotechnol. 33: 730-32; U.S. Pat. App. Pub. No. 20160046988; Int'l Pat. App. No. PCT/US2017/016977, each of which is incorporated herein by reference in its entirety). In contrast, the present technology provides in some embodiments a new method for detecting target analytes bound stably to a surface by determining the spatial and temporal coordinates of each binding and dissociation event of a low-affinity query probe, then clustering binding events by position, and subjecting each cluster event to a kinetic analysis (e.g., determining the mean, median, maximum, minimum, standard deviation, or other metrics of dwell times between events or number of events per target molecule per unit time). This method permits discrimination between nonspecific probe binding and binding of the probe to the target analyte because the accumulation of position-vs-time statistics over multiple binding events of a query probe to a single target analyte yields greater confidence in the identity of the analyte than any of the following: a single binding event, a cumulative count of binding events across an observation area, or a cumulative count of probe signal across an observation area. Moreover, the method uses spatial position information and clustering based on intensity fluctuations (rather than overall signal intensity) to surpass the resolution limits of the detection apparatus to provide "super-resolution" measurements. Accordingly, in some embodiments the technology provides a higher dynamic range than previously published methods (Figure 2) and improved discrimination of specific binding to a target analyte from nonspecific binding of detection probes to the imaging surface or other surface-bound analytes (Figure 3). In particular, a tightly clustered spatial localization of signals from target binding and distinct kinetic behavior of probe binding to target are used to provide improved discrimination of target signal from nonspecific

(e.g., false positive) signal. In some embodiments, when applied to query probe binding events over a large detector area, the method finds use in digitally counting one or more target analytes at the level of single molecules.

The technology provides advantages over prior technologies including, but not limited to, improved discrimination of analyte relative to background binding, improved discrimination between closely related analytes, and analysis of very dilute or low-volume specimens (e.g., with improved sensitivity and/or specificity relative to prior technologies for analysis of the same analytes).

Accordingly, in some embodiments the technology provides a method of characterizing an analyte in a sample.

The technology is not limited in the analyte that is detected. For example, embodiments provide for detection of an analyte that is a nucleic acid, a polypeptide, a carbohydrate, a polysaccharide, a fatty acid, a phospholipid, a glycolipid, a sphingolipid, a small molecule, a metabolite, a cofactor, etc.

In some embodiments, the query and/or capture probe is a nucleic acid, a polypeptide (e.g., an antibody, antibody fragment, linear antibody, single-chain antibody, or other antigen-binding antibody derivative; an enzyme; a binding protein that recognizes the analyte with specificity); in some embodiments in which the analyte comprises a carbohydrate or polysaccharide, the query probe comprises a carbohydrate-binding protein such as a lectin or a carbohydrate-binding antibody. In some embodiments, the presence of a specific glycosidic linkage or set of glycosidic linkages between carbohydrate monomers yields a distinguishable pattern of query probe binding. In some embodiments, the capture probe is a rabbit monoclonal antibody; and in some embodiments, the query probe is a mouse monoclonal antibody.

For example, in some embodiments the method comprises recording a time-dependent signal of query probe events for analytes immobilized to a surface as a function of (x, y) position on the surface; clustering events into local clusters by (x, y) position; and calculating a kinetic parameter for each event cluster to characterize the analyte. In some embodiments, the surface is a solid support. In some embodiments, the surface is diffusible. In some embodiments, recording a time-dependent signal of query probe events comprises measuring the signal for an analyte with single-molecule sensitivity. In some embodiments, methods further comprise calculating a differential intensity map comprising the time-dependent signal intensity changes at the surface as a function of (x, y) position. In some embodiments, (x, y) position is determined with sub-pixel accuracy.

In some embodiments, clustered events represent binding events for a single analyte molecule. In some embodiments, characterizing the analyte comprises indicating the presence, absence, concentration, or number of the analyte in the sample. In some embodiments, the analyte comprises a polypeptide. In some embodiments, the method indicates the presence or absence of a post-translational modification on the polypeptide. In some embodiments, the post-translational modification mediates a transient association of the query probe with the polypeptide. In some embodiments, a chemical affinity tag mediates a transient association between the post-translational modification and the query probe. In some embodiments, the chemical affinity tag is a nucleic acid. In some embodiments, the analyte is a nucleic acid. In some embodiments, a transient association of the query probe with the analyte is distinguishably affected by a covalent modification of the analyte. In some embodiments, the query probe is a nucleic acid or aptamer. In some embodiments, the query probe is a low-affinity antibody, an antibody fragment, or a nanobody. In some embodiments, the query probe is a DNA-binding protein, an RNA-binding protein, or a DNA-binding ribonucleoprotein complex.

In some embodiments, the (x, y) position of each query probe event is determined by processing the differential intensity profile using centroid determination, least-squares fitting to a Gaussian function, least-squares fitting to an airy disk function, least-squares fitting to a polynomial function (e.g., a parabola), or maximum likelihood estimation. In some embodiments, the analyte is subjected to thermal denaturation in the presence of a carrier prior to surface immobilization. In some embodiments, the analyte is subjected to chemical denaturation in the presence of a carrier prior to surface immobilization, e.g., the analyte is denatured with a denaturant such as urea, formamide, guanidinium chloride, high ionic strength, low ionic strength, high pH, low pH, or sodium dodecyl sulfate (SDS).

Further embodiments provide a system for quantifying an analyte in a sample. For example in some embodiments, systems comprise a functionality to stably immobilize an analyte to a surface; a freely diffusing query probe that binds to the target analyte with a low affinity; and a detection system that records query probe events and the spatial position of query probe events for analytes. In some embodiments, systems further comprise analytical procedures to identify an individual molecular copy of the target analyte according to the spatial position and timing of repeated binding and dissociation events to said target analyte. In some embodiments, the query probe is a nucleic acid or aptamer. In some embodiments, the query probe is a low-affinity antibody, an antibody fragment, or a nanobody. In some embodiments of

systems, the query probe and/or the capture probe is a DNA-binding protein, an RNA-binding protein, or a DNA-binding ribonucleoprotein complex. In some embodiments, the query and/or capture probe is a nucleic acid, a polypeptide (e.g., an antibody, antibody fragment, linear antibody, single-chain antibody, or other antigen-binding antibody derivative; an enzyme; a binding protein that recognizes the analyte with specificity); in some embodiments in which the analyte comprises a carbohydrate or polysaccharide, the query probe comprises a carbohydrate-binding protein such as a lectin or a carbohydrate-binding antibody. In some embodiments, the presence of a specific glycosidic linkage or set of glycosidic linkages between carbohydrate monomers yields a distinguishable pattern of query probe binding. In some embodiments, the capture probe is a rabbit monoclonal antibody; and in some embodiments, the query probe is a mouse monoclonal antibody.

In some system embodiments, the analyte is stably immobilized to the surface by a surface-bound capture probe that stably binds the target analyte. In some embodiments, the capture probe is a high-affinity antibody, an antibody fragment, or a nanobody. In some embodiments, the analyte is stably immobilized to the surface by a covalent bond cross-linking the target analyte to the surface. In some embodiments, the analyte is subjected to thermal denaturation in the presence of a carrier prior to surface immobilization. In some embodiments, the analyte is subjected to chemical denaturation in the presence of a carrier prior to surface immobilization, e.g., the analyte is denatured with a denaturant such as urea, formamide, guanidinium chloride, high ionic strength, low ionic strength, high pH, low pH, or sodium dodecyl sulfate (SDS). Additional embodiments will be apparent to persons skilled in the relevant art based on the teachings contained herein.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present technology will become better understood with regard to the following drawings:

FIG. 1 is schematic drawing showing steps of a method embodiment of the technology described herein and experimental data collected during the development of embodiments of the technology described herein.

FIG. 2 is a plot of signal ("Accepted counts") versus concentration in picomolar units for data collected during experiments described herein. The plot indicates that the dynamic range of the technology as described herein (filled squares) is improved relative to prior methods (open circles).

FIG.3A-B shows a representation of binding event clusters identified during experiments described herein in the presence of target analyte only (FIG. 3A) and in the presence of an interfering background analyte, but in the absence of the target analyte (FIG. 3B). FIG. 3A shows significant positive signal from the target analyte. Due to the specificity of the method, there was no target analyte signal detected in FIG. 3B. The experiment indicates the high specificity of embodiments of the technology described herein.

It is to be understood that the figures are not necessarily drawn to scale, nor are the objects in the figures necessarily drawn to scale in relationship to one another. The figures are depictions that are intended to bring clarity and understanding to various embodiments of apparatuses, systems, and methods disclosed herein. Wherever possible, the same reference numbers will be used throughout the drawings to refer to the same or like parts. Moreover, it should be appreciated that the drawings are not intended to limit the scope of the present teachings in any way.

DETAILED DESCRIPTION

In some embodiments, the technology provided herein relates to detecting biomolecular analytes with transient (e.g., kinetic), rather than stable (equilibrium, thermodynamic), interactions with one or more query probes. The analytes are immobilized on a surface with a capture probe, then detected with the transiently binding query probe. The technology is not limited in the analyte, capture probe, or query probe. For example, in some embodiments, the query probe is an antibody, nanobody, polypeptide, oligopeptide, or aptamer; for example, in some embodiments, the analyte is a nucleic acid, protein, peptide, lipid, small molecule, metabolite, or any molecule or compound.

In contrast to prior technologies, the technology described herein distinguishes between closely related analytes (e.g., phosphorylated and non-phosphorylated protein targets) with arbitrary precision by analyzing the kinetic behavior of the probe-target interaction.

In various embodiments, the assay conditions are controlled such that the interactions of the query probe with the target analyte are made transient. For example, in some embodiments the technology comprises one or more of the following to provide conditions in which a transient interaction of probe and analyte occurs: (1) engineering a query probe such that it interacts weakly with the target (e.g., in the nanomolar affinity range); (2) controlling the temperature such that the query probe interacts weakly with the target analyte; (3) controlling the solution conditions, e.g., ionic strength, ionic

composition, addition of chaotropic agents, addition of competing probes, etc., such that the query probe interacts weakly with the target analyte.

In some embodiments, the technology comprises use of, e.g., photonic forces and/or ultrasound energy. For example, in some embodiments photonic forces promote the concentration of material, especially larger particles, in a particular location. In some embodiments, ultrasound promotes mixing, e.g., to modulate the kinetics association, e.g., by increasing mixing rate beyond simple diffusion.

In some embodiments, binding of the query probe to the target analyte is measured by total internal reflection fluorescence microscopy or another technique capable of single-molecule sensitivity.

In this detailed description of the various embodiments, for purposes of explanation, numerous specific details are set forth to provide a thorough understanding of the embodiments disclosed. One skilled in the art will appreciate, however, that these various embodiments may be practiced with or without these specific details. In other instances, structures and devices are shown in block diagram form. Furthermore, one skilled in the art can readily appreciate that the specific sequences in which methods are presented and performed are illustrative and it is contemplated that the sequences can be varied and still remain within the spirit and scope of the various embodiments disclosed herein.

All literature and similar materials cited in this application, including but not limited to, patents, patent applications, articles, books, treatises, and internet web pages are expressly incorporated by reference in their entirety for any purpose. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which the various embodiments described herein belongs. When definitions of terms in incorporated references appear to differ from the definitions provided in the present teachings, the definition provided in the present teachings shall control. The section headings used herein are for organizational purposes only and are not to be construed as limiting the described subject matter in any way.

Definitions

To facilitate an understanding of the present technology, a number of terms and phrases are defined below. Additional definitions are set forth throughout the detailed description.

Throughout the specification and claims, the following terms take the meanings explicitly associated herein, unless the context clearly dictates otherwise. The phrase "in one embodiment" as used herein does not necessarily refer to the same embodiment, though it may. Furthermore, the phrase "in another embodiment" as used herein does not necessarily refer to a different embodiment, although it may. Thus, as described below, various embodiments of the invention may be readily combined, without departing from the scope or spirit of the invention.

In addition, as used herein, the term "or" is an inclusive "or" operator and is equivalent to the term "and/or" unless the context clearly dictates otherwise. The term "based on" is not exclusive and allows for being based on additional factors not described, unless the context clearly dictates otherwise. In addition, throughout the specification, the meaning of "a", "an", and "the" include plural references. The meaning of "in" includes "in" and "on."

As used herein, the terms "subject" and "patient" refer to any organisms including plants, microorganisms, and animals (e.g., mammals such as dogs, cats, livestock, and humans).

The term "sample" in the present specification and claims is used in its broadest sense. In some embodiments, a sample is or comprises an animal cell or tissue. In some embodiments, a sample includes a specimen or a culture (e.g., a microbiological culture) obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from plants or animals (including humans) and encompass fluids, solids, tissues, and gases. Environmental samples include environmental material such as surface matter, soil, water, and industrial samples. These examples are not to be construed as limiting the sample types applicable to the present technology.

As used herein, a "biological sample" refers to a sample of biological tissue or fluid. For instance, a biological sample may be a sample obtained from an animal (including a human); a fluid, solid, or tissue sample; as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, lagomorphs, rodents, etc. Examples of biological samples include sections of tissues, blood, blood fractions, plasma, serum, urine, or samples from other peripheral sources or cell cultures, cell colonies, single cells, or a collection of single cells. Furthermore, a biological sample includes pools or mixtures of the above mentioned samples. A biological sample may be provided by removing a

sample of cells from a subject, but can also be provided by using a previously isolated sample. For example, a tissue sample can be removed from a subject suspected of having a disease by conventional biopsy techniques. In some embodiments, a blood sample is taken from a subject. A biological sample from a patient means a sample from a subject suspected to be affected by a disease.

Environmental samples include environmental material such as surface matter, soil, water, and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The term "label" as used herein refers to any atom, molecule, molecular complex (e.g., metal chelate), or colloidal particle (e.g., quantum dot, nanoparticle, microparticle, etc.) that can be used to provide a detectable (preferably quantifiable) effect, and that can be attached to a nucleic acid or protein. Labels include, but are not limited to, dyes (e.g., optically-detectable labels, fluorescent dyes or moieties, etc.); radiolabels such as ^{32}P; binding moieties such as biotin; haptens such as digoxgenin; luminogenic, phosphorescent, optically-detectable, or fluorogenic moieties; mass tags; and fluorescent dyes alone or in combination with moieties that can suppress or shift emission spectra by fluorescence resonance energy transfer (FRET). Labels may provide signals detectable by fluorescence, luminescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, characteristics of mass or behavior affected by mass (e.g., MALDI time-of-flight mass spectrometry; fluorescence polarization), and the like. A label may be a charged moiety (positive or negative charge) or, alternatively, may be charge neutral. Labels can include or consist of nucleic acid or protein sequence, so long as the sequence comprising the label is detectable.

"Support" or "solid support", as used herein, refers to a matrix on or in which nucleic acid molecules, microparticles, and the like may be immobilized, e.g., to which they may be covalently or noncovalently attached or in or on which they may be partially or completely embedded so that they are largely or entirely prevented from diffusing freely or moving with respect to one another.

As used herein, "moiety" refers to one of two or more parts into which something may be divided, such as, for example, the various parts of an oligonucleotide, a molecule, a chemical group, a domain, a probe, etc.

As used herein, a "nucleic acid" or a "nucleic acid sequence" refers to a polymer or oligomer of pyrimidine and/or purine bases, preferably cytosine, thymine, and uracil,

and adenine and guanine, respectively (See Albert L. Lehninger, Principles of Biochemistry, at 793-800 (Worth Pub. 1982)). The present technology contemplates any deoxyribonucleotide, ribonucleotide, or peptide nucleic acid component, and any chemical variants thereof, such as methylated, hydroxymethylated, or glycosylated forms of these bases, and the like. The polymers or oligomers may be heterogenous or homogenous in composition, and may be isolated from naturally occurring sources or may be artificially or synthetically produced. In addition, the nucleic acids may be DNA or RNA, or a mixture thereof, and may exist permanently or transitionally in single-stranded or double-stranded form, including homoduplex, heteroduplex, and hybrid states. In some embodiments, a nucleic acid or nucleic acid sequence comprises other kinds of nucleic acid structures such as, for instance, a DNA/RNA helix, peptide nucleic acid (PNA), morpholino, locked nucleic acid (LNA), and/or a ribozyme. Hence, the term "nucleic acid" or "nucleic acid sequence" may also encompass a chain comprising non-natural nucleotides, modified nucleotides, and/or non- nucleotide building blocks that can exhibit the same function as natural nucleotides (e.g., "nucleotide analogs"); further, the term "nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin, which may be single or double-stranded, and represent the sense or antisense strand.

The term "nucleotide analog" as used herein refers to modified or non-naturally occurring nucleotides including but not limited to analogs that have altered stacking interactions such as 7-deaza purines (i.e., 7-deaza-dATP and 7-deaza-dGTP); base analogs with alternative hydrogen bonding configurations (e.g., such as Iso-C and Iso-G and other non-standard base pairs described in U.S. Pat. No. 6,001,983 to S. Benner and herein incorporated by reference); non-hydrogen bonding analogs (e.g., non-polar, aromatic nucleoside analogs such as 2,4-difluorotoluene, described by B. A. Schweitzer and E. T. Kool, J. Org. Chem., 1994, 59, 7238-7242, B. A. Schweitzer and E. T. Kool, J. Am. Chem. Soc., 1995, 117, 1863-1872; each of which is herein incorporated by reference); "universal" bases such as 5-nitroindole and 3-nitropyrrole; and universal purines and pyrimidines (such as "K" and "P" nucleotides, respectively; P. Kong, et al., Nucleic Acids Res., 1989, 17, 10373-10383, P. Kong et al., Nucleic Acids Res., 1992, 20, 5149-5152). Nucleotide analogs include nucleotides having modification on the sugar moiety, such as dideoxy nucleotides and 2'-O-methyl nucleotides. Nucleotide analogs include modified forms of deoxyribonucleotides as well as ribonucleotides.

"Peptide nucleic acid" means a DNA mimic that incorporates a peptide-like polyamide backbone.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (e.g., a sequence of nucleotides such as an oligonucleotide capture probe, query probe or a target analyte that is a nucleic acid) related by the base-pairing rules. For example, for the sequence "5'-A-G-T-3'" is complementary to the sequence "3'-T-C-A-5'." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids. Either term may also be used in reference to individual nucleotides, especially within the context of polynucleotides. For example, a particular nucleotide within an oligonucleotide may be noted for its complementarity, or lack thereof, to a nucleotide within another nucleic acid strand, in contrast or comparison to the complementarity between the rest of the oligonucleotide and the nucleic acid strand.

In some contexts, the term "complementarity" and related terms (e.g., "complementary", "complement") refers to the nucleotides of a nucleic acid sequence that can bind to another nucleic acid sequence through hydrogen bonds, e.g., nucleotides that are capable of base pairing, e.g., by Watson-Crick base pairing or other base pairing. Nucleotides that can form base pairs, e.g., that are complementary to one another, are the pairs: cytosine and guanine, thymine and adenine, adenine and uracil, and guanine and uracil. The percentage complementarity need not be calculated over the entire length of a nucleic acid sequence. The percentage of complementarity may be limited to a specific region of which the nucleic acid sequences that are base-paired, e.g., starting from a first base-paired nucleotide and ending at a last base-paired nucleotide. The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for

example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

Thus, in some embodiments, "complementary" refers to a first nucleobase sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the complement of a second nucleobase sequence over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more nucleobases, or that the two sequences hybridize under stringent hybridization conditions. "Fully complementary" means each nucleobase of a first nucleic acid is capable of pairing with each nucleobase at a corresponding position in a second nucleic acid. For example, in certain embodiments, an oligonucleotide wherein each nucleobase has complementarity to a nucleic acid has a nucleobase sequence that is identical to the complement of the nucleic acid over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more nucleobases.

"Mismatch" means a nucleobase of a first nucleic acid that is not capable of pairing with a nucleobase at a corresponding position of a second nucleic acid.

The term "domain" when used in reference to a polypeptide refers to a subsection of the polypeptide which possesses a unique structural and/or functional characteristic; typically, this characteristic is similar across diverse polypeptides. The subsection typically comprises contiguous amino acids, although it may also comprise amino acids which act in concert or which are in close proximity due to folding or other configurations. Examples of a protein domain include transmembrane domains, glycosylation sites, etc.

The term "gene" refers to a nucleic acid (e.g., DNA or RNA) sequence that comprises coding sequences necessary for the production of an RNA, or a polypeptide or its precursor (e.g., proinsulin). A functional polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence as long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction, etc.) of the polypeptide are retained. The term "portion" when used in reference to a gene refers to fragments of that gene. The fragments may range in size from a few nucleotides to the entire gene sequence minus one nucleotide. Thus, "a nucleotide comprising at least a portion of a gene" may comprise fragments of the gene or the entire gene.

The term "gene" also encompasses the coding regions of a structural gene and includes sequences located adjacent to the coding region on both the 5′ and 3′ ends for a

distance of about 1 kb on either end such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5′ of the coding region and which are present on the mRNA are referred to as 5′ non-translated sequences. The sequences which are located 3′ or downstream of the coding region and which are present on the mRNA are referred to as 3′ non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5′ and 3′ end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5′ or 3′ to the non-translated sequences present on the mRNA transcript). The 5′ flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3′ flanking region may contain sequences which direct the termination of transcription, posttranscriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or a gene product that has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified," "mutant," or "polymorphic" refers to a gene or gene product that displays modifications in sequence and or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product. Thus, the terms "variant" and "mutant" when used in reference to a nucleotide sequence refer to an nucleic acid sequence that differs by one or more nucleotides from another, usually related nucleotide acid sequence. A "variation" is a difference between two different nucleotide sequences; in some embodiments, one sequence is a reference sequence.

The term "allele" refers to different variations in a gene; the variations include but are not limited to variants and mutants, polymorphic loci and single nucleotide polymorphic loci, frameshift and splice mutations. An allele may occur naturally in a population, or it might arise during the lifetime of any particular individual of the population.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (e.g., the strength of the association between the nucleic acids) is influenced by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, and the T_m of the formed hybrid. "Hybridization" methods involve the annealing of one nucleic acid to another, complementary nucleic acid, e.g., a nucleic acid having a complementary nucleotide sequence. The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, Proc. Natl. Acad. Sci. USA 46:453 (1960) and Doty et al., Proc. Natl. Acad. Sci. USA 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41 * (\% G+C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (see, e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi and SantaLucia, Biochemistry 36: 10581-94 (1997) include more sophisticated computations which account for structural, environmental, and sequence characteristics to calculate T_m. For example, in some embodiments these computations provide an improved estimate of T_m for short nucleic acid probes and targets (e.g., as used in the examples).

The terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably. A "protein" or "polypeptide" encoded by a gene is not limited to the amino acid sequence encoded by the gene, but includes post-translational modifications of the protein. Where the term "amino acid sequence" is recited herein to refer to an amino acid sequence of a protein molecule, "amino acid sequence" and like terms such as "polypeptide" or "protein" are

not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule. Furthermore, an "amino acid sequence" can be deduced from the nucleic acid sequence encoding the protein. Conventional one and three-letter amino acid codes are used herein as follows – Alanine: Ala, A; Arginine: Arg, R; Asparagine: Asn, N; Aspartate: Asp, D; Cysteine: Cys, C; Glutamate: Glu, E; Glutamine: Gln, Q; Glycine: Gly, G; Histidine: His, H; Isoleucine: Ile, I; Leucine: Leu, L; Lysine: Lys, K; Methionine: Met, M; Phenylalanine: Phe, F; Proline: Pro, P; Serine: Ser, S; Threonine: Thr, T; Tryptophan: Trp, W; Tyrosine: Tyr, Y; Valine: Val, V. As used herein, the codes Xaa and X refer to any amino acid.

The terms "variant" and "mutant" when used in reference to a polypeptide refer to an amino acid sequence that differs by one or more amino acids from another, usually related polypeptide.

As used herein, the term "melting" when used in reference to a nucleic acid refers to the dissociation of a double-stranded nucleic acid or region of a nucleic acid into a single-stranded nucleic acid or region of a nucleic acid.

As used herein, a "query probe" or "reader probe" is any entity (e.g., molecule, biomolecule, etc.) that recognizes an analyte (e.g., binds to an analyte, e.g., binds specifically to an analyte). In exemplary embodiments, the query probe is a protein that recognizes an analyte. In some other exemplary embodiments, the query probe is a nucleic acid that recognizes an analyte (e.g., a DNA, an RNA, a nucleic acid comprising DNA and RNA, a nucleic acid comprising modified bases and/or modified linkages between bases; e.g., a nucleic acid as described hereinabove, a nucleic acid aptamer). In some embodiments, the query probe is labeled, e.g., with a detectable label such as, e.g., a fluorescent moiety as described herein. In some embodiments, the query probe comprises more than one type of molecule (e.g., more than one of a protein, a nucleic acid, a chemical linker or a chemical moiety).

As used herein, an "event" refers to an instance of a query probe binding to an analyte or an instance of query probe dissociation from an analyte, e.g., as measured by monitoring a detectable property indicating the binding of a query probe to an analyte and/or the dissociation of a query probe from an analyte.

As used herein, a "capture probe" is any entity (e.g., molecule, biomolecule, etc.) that recognizes an analyte (e.g., binds to an analyte, e.g., binds specifically to an analyte) and links the analyte to a solid support. In exemplary embodiments, the capture probe is a protein that recognizes an analyte. In some other exemplary embodiments, a capture probe is a nucleic acid that recognizes an analyte (e.g., a DNA,

an RNA, a nucleic acid comprising DNA and RNA, a nucleic acid comprising modified bases and/or modified linkages between bases; e.g., a nucleic acid as described hereinabove, a nucleic acid aptamer). In some embodiments, a capture probe is labeled, e.g., with a detectable label such as, e.g., a fluorescent moiety as described herein. In some embodiments, the capture probe comprises more than one type of molecule (e.g., more than one of a protein, a nucleic acid, a chemical linker or a chemical moiety).

As used herein, the term "sensitivity" refers to the probability that an assay gives a positive result for the analyte when the sample comprises the analyte. Sensitivity is calculated as the number of true positive results divided by the sum of the true positives and false negatives. Sensitivity is a measure of how well an assay detects an analyte.

As used herein, the term "specificity" refers to the probability that an assay gives a negative result when the sample does not comprise the analyte. Specificity is calculated as the number of true negative results divided by the sum of the true negatives and false positives. Specificity is a measure of how well a method of the present invention excludes samples that do not comprise an analyte from those that do comprise the analyte.

As used herein, the "equilibrium constant" (K_{eq}), the "equilibrium association constant" (K_a), and "association binding constant" (or "binding constant"(K_B)) are used interchangeably for the following binding reaction of A and B at equilibrium:

where A and B are two entities that associate with each other (e.g., capture probe and analyte, query probe and analyte) and $K_{eq} = [AB] / ([A] \times [B])$. The dissociation constant $K_D = 1/K_B$. The K_D is a useful way to describe the affinity of a one binding partner A for a partner B with which it associates, e.g., the number K_D represents the concentration of A or B that is required to yield a significant amount of AB. $K_{eq} = k_{off} / k_{on}$; $K_D = k_{off} / k_{on}$.

As used herein, a "significant amount" of the product of two entities that associate with each other, e.g., formation of AB from A and B according to the equation above, refers to a concentration of AB that is equal to or greater than the free concentration of A or B, whichever is smaller.

As used herein, "nanomolar affinity range" refers to the association of two components that has an equilibrium dissociation constant K_D (e.g., ratio of k_{off} / k_{on}) in the nanomolar range, e.g., a dissociation constant (K_D) of 1×10^{-10} to 1×10^{-5} M (e.g., in some embodiments 1×10^{-9} to 1×10^{-6} M). The dissociation constant has molar units

(M). The smaller the dissociation constant, the higher the affinity between two components (e.g., capture probe and analyte; query probe and analyte).

As used herein, a "weak affinity" or "weak binding" or "weak association" refers to an association having a K_D of approximately 100 nanomolar (e.g., approximately 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 300, 400, or 500 nanomolar) and/or, in some embodiments, in the range of 1 nanomolar to 10 micromolar.

The terms "specific binding" or "specifically binding" when used in reference to the interaction of two components A and B that associate with one another refers to an association of A and B having a K_D that is smaller than the K_D for the interaction of A or B with other similar components in the solution, e.g., at least one other molecular species in the solution that is not A or B.

As used herein, the word "presence" or "absence" (or, alternatively, "present" or "absent") is used in a relative sense to describe the amount or level of a particular entity (e.g., an analyte). For example, when an analyte is said to be "present" in a sample, it means the level or amount of this analyte is above a pre-determined threshold; conversely, when an analyte is said to be "absent" in a sample, it means the level or amount of this analyte is below a pre-determined threshold. The pre-determined threshold may be the threshold for detectability associated with the particular test used to detect the analyte or any other threshold. When an analyte is "detected" in a sample it is "present" in the sample; when an analyte is "not detected" it is "absent" from the sample. Further, a sample in which an analyte is "detected" or in which the analyte is "present" is a sample that is "positive" for the analyte. A sample in which an analyte is "not detected" or in which the analyte is "absent" is a sample that is "negative" for the analyte.

As used herein, an "increase" or a "decrease" refers to a detectable (e.g., measured) positive or negative change in the value of a variable relative to a previously measured value of the variable, relative to a pre-established value, and/or relative to a value of a standard control. An increase is a positive change preferably at least 10%, more preferably 50%, still more preferably 2-fold, even more preferably at least 5-fold, and most preferably at least 10-fold relative to the previously measured value of the variable, the pre-established value, and/or the value of a standard control. Similarly, a decrease is a negative change preferably at least 10%, more preferably 50%, still more preferably at least 80%, and most preferably at least 90% of the previously measured value of the variable, the pre-established value, and/or the value of a standard control.

Other terms indicating quantitative changes or differences, such as "more" or "less," are used herein in the same fashion as described above.

The term "detection assay" refers to an assay for detecting the presence or absence of an analyte or the activity or effect of an analyte or for detecting the presence or absence of a variant of an analyte.

A "system" denotes a set of components, real or abstract, comprising a whole where each component interacts with or is related to at least one other component within the whole.

In some embodiments the technology comprises an antibody component or moiety, e.g., an antibody or fragments or derivatives thereof. As used herein, an "antibody", also known as an "immunoglobulin" (e.g., IgG, IgM, IgA, IgD, IgE), comprises two heavy chains linked to each other by disulfide bonds and two light chains, each of which is linked to a heavy chain by a disulfide bond. The specificity of an antibody resides in the structural complementarity between the antigen combining site of the antibody (or paratope) and the antigen determinant (or epitope). Antigen combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions influence the overall domain structure and hence the combining site. Some embodiments comprise a fragment of an antibody, e.g., any protein or polypeptide-containing molecule that comprises at least a portion of an immunoglobulin molecule such as to permit specific interaction between said molecule and an antigen. The portion of an immunoglobulin molecule may include, but is not limited to, at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof. Such fragments may be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

Fragments of antibodies include, but are not limited to, Fab (e.g., by papain digestion), $F(ab')_2$ (e.g., by pepsin digestion), Fab' (e.g., by pepsin digestion and partial reduction) and Fv or scFv (e.g., by molecular biology techniques) fragments.

A Fab fragment can be obtained by treating an antibody with the protease papaine. Also, the Fab may be produced by inserting DNA encoding a Fab of the antibody into a vector for prokaryotic expression system or for eukaryotic expression system, and introducing the vector into a prokaryote or eukaryote to express the Fab. A $F(ab')_2$ may be obtained by treating an antibody with the protease pepsin. Also, the $F(ab')_2$ can be produced by binding a Fab' via a thioether bond or a disulfide bond. A Fab may be obtained by treating $F(ab')_2$ with a reducing agent, e.g., dithiothreitol. Also, a Fab' can be produced by inserting DNA encoding a Fab' fragment of the antibody into an expression vector for a prokaryote or an expression vector for a eukaryote, and introducing the vector into a prokaryote or eukaryote for its expression. A Fv fragment may be produced by restricted cleavage by pepsin, e.g., at 4°C and pH 4.0. (a method called "cold pepsin digestion"). The Fv fragment consists of the heavy chain variable domain (V_H) and the light chain variable domain (V_L) held together by strong noncovalent interaction. A scFv fragment may be produced by obtaining cDNA encoding the V_H and V_L domains as previously described, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv.

In general, antibodies can usually be raised to any antigen, using the many conventional techniques now well known in the art.

As used herein, the term "conjugated" refers to when one molecule or agent is physically or chemically coupled or adhered to another molecule or agent. Examples of conjugation include covalent linkage and electrostatic complexation. The terms "complexed," "complexed with," and "conjugated" are used interchangeably herein.

As used herein, a "stable interaction" or referring to a "stably bound" interaction refers to an association that is relatively persistent under the thermodynamic equilibrium conditions of the interaction. In some embodiments, a "stable interaction" is an interaction between two components having a K_D that is smaller than approximately 10^{-9} M or, in some embodiments a K_D that is smaller than 10^{-8} M. In some embodiments, a "stable interaction" has a dissociation rate constant k_{off} that is smaller than 1 per hour or, in some embodiments, a dissociation rate constant k_{off} that is smaller than 1 per minute. In some embodiments, a "stable interaction" is defined as not being a "transient interaction". In some embodiments, a "stable interaction" includes interactions mediated by covalent bonds and other interactions that are not typically described by a K_D value but that involve an average association lifetime between two entities that is longer than

approximately 1 minute (e.g., 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, or 180 seconds) per each interaction.

In some embodiments, the distinction between a "stable interaction" and a "transient interaction" is determined by a cutoff value of K_D and/or k_{off} and/or another kinetic or thermodynamic value describing the associations, wherein the cutoff is used to discriminate between stable and transient interactions that might otherwise be characterized differently if described in absolute terms of a K_D and/or k_{off} or another kinetic or thermodynamic value describing the associations. For example, a "stable interaction" characterized by a K_D value might also be characterized as a "transient interaction" in the context of another interaction that is even more stable. One of skill in the art would understand other relative comparisons of stable and transient interactions, e.g., that a "transient interaction" characterized by a K_D value might also be characterized as a "stable interaction" in the context of another interaction that is even more transient (less stable).

As used herein, "moiety" refers to one of two or more parts into which something may be divided, such as, for example, the various parts of an oligonucleotide, a molecule, a chemical group, a domain, a probe, an "R" group, a polypeptide, etc.

As used herein, in some embodiments a "signal" is a time-varying quantity associated with one or more properties of a sample that is assayed, e.g., the binding of a query probe to an analyte and/or dissociation of a query probe from an analyte. A signal can be continuous in the time domain or discrete in the time domain. As a mathematical abstraction, the domain of a continuous-time signal is the set of real numbers (or an interval thereof) and the domain of a discrete-time signal is the set of integers (or an interval thereof). Discrete signals often arise via "digital sampling" of continuous signals. For example, an audio signal consists of a continually fluctuating voltage on a line that can be digitized by reading the voltage level on the line at a regular interval, e.g., every 50 microseconds. The resulting stream of numbers is stored as a discrete-time digital signal. In some embodiments, the signal is recorded as a function of location is space (e.g., x, y coordinates; e.g., x, y, z coordinates). In some embodiments, the signal is recorded as a function of time. In some embodiments, the signal is recorded as a function of time and location.

The term "substantially" as used herein, is a broad term and is used in its ordinary sense, including, but not limited to, being largely but not necessarily wholly that which is specified.

The term "algorithm," as used herein, is a broad term and is used in its ordinary sense, including, but not limited to, the computational processes (for example, programs) involved in transforming information from one state to another, for example using computer processing.

Description

Although the disclosure herein refers to certain illustrated embodiments, it is to be understood that these embodiments are presented by way of example and not by way of limitation.

Poisson processes

Embodiments of the technology are related to single-molecule recognition by recording the characteristic kinetics of a query probe binding to a target analyte. In particular embodiments, this process is a Poisson process. A Poisson process is a continuous-time stochastic process that counts the number of events and the time that events (e.g., transient binding of a detectably labeled (e.g., fluorescent) query probe to an immobilized target analyte) occur in a given time interval. The time interval between each pair of consecutive events has an exponential distribution and each interval is assumed to be independent of other intervals. The Poisson distribution is a discrete probability distribution that expresses the probability of a given number of the events occurring in the given time interval if these events occur with a known average rate and independently of the time since the last event. The Poisson distribution can also be used for the number of events in other specified intervals such as distance, area, or volume.

A Poisson distribution is a special case of the general binomial distribution where the number of trials n is large, the probability of success p is small, and the product $np = \lambda$ is moderate. In a Poisson process, the probability that a number of events N is j at any arbitrary time t follows the Poisson probability distribution $P_j(t)$:

$$P_j(t) = \frac{e^{-\lambda t}(\lambda t)^j}{j!},\ j = 0,\ 1,\ 2,\ \ldots \qquad (1).$$

That is, the number N of events that occur up to time t has a Poisson distribution with parameter λt. Statistical and mathematical methods relevant to Poisson processes and Poisson distributions are known in the art. See, e.g., "Stochastic Processes (i): Poisson Processes and Markov Chains" in *Statistics for Biology and Health – Statistical Methods*

in Bioinformatics (Ewans and Grant, eds.), Springer (New York, 2001), page 129 et seq., incorporated herein by reference in its entirety. Software packages such as Matlab and R may be used to perform mathematical and statistical methods associated with Poisson processes, probabilities, and distributions.

Kinetics of detection

Particular embodiments of the technology are related to detecting an analyte by analyzing the kinetics of the interaction of a query probe with the analyte to be detected. For the interaction of a query probe Q (e.g., at an equilibrium concentration [Q]) with a target analyte T (e.g., at an equilibrium concentration [T]), the kinetic rate constant k_{on} describes the time-dependent formation of the complex QT comprising the probe Q hybridized to the analyte T. In particular embodiments, while the formation of the QT complex is associated with a second order rate constant that is dependent on the concentration of query probe and has units of $M^{-1}min^{-1}$ (or the like), the formation of the QT complex is sufficiently described by a k_{on} that is a pseudo-first order rate constant associated with the formation of the QT complex. Thus, as used herein, k_{on} is an apparent ("pseudo") first-order rate constant.

Likewise, the kinetic rate constant k_{off} describes the time-dependent dissociation of the complex QT into the probe Q and the analyte T. Kinetic rates are typically provided herein in units of min^{-1} or s^{-1}. The "dwell time" of the query probe Q in the bound state (τ_{on}) is the time interval (e.g., length of time) that the probe Q is hybridized to the analyte T during each instance of query probe Q binding to the analyte T to form the QT complex. The "dwell time" of the query probe Q in the unbound state (τ_{off}) is the time interval (e.g., length of time) that the probe Q is not hybridized to the analyte T between each instance of query probe Q binding to the analyte to form the QT complex (e.g., the time the query probe Q is dissociated from the target analyte T between successive binding events of the query probe Q to the target analyte T). Dwell times may be provided as averages or weighted averages integrating over numerous binding and non-binding events.

Further, in some embodiments, the repeated, stochastic binding of probes (e.g., detectably labeled query probes (e.g., fluorescent probes) to target analytes is modeled as a Poisson process occurring with constant probability per unit time and in which the standard deviation in the number of binding and dissociation events per unit time (N_{b+d}) increases as $(N_{b+d})^{1/2}$. Thus, the statistical noise becomes a smaller fraction of N_{b+d} as the observation time is increased. Accordingly, the observation is lengthened as needed in

some embodiments to achieve discrimination between target and off-target binding. And, as the acquisition time is increased, the signal and background peaks in the N_{b+d} histogram become increasingly separated and the width of the signal distribution increases as the square root of N_{b+d}, consistent with kinetic Monte Carlo simulations.

Further, in some embodiments assay conditions are controlled to tune the kinetic behavior to improve discrimination of query probe binding events to the target analyte from background binding. For example, in some embodiments the technology comprises control of assay conditions such as, e.g., using a query probe that is designed to interact weakly with the target analyte (e.g., in the nanomolar affinity range); controlling the temperature such that the query probe interacts weakly with the target analyte; controlling the solution conditions, e.g., ionic strength, ionic composition, addition of chaotropic agents, and addition of competing probes.

Analytes

The technology is not limited in the analyte that is detected, quantified, identified, or otherwise characterized (e.g., presence, absence, amount, concentration, state). The term "analyte" as used herein is a broad term and is used in its ordinary sense, including, without limitation, to refer to a substance or chemical constituent in a sample such as a biological fluid (for example, blood, interstitial fluid, cerebral spinal fluid, lymph fluid or urine) that can be analyzed. Analytes can include naturally occurring substances, artificial substances, metabolites, and/or reaction products. In some embodiments, the analyte comprises a salt, sugars, protein, fat, vitamin, or hormone. In some embodiments, the analyte is naturally present in a biological sample (e.g., is "endogenous"); for example, in some embodiments, the analyte is a metabolic product, a hormone, an antigen, an antibody, and the like. Alternatively, in some embodiments, the analyte is introduced into a biological organism (e.g., is "exogenous), for example, a drug, drug metabolite, a drug precursor (e.g., prodrug), a contrast agent for imaging, a radioisotope, a chemical agent, etc. The metabolic products of drugs and pharmaceutical compositions are also contemplated analytes.

In some embodiments, the analyte is a polypeptide, a nucleic acid, a small molecule, a lipid, a carbohydrate, a polysaccharide, a fatty acid, a phospholipid, a glycolipid, a sphingolipid, an organic molecule, an inorganic molecule, cofactor, pharmaceutical, bioactive agent, a cell, a tissue, an organism, etc. In some embodiments, the analyte comprises a polypeptide, a nucleic acid, a small molecule, a lipid, a carbohydrate, a polysaccharide, a fatty acid, a phospholipid, a glycolipid, a sphingolipid,

an organic molecule, an inorganic molecule, cofactor, pharmaceutical, bioactive agent, a cell, a tissue, an organism, etc. In some embodiments, the analyte comprises a combination of one or more of a polypeptide, a nucleic acid, a small molecule, a lipid, a carbohydrate, a polysaccharide, a fatty acid, a phospholipid, a glycolipid, a sphingolipid, an organic molecule, an inorganic molecule, cofactor, pharmaceutical, bioactive agent, a cell, a tissue, an organism, etc.

In some embodiments, the analyte is part of a multimolecular complex, e.g., a multiprotein complex, a nucleic acid/protein complex, a molecular machine, an organelle (e.g., a cell-free mitochondrion, e.g., in plasma; a plastid; golgi, endoplasmic reticulum, vacuole, peroxisome, lysosome, and/or nucleus), cell, virus particle, tissue, organism, or any macromolecular complex or structure or other entity that can be captured and is amenable to analysis by the technology described herein (e.g., a ribosome, spliceosome, vault, proteasome, DNA polymerase III holoenzyme, RNA polymerase II holoenzyme, symmetric viral capsids, GroEL / GroES; membrane protein complexes: photosystem I, ATP synthase, nucleosome, centriole and microtubule-organizing center (MTOC), cytoskeleton, flagellum, nucleolus, stress granule, germ cell granule, or neuronal transport granule). For example, in some embodiments a multimolecular complex is isolated and the technology finds use in characterizing, identifying, quantifying, and/or detecting one or more molecules (analytes) associated with (e.g., that is a component of) the multimolecular complex. In some embodiments an extracellular vesicle is isolated and the technology finds use in characterizing, identifying, quantifying, and/or detecting one or more molecules (analytes) associated with the vesicle. In some embodiments, the technology finds use in characterizing, identifying, quantifying, and/or detecting a protein (e.g., a surface protein) and/or an analytes present inside the vesicle, e.g., a protein, nucleic acid, or other analyte described herein. In some embodiments, the vesicle is fixed and permeabilized prior to analysis.

In some embodiments, the analyte is chemically modified to provide a site for query probe binding. For instance, in some embodiments, beta-elimination of phosphoserine and phosphothreonine under strongly basic conditions is used to introduce an alkene, followed by Michael addition of a nucleophile such as a dithiol to the alkene. The remaining free thiol is then used for conjugation to a maleimide-containing oligonucleotide with a sequence complementary to an oligonucleotide query probe. The post-translational modifications phosphoserine and phosphothreonine may then be probed using the query probe and analyzed as described herein.

As used herein "detect an analyte" or "detect a substance" will be understood to encompass direct detection of the analyte itself or indirect detection of the analyte by detecting its by-product(s).

Capture

Embodiments of the technology comprise capture of an analyte. In some embodiments, the analyte is captured and immobilized. In some embodiments, the analyte is stably attached to a solid support. In some embodiments, the solid support is immobile relative to a bulk liquid phase contacting the solid support. In some embodiments, the solid support is diffusible within a bulk liquid phase contacting the solid support.

In some embodiments, stable attachment of the target analyte to a surface or other solid substrate is provided by a high-affinity or irreversible interaction (e.g., as used herein, an "irreversible interaction" refers to an interaction having a dissociation half-life longer than the observation time, e.g., in some embodiments, a time that is 1 to 5 minutes (e.g., 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, or 600 seconds, or longer). The technology is not limited in the components and/or methods used for capture of the analyte. For example, the stable attachment is provided by a variety of methods, including but not limited to one or more of the following.

In some embodiments, an analyte is immobilized by a surface-bound capture probe with a dissociation constant (K_D) for the analyte smaller than approximately 1 nanomolar (nM) (e.g., less than 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5 nanomolar) and a dissociation rate constant for the analyte that is smaller than approximately 1 min^{-1} (e.g., less than approximately 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.8, 0.7, 0.6, 0.5 min^{-1}). Exemplary surface-bound capture probes include, e.g., an antibody, antibody fragment, nanobody, or other protein; a high-affinity DNA-binding protein or ribonucleoprotein complex such as Cas9, dCas9, Cpf1, transcription factors, or transcription activator-like effector nucleases (TALENs); an oligonucleotide; a small organic molecule; or a metal ion complex.

In some embodiments, an analyte is immobilized by direct noncovalent attachment to a surface (e.g., by interactions between the analyte and the surface, e.g., a glass surface or a nylon, nitrocellulose, or polyvinylidene difluoride membrane).

In some embodiments, an analyte is immobilized by chemical linking (e.g., by a covalent bond) of the analyte to the solid support. In some embodiments, the analyte is chemically linked to the solid support by, e.g., a carbodiimide, a N-hydroxysuccinimide esters (NHS) ester, a maleimide, a haloacetyl group, a hydrazide, or an alkoxyamine. In some embodiments, an analyte is immobilized by radiation (e.g., ultraviolet light)-induced cross-linking of the target analyte to the surface and/or to a capture probe attached to the surface. In some embodiments, the capture probe is a rabbit monoclonal antibody. In some embodiments in which the analyte comprises a carbohydrate or polysaccharide, the capture probe comprises a carbohydrate-binding protein such as a lectin or a carbohydrate-binding antibody.

Alternatively, instead of immobilizing the target analyte to a solid support that is relatively stationary with respect to a bulk phase that contacts the solid support as described above, some embodiments provide that the target analyte is associated with a freely diffusing particle that diffuses within the bulk fluid phase contacting the freely diffusing particle. Accordingly, in some embodiments, the target analyte is covalently or noncovalently bound to a freely diffusing substrate. In some embodiments, the freely diffusing substrate is, e.g., a colloidal particle (e.g., a particle having a diameter of approximately 10-1000 nm (e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 nm)). In some embodiments, the freely diffusing substrate comprises and/or is made of, e.g., polystyrene, silica, dextran, gold, or DNA origami. In some embodiments, the target analyte is associated with a freely diffusing particle that diffuses slowly relative to the diffusion of the query probe, e.g., the target analyte has a diffusion coefficient that is less than approximately 10% (e.g., less than 15, 14, 13, 12, 11, 10.5, 10.4, 10.3, 10.2, 10.1, 10.0, 9.9, 9.8, 9.7, 9.6, 9.5, or 9.0% or less) of the diffusion coefficient of the query probe.

Furthermore, in some embodiments the target analyte is associated with a freely diffusing particle and the location of the target analyte is observable and/or recordable independently of observing and/or recording query probe binding. For example, in some embodiments a detectable label (e.g., a fluorophore, fluorescent protein, quantum dot) is covalently or noncovalently attached to the target analyte, e.g., for detection and localization of the target analyte. Accordingly, in some embodiments the position of the target analyte and the position of query probe binding events are simultaneously and independently measured.

Query

Embodiments of the technology comprise a query probe (e.g., a detectably labeled query probe) that binds transiently and repeatedly to the analyte, e.g., a query probe that binds to and dissociates from the target analyte several (e.g., greater than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) times per observation window. In some embodiments, the query probe has a dissociation constant (K_D) for the analyte of larger than approximately 1 nanomolar (e.g., greater than 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0 or more nanomolar) under the assay conditions. In some embodiments, the query probe has a binding and/or a dissociation constant for the analyte that is larger than approximately 1 min^{-1} (e.g., greater than 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0 or more min^{-1}).

The technology is not limited in the query probe. In some embodiments, the query probe is an antibody or antibody fragment. In some embodiments, the query probe is a low-affinity antibody or antibody fragment. In some embodiments, the query probe is a nanobody, a DNA-binding protein or protein domain, a methylation binding domain (MBD), a kinase, a phosphatase, an acetylase, a deacetylase, an enzyme, or a polypeptide. In some embodiments, the query probe is an oligonucleotide that interacts with the target analyte. For example, in some embodiments the query probe is an oligonucleotide that hybridizes to the target analyte to form a duplex that has a melting temperature that is within approximately 10 degrees Celsius of the temperature at which the observations are made (e.g., approximately 7-12 nucleotides for observation that is performed at room temperature). In some embodiments, the query probe is a mononucleotide. In some embodiments, the query probe is a small organic molecule (e.g., a molecule having a molecular weight that is less than approximately 2000 daltons, e.g., less than 2100, 2050, 2000, 1950, 1900, 1850, 1800, 1750, 1700, 1650, 1600, 1550, 1500 daltons, or less). In some embodiments, the query probe is a pharmaceutical agent, e.g., a drug or other bioactive molecule. In some embodiments, the query probe is a metal ion complex. In some embodiments, the query probe is a methyl-binding domain (e.g., MBD1). In some embodiments, the query probe is labeled with a detectable label as described herein. In some embodiments, the query probe is covalently linked to the detectable label. In some embodiments, the query probe is indirectly and/or non-

covalently linked and/or associated with the detectable label. In some embodiments, the detectable label is fluorescent.

In some embodiments, the query probe is a mouse monoclonal antibody.

In some embodiments in which the analyte comprises a carbohydrate or polysaccharide, the query probe comprises a carbohydrate-binding protein such as a lectin or a carbohydrate-binding antibody.

Detection

The technology provides for the detection of target analytes, e.g., in the presence of similar analytes and, in some embodiments, background noise. In some embodiments, signal originating from the transient binding of the query probe to the target analyte is distinguishable from the signal produced by unbound query probe (e.g., by observing, monitoring, and/or recording a localized change in signal intensity during the binding event). In some embodiments, observing the transient binding of the query probe (e.g., a fluorescently labeled query probe) to the target analyte is provided by a technology such as, e.g., total internal reflection fluorescence (TIRF) or near-TIRF microscopy, zero-mode waveguides (ZMWs), light sheet microscopy, stimulated emission depletion (STED) microscopy, or confocal microscopy. In some embodiments, the technology provided herein uses query probes having a fluorescence emission that is quenched when not bound to the target analyte and/or a fluorescence emission that is dequenched when bound to the target analyte.

The technology comprises locating and/or observing the transient binding of a query probe to an analyte within a discrete region of an area and/or a discreet region of a volume that is observed, e.g., at particular spatial coordinates in a plane or a volume. In some embodiments, the error in determining the spatial coordinates of a binding or dissociation event (e.g., due to limited signal, detector noise, or spatial binning in the detector) is small (e.g., minimized, eliminated) relative to the average spacing between immobilized (e.g., surface-bound) target analytes. In some embodiments comprising use of wide-field fluorescence microscopy, measurement errors are minimized and/or eliminated by use of effective detector pixel dimensions in the specimen plane that are not larger than the average distance between immobilized (e.g., surface-bound) target analytes and that many fluorescent photons (in some embodiments, more than 100, e.g., more than 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, or 130 or more) are collected per time point of detection.

In some embodiments, the detectable (e.g., fluorescent) query probe produces a fluorescence emission signal when it is close to the surface of the solid support (e.g., within about 100 nm of the surface of the solid support). When unbound, query probes quickly diffuse and thus are not individually detected; accordingly, when in the unbound state, the query probes produce a low level of diffuse background fluorescence. Consequently, in some embodiments detection of bound query probes comprises use of total internal reflection fluorescence microscopy (TIRF), HiLo microscopy (see, e.g., US20090084980, EP2300983 B1, WO2014018584 A1, WO2014018584 A1, incorporated herein by reference), confocal scanning microscopy, or other technologies comprising illumination schemes that illuminate (e.g., excite) only those query probe molecules near or on the surface of the solid support. Thus, in some embodiments, only query probes that are bound to an immobilized target near or on the surface produce a point-like emission signal (e.g., a "spot") that can be confirmed as originating from a single molecule.

In some embodiments, the query probe comprises a fluorescent label having an emission wavelength. Detection of fluorescence emission at the emission wavelength of the fluorescent label indicates that the query probe is bound to an immobilized target analyte. Binding of the query probe to the target analyte is a "binding event". In some embodiments of the technology, a binding event has a fluorescence emission having a measured intensity greater than a defined threshold. For example, in some embodiments a binding event has a fluorescence intensity that is above the background fluorescence intensity (e.g., the fluorescence intensity observed in the absence of a target analyte). In some embodiments, a binding event has a fluorescence intensity that is at least 1, 2, 3, 4 or more standard deviations above the background fluorescence intensity (e.g., the fluorescence intensity observed in the absence of a target analyte). In some embodiments, a binding event has a fluorescence intensity that is at least 2 standard deviations above the background fluorescence intensity (e.g., the fluorescence intensity observed in the absence of a target analyte). In some embodiments, a binding event has a fluorescence intensity that is at least 1.5, 2, 3, 4, or 5 times the background fluorescence intensity (e.g., the mean fluorescence intensity observed in the absence of a target analyte).

Accordingly, in some embodiments detecting fluorescence at the emission wavelength of the query probe that has an intensity above the defined threshold (e.g., at least 2 standard deviations greater than background intensity) indicates that a binding event has occurred (e.g., at a discrete location on the solid support where a target

analyte is immobilized). Also, in some embodiments detecting fluorescence at the emission wavelength of the query probe that has an intensity above the defined threshold (e.g., at least 2 standard deviations greater than background intensity) indicates that a binding event has started. Accordingly, in some embodiments detecting an absence of fluorescence at the emission wavelength of the query probe that has an intensity above the defined threshold (e.g., at least 2 standard deviations greater than background intensity) indicates that a binding event has ended (e.g., the query probe has dissociated from the target analyte). The length of time between when the binding event started and when the binding event ended (e.g., the length of time that fluorescence at the emission wavelength of the fluorescent probe having an intensity above the defined threshold (e.g., at least 2 standard deviations greater than background intensity) is detected) is the dwell time of the binding event. A "transition" refers to the binding and dissociation of a query probe to the target analyte (e.g., an on/off event), e.g., a query probe dissociating from a bound state or a query probe associating with a target analyte from the unbound state.

Methods according to the technology comprise counting the number of query probe binding events that occur at each discrete location (e.g., at a position identified by x, y coordinates) on the solid support during a defined time interval that is the "acquisition time" (e.g., a time interval that is tens to hundreds to thousands of seconds, e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 seconds; e.g., 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 0 minutes; e.g., 1, 1.5, 2, 2.5, or 3 hours). In some embodiments, the acquisition time is approximately 1 to 10 seconds to 1 to 10 minutes (e.g., approximately 1 to 100 seconds, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, or 100 seconds, e.g., 1 to 100 minutes, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 60, 70, 80, 90, or 100 minutes).

Further, the length of time the query probe remains bound to the target analyte during a binding event is the "dwell time" of the binding event. The number of binding events detected during the acquisition time and/or the lengths of the dwell times recorded for the binding events is/are characteristic of a query probe binding to a target analyte and thus provide an indication that the target analyte is immobilized at said discrete location and thus that the target analyte is present in the sample.

Binding of the query probe to the immobilized target analyte and/or and dissociation of the query probe from the immobilized target analyte is/are monitored (e.g., using a light source to excite the fluorescent probe and detecting fluorescence emission from a bound query probe, e.g., using a fluorescence microscope) and/or

recorded during a defined time interval (e.g., during the acquisition time). The number of times the query probe binds to the nucleic acid during the acquisition time and/or the length of time the query probe remains bound to the nucleic acid during each binding event and the length of time the query probe remains unbound to the nucleic acid between each binding event (e.g., the "dwell times" in the bound and unbound states, respectively) are determined, e.g., by the use of a computer and software (e.g., to analyze the data using a hidden Markov model and Poisson statistics).

In some embodiments, positive and/or negative control samples are measured (e.g., a control sample known to comprise or not to comprise a target). Fluorescence detected in a negative control sample is "background fluorescence" or "background (fluorescence) intensity" or "baseline".

In some embodiments, data comprising measurements of fluorescence intensity at the emission wavelength of the query probe are recorded as a function of time. In some embodiments, the number of binding events and the dwell times of binding events (e.g. for each immobilized analyte) are determined from the data (e.g., by determining the number of times and the lengths of time the fluorescence intensity is above a threshold background fluorescence intensity). In some embodiments, transitions (e.g., binding and dissociation of a query probe) are counted for each discrete location on the solid support where a target analyte is immobilized. In some embodiments, a threshold number of transitions is used to discriminate the presence of a target analyte at a discrete location on the solid support from background signal, non-target analyte, and/or spurious binding of the query probe.

In some embodiments, a distribution of the number of transitions for each immobilized target is determined – e.g., the number of transitions is counted for each immobilized analyte observed. In some embodiments a histogram is produced. In some embodiments, characteristic parameters of the distribution are determined, e.g., the mean, median, peak, shape, etc. of the distribution are determined. In some embodiments, data and/or parameters (e.g., fluorescence data (e.g., fluorescence data in the time domain), kinetic data, characteristic parameters of the distribution, etc.) are analyzed by algorithms that recognize patterns and regularities in data, e.g., using artificial intelligence, pattern recognition, machine learning, statistical inference, neural nets, etc. In some embodiments, the analysis comprises use of a frequentist analysis and in some embodiments the analysis comprises use of a Bayesian analysis. In some embodiments, pattern recognition systems are trained using known "training" data (e.g., using supervised learning) and in some embodiments algorithms are used to discover

previously unknown patterns (e.g., unsupervised learning). See, e.g., Duda, et al. (2001) *Pattern classification* (2nd edition), Wiley, New York; Bishop (2006) *Pattern Recognition and Machine Learning*, Springer.

Pattern recognition (e.g., using training sets, supervised learning, unsupervised learning, and analysis of unknown samples) associates identified patterns with analytes such that particular patterns provide a "fingerprint" of particular analytes that find use in detection, quantification, and identification of analytes.

In some embodiments, the distribution produced from a target analyte is significantly different than a distribution produced from a non-target analyte or the distribution produced in the absence of a target analyte. In some embodiments, a mean number of transitions is determined for the plurality of immobilized target analytes. In some embodiments, the mean number of transitions observed for a sample comprising a target analyte is approximately linearly related as a function of time and has a positive slope (e.g., the mean number of transitions increases approximately linearly as a function of time).

In some embodiments, the data are treated using statistics (e.g., Poisson statistics) to determine the probability of a transition occurring as a function of time at each discrete location on the solid support. In some particular embodiments, a relatively constant probability of a transition event occurring as a function of time at a discrete location on the solid support indicates the presence of a target analyte at said discrete location on the solid support. In some embodiments, a correlation coefficient relating event number and elapsed time is calculated from the probability of a transition event occurring as a function of time at a discrete location on the solid support. In some embodiments, a correlation coefficient relating event number and elapsed time greater than 0.95 when calculated from the probability of a transition event occurring as a function of time at a discrete location on the solid support indicates the presence of a target analyte at said discrete location on the solid support.

In some embodiments, dwell times of bound query probe (τ_{on}) and unbound query probe (τ_{off}) are used to identify the presence of a target analyte in a sample and/or to distinguish a sample comprising a target analyte from a sample comprising a non-target analyte and/or not comprising the target analyte. For example, the τ_{on} for a target analyte is greater than the τ_{on} for a non-target analyte; and, the τ_{off} for a target analyte is smaller than the τ_{off} for a non-target analyte. In some embodiments, measuring τ_{on} and τ_{off} for a negative control and for a sample indicates the presence or absence of the target analyte in the sample. In some embodiments, a plurality of τ_{on} and τ_{off} values is

determined for each of a plurality of spots imaged on a solid support, e.g., for a control (e.g., positive and/or negative control) and a sample suspected of comprising a target analyte. In some embodiments, a mean τ_{on} and/or τ_{off} is determined for each of a plurality of spots imaged on a solid support, e.g., for a control (e.g., positive and/or negative control) and a sample suspected of comprising a target analyte. In some embodiments, a plot of τ_{on} versus τ_{off} (e.g., mean τ_{on} and τ_{off}, time-averaged τ_{on} and τ_{off}, etc.) for all imaged spots indicates the presence or absence of the target analyte in the sample.

Fluorescent moieties

In some embodiments, a query probe and/or an analyte comprises a fluorescent moiety (e.g., a fluorogenic dye, also referred to as a "fluorophore" or a "fluor"). A wide variety of fluorescent moieties is known in the art and methods are known for linking a fluorescent moiety to analytes and/or query probes.

Examples of compounds that may be used as the fluorescent moiety include but are not limited to xanthene, anthracene, cyanine, porphyrin, and coumarin dyes. Examples of xanthene dyes that find use with the present technology include but are not limited to fluorescein, 6-carboxyfluorescein (6-FAM), 5-carboxyfluorescein (5-FAM), 5- or 6-carboxy-4, 7, 2', 7'- tetrachlorofluorescein (TET), 5- or 6-carboxy-4'5'2'4'5'7' hexachlorofluorescein (HEX), 5' or 6'-carboxy-4',5'-dichloro-2,'7'-dimethoxyfluorescein (JOE), 5-carboxy-2',4',5',7'-tetrachlorofluorescein (ZOE), rhodol, rhodamine, tetramethylrhodamine (TAMRA), 4,7-dlchlorotetramethyl rhodamine (DTAMRA), rhodamine X (ROX), and Texas Red. Examples of cyanine dyes that may find use with the present invention include but are not limited to Cy 3, Cy 3B, Cy 3.5, Cy 5, Cy 5.5, Cy 7, and Cy 7.5. Other fluorescent moieties and/or dyes that find use with the present technology include but are not limited to energy transfer dyes, composite dyes, and other aromatic compounds that give fluorescent signals. In some embodiments, the fluorescent moiety comprises a quantum dot.

In some embodiments, the fluorescent moiety comprises a fluorescent protein (e.g., a green fluorescent protein (GFP), a modified derivative of GFP (e.g., a GFP comprising S65T, an enhanced GFP (e.g., comprising F64L)), or others known in the art such as, e.g., blue fluorescent protein (e.g., EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (e.g., ECFP, Cerulean, CyPet, mTurquoise2), and yellow fluorescent protein derivatives (e.g., YFP, Citrine, Venus, YPet). Embodiments provide that the

fluorescent protein may be covalently or noncovalently bonded to one or more query probes, analytes, and/or capture probes.

Fluorescent dyes include, without limitation, d-Rhodamine acceptor dyes including Cy 5, dichloro[R110], dichloro[R6G], dichloro[TAMRA], dichloro[ROX] or the like, fluorescein donor dyes including fluorescein, 6-FAM, 5-FAM, or the like; Acridine including Acridine orange, Acridine yellow, Proflavin, pH 7, or the like; Aromatic Hydrocarbons including 2-Methylbenzoxazole, Ethyl p-dimethylaminobenzoate, Phenol, Pyrrole, benzene, toluene, or the like; Arylmethine Dyes including Auramine O, Crystal violet, Crystal violet, glycerol, Malachite Green or the like; Coumarin dyes including 7-Methoxycoumarin-4-acetic acid, Coumarin 1, Coumarin 30, Coumarin 314, Coumarin 343, Coumarin 6 or the like; Cyanine Dyes including 1,1'-diethyl-2,2'-cyanine iodide, Cryptocyanine, Indocarbocyanine (C3) dye, Indodicarbocyanine (C5) dye, Indotricarbocyanine (C7) dye, Oxacarbocyanine (C3) dye, Oxadicarbocyanine (C5) dye, Oxatricarbocyanine (C7) dye, Pinacyanol iodide, Stains all, Thiacarbocyanine (C3) dye, ethanol, Thiacarbocyanine (C3) dye, n-propanol, Thiadicarbocyanine (C5) dye, Thiatricarbocyanine (C7) dye, or the like; Dipyrrin dyes including N,N'-Difluoroboryl-1,9-dimethyl-5-(4-iodophenyl)-dipyrrin, N,N'-Difluoroboryl-1,9-dimethyl-5-[(4-(2-trimethylsilylethynyl), N,N'-Difluoroboryl-1,9-dimethyl-5-phenydipyrrin, or the like; Merocyanines including 4-(dicyanomethylene)-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran (DCM), acetonitrile, 4-(dicyanomethylene)-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran (DCM), methanol, 4-Dimethylamino-4'-nitrostilbene, Merocyanine 540, or the like; Miscellaneous Dyes including 4',6-Diamidino-2-phenylindole (DAPI), dimethylsulfoxide, 7-Benzylamino-4-nitrobenz-2-oxa-1,3-diazole, Dansyl glycine, Dansyl glycine, dioxane, Hoechst 33258, DMF, Hoechst 33258, Lucifer yellow CH, Piroxicam, Quinine sulfate, Quinine sulfate, Squarylium dye III, or the like; Oligophenylenes including 2,5-Diphenyloxazole (PPO), Biphenyl, POPOP, p-Quaterphenyl, p-Terphenyl, or the like; Oxazines including Cresyl violet perchlorate, Nile Blue, methanol, Nile Red, ethanol, Oxazine 1, Oxazine 170, or the like; Polycyclic Aromatic Hydrocarbons including 9,10-Bis(phenylethynyl)anthracene, 9,10-Diphenylanthracene, Anthracene, Naphthalene, Perylene, Pyrene, or the like; polyene/polyynes including 1,2-diphenylacetylene, 1,4-diphenylbutadiene, 1,4-diphenylbutadiyne, 1,6-Diphenylhexatriene, Beta-carotene, Stilbene, or the like; Redox-active Chromophores including Anthraquinone, Azobenzene, Benzoquinone, Ferrocene, Riboflavin, Tris(2,2'-bipyridypruthenium(II), Tetrapyrrole, Bilirubin, Chlorophyll a, diethyl ether, Chlorophyll a, methanol, Chlorophyll b, Diprotonated-tetraphenylporphyrin, Hematin,

Magnesium octaethylporphyrin, Magnesium octaethylporphyrin (MgOEP), Magnesium phthalocyanine (MgPc), PrOH, Magnesium phthalocyanine (MgPc), pyridine, Magnesium tetramesitylporphyrin (MgTMP), Magnesium tetraphenylporphyrin (MgTPP), Octaethylporphyrin, Phthalocyanine (Pc), Porphin, ROX, TAMRA, Tetra-t-butylazaporphine, Tetra-t-butylnaphthalocyanine, Tetrakis(2,6-dichlorophenyl)porphyrin, Tetrakis(o-aminophenyl)porphyrin, Tetramesitylporphyrin (TMP), Tetraphenylporphyrin (TPP), Vitamin B12, Zinc octaethylporphyrin (ZnOEP), Zinc phthalocyanine (ZnPc), pyridine, Zinc tetramesitylporphyrin (ZnTMP), Zinc tetramesitylporphyrin radical cation, Zinc tetraphenylporphyrin (ZnTPP), or the like; Xanthenes including Eosin Y, Fluorescein, basic ethanol, Fluorescein, ethanol, Rhodamine 123, Rhodamine 6G, Rhodamine B, Rose bengal, Sulforhodamine 101, or the like; or mixtures or combination thereof or synthetic derivatives thereof.

Several classes of fluorogenic dyes and specific compounds are known that are appropriate for particular embodiments of the technology: xanthene derivatives such as fluorescein, rhodamine, Oregon green, eosin, and Texas red; cyanine derivatives such as cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, and merocyanine; naphthalene derivatives (dansyl and prodan derivatives); coumarin derivatives; oxadiazole derivatives such as pyridyloxazole, nitrobenzoxadiazole, and benzoxadiazole; pyrene derivatives such as cascade blue; oxazine derivatives such as Nile red, Nile blue, cresyl violet, and oxazine 170; acridine derivatives such as proflavin, acridine orange, and acridine yellow; arylmethine derivatives such as auramine, crystal violet, and malachite green; and tetrapyrrole derivatives such as porphin, phtalocyanine, bilirubin. In some embodiments the fluorescent moiety a dye that is xanthene, fluorescein, rhodamine, BODIPY, cyanine, coumarin, pyrene, phthalocyanine, phycobiliprotein, ALEXA FLUOR® 350, ALEXA FLUOR® 405, ALEXA FLUOR® 430, ALEXA FLUOR® 488, ALEXA FLUOR® 514, ALEXA FLUOR® 532, ALEXA FLUOR® 546, ALEXA FLUOR® 555, ALEXA FLUOR® 568, ALEXA FLUOR® 568, ALEXA FLUOR® 594, ALEXA FLUOR® 610, ALEXA FLUOR® 633, ALEXA FLUOR® 647, ALEXA FLUOR® 660, ALEXA FLUOR® 680, ALEXA FLUOR® 700, ALEXA FLUOR® 750, or a squaraine dye. In some embodiments, the label is a fluorescently detectable moiety as described in, e.g., Haugland (September 2005) MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (10th ed.), which is herein incorporated by reference in its entirety.

In some embodiments the label (e.g., a fluorescently detectable label) is one available from ATTO-TEC GmbH (Am Eichenhang 50, 57076 Siegen, Germany), e.g., as

described in U.S. Pat. Appl. Pub. Nos. 20110223677, 20110190486, 20110172420, 20060179585, and 20030003486; and in U.S. Pat. No. 7,935,822, all of which are incorporated herein by reference (e.g., ATTO 390, ATTO 425, ATTO 465, ATTO 488, ATTO 495, ATTO 514, ATTO 520, ATTO 532, ATTO Rho6G, ATTO 542, ATTO 550, ATTO 565, ATTO Rho3B, ATTO Rho11, ATTO Rho12, ATTO Thio12, ATTO Rho101, ATTO 590, ATTO 594, ATTO Rho13, ATTO 610, ATTO 620, ATTO Rho14, ATTO 633, ATTO 647, ATTO 647N, ATTO 655, ATTO Oxa12, ATTO 665, ATTO 680, ATTO 700, ATTO 725, ATTO740).

One of ordinary skill in the art will recognize that dyes having emission maxima outside these ranges may be used as well. In some cases, dyes ranging between 500 nm to 700 nm have the advantage of being in the visible spectrum and can be detected using existing photomultiplier tubes. In some embodiments, the broad range of available dyes allows selection of dye sets that have emission wavelengths that are spread across the detection range. Detection systems capable of distinguishing many dyes are known in the art.

Methods

Some embodiments provide a method of identifying an analyte by repetitive query probe binding. In some embodiments, methods comprise immobilizing an analyte to a solid support. In some embodiments, the solid support is a surface (e.g., a substantially planar surface, a rounded surface), e.g., a surface in contact with a bulk solution, e.g., a bulk solution comprising analyte. In some embodiments, the solid support is a freely diffusible solid support (e.g., a bead, a colloidal particle, e.g., a colloidal particle having a diameter of approximately 10-1000 nm (e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 nm)), e.g., that freely diffuses within the bulk solution, e.g., a bulk solution comprising the analyte. In some embodiments, immobilizing an analyte to a solid support comprises covalent interaction between the solid support and analyte. In some embodiments, immobilizing an analyte to a solid support comprises non-covalent interaction between the solid support and analyte. In some embodiments, the analyte (e.g., a molecule, e.g., a molecule such as, e.g., a protein, peptide, nucleic acid, small molecule, lipid, metabolite, drug, etc.) is stably immobilized to a surface and methods comprise repetitive (e.g., transient, low-affinity) binding of a query probe to the target analyte. In some embodiments, methods comprise detecting the repetitive (e.g., transient, low-affinity) binding of a query probe to the target analyte. In some

embodiments, methods comprise generating a dataset comprising a signal produced from query probe binding to the analyte (e.g., a dataset of query probe signal as a function of time) and information (e.g., coordinates, e.g., x, y coordinates) describing the spatial position on the surface of the query probe binding to the analyte. In some embodiments, the dataset is processed (e.g., manipulated, transformed, visualized, etc.), e.g., to improve the spatial resolution of the query probe binding events. For example, in particular embodiments, the dataset (e.g., comprising query probe signal as a function of time and information (e.g., coordinates, e.g., x, y coordinates) describing the spatial position on the surface of the query probe binding to the analyte) is subjected to processing. In some embodiments, the processing comprises a frame-by-frame subtraction process to generate differential intensity profiles showing query probe binding or dissociation events within each frame of the time series data. Data collected during the development of the technology described herein indicate that the differential intensity profiles have a higher resolution than the query probe binding signal vs. position map. In some embodiments, after determining the spatial position (e.g., x, y coordinates) of each query probe binding and/or dissociation event, a plurality of events is clustered according to spatial position and the kinetics of the events within each cluster are subjected to statistical analysis to determine whether the cluster of events originates from a given target analyte.

For instance, some embodiments of methods for quantifying one or more surface-immobilized or diffusing target analytes comprise one or more steps including, e.g., measuring the signal of one or more transiently binding query probes to the immobilized target analyte(s) with single-molecule sensitivity. In some embodiments, methods comprise tracking (e.g., detecting and/or recording the position of) target analytes independently from query probe binding. In some embodiments, the methods further comprise calculating the time-dependent probe binding signal intensity changes at the surface as a function of position (e.g., x, y position). In some embodiments, calculating the time-dependent query probe binding signal intensity changes at the surface as a function of position (e.g., x, y position) produces a "differential intensity profile" for query probe binding to the analyte. In some embodiments, the methods comprise determining the position (e.g., x, y position) of each query probe binding and dissociation event ("event") with sub-pixel accuracy from a differential intensity profile. In some embodiments, methods comprise grouping events into local clusters by position (e.g., x, y position) on the surface, e.g., to associate events for a single immobilized target analyte. In some embodiments, the methods comprise calculating kinetic parameters from each

local cluster of events to determine whether the cluster originates from a particular analyte, e.g., from transient probe binding to a particular analyte.

Embodiments of methods are not limited in the analyte that is detected. For example, in some embodiments the analyte is polypeptide, e.g., a protein or a peptide. In some embodiments, the target analyte is a nucleic acid. In some embodiments, the target analyte is a small molecule.

In some embodiments, the interaction between the target analyte and the query probe is distinguishably influenced by a covalent modification of the target analyte. For example, in some embodiments, the analyte is a polypeptide comprising a post-translational modification, e.g., a protein or a peptide comprising a post-translational modification. In some embodiments, a post-translational modification of a polypeptide affects the transient binding of a query probe with the analyte, e.g., the query probe signal is a function of the presence or absence of the post-translational modification on the polypeptide. For example, in some embodiments, the analyte is a nucleic acid comprising an epigenetic modification, e.g., a nucleic acid comprising a methylated base. In some embodiments, the analyte is a nucleic acid comprising a covalent modification to a nucleobase, a ribose, or a deoxyribose moiety of the target analyte.

In some embodiments, a modification of a nucleic acid affects the transient binding of a query probe with the analyte, e.g., the query probe signal is a function of the presence or absence of the modification on the nucleic acid.

In some embodiments, the transient interaction between the post-translational modification and the query probe is mediated by a chemical affinity tag, e.g., a chemical affinity tag comprising a nucleic acid.

In some embodiments, the query probe is a nucleic acid or an aptamer.

In some embodiments, the query probe is a low-affinity antibody, antibody fragment, or nanobody.

In some embodiments, the query probe is a DNA-binding protein, RNA-binding protein, or a DNA-binding ribonucleoprotein complex.

In some embodiments, the positon, e.g., the (x,y) position, of each binding or dissociation event is determined by subjecting the differential intensity profile to centroid determination, least-squares fitting to a Gaussian function, least-square fitting to an airy disk function, least-squares fitting to a polynomial function (e.g., a parabola), or maximum likelihood estimation.

In some embodiments, the capture probe is a high-affinity antibody, antibody fragment, or nanobody. In some embodiments, the capture probe is a nucleic acid. In

some embodiments, capture is mediated by a covalent bond cross-linking the target analyte to the surface. In some embodiments, the target analyte is subjected to thermal denaturation in the presence of a carrier prior to surface immobilization. In some embodiments, the analyte is subjected to chemical denaturation in the presence of a carrier prior to surface immobilization, e.g., the analyte is denatured with a denaturant such as urea, formamide, guanidinium chloride, high ionic strength, low ionic strength, high pH, low pH, or sodium dodecyl sulfate (SDS).

Super-resolution imaging

The technology provides a super-resolution identification, detection, quantification, and/or characterization of analytes based on transformation of signals produced by transient binding of query probes to analytes into data providing information relating to the identification, detection, quantification, and/or characterization of analytes. Some embodiments comprise one or more steps as described herein, e.g., one or more ordered steps as described herein. In some embodiments, one or more steps depend on and follow one or more other steps; however, some embodiments comprise one or more of the described steps without respect to any particular order.

As used herein, the term “dataset” or “movie” relates to data comprising a time series of sensor array data (e.g., from a CCD, intensified CCD, electron multiplying CCD, CMOS sensor, or the like), wherein each time point of the time series of sensor array data (or “frame”) comprises a set of signal intensity values as a function of position (e.g., x, y coordinates) within the sensor array. In some embodiments, the (x, y) position refers to the coordinates of sensor elements (e.g., pixels) in the horizontal (x) and vertical (y) directions within the two-dimensional sensor array, e.g., in a frame of a movie dataset.

In some embodiments, a dataset as described is collected from a sample comprising query probes (e.g., some embodiments comprise collecting a dataset from a sample comprising query probes). In some embodiments, the sample comprises analyte. In some embodiments, the sample is believed to comprise analyte. In some embodiments, it is not known if the sample comprises analyte.

In some embodiments, one or more frames of a dataset are corrected, e.g., for lateral drift of the imaging surface with respect to the sensor that results in apparent (x, y) movement of the imaging surface (e.g., some embodiments optionally comprise correcting for drift of the imaging surface with respect to the sensor). For example, most optical microscopes exhibit stage drift that causes a projected image to move (e.g.,

several nanometers to several micrometers) during the course of acquiring a movie, resulting in apparent movement of an object that is in fact stationary with respect to the imaging surface (e.g., one or more frames of the movie are out of register with one or more other frames of the movie). In some embodiments, when datasets are not corrected, the resolution of the technology is decreased, which renders subsequent clustering operations more challenging by broadening the distribution of apparent (x, y) positions of query probe binding events to a single immobilized target analyte. In some embodiments, methods for drift correction include, but are not limited to, e.g., use of fiducial markers whose position is independently measured (e.g., using the same sensor used to detect query probe binding and/or using other, independent sensors that are not used to detect query probe binding). In some embodiments, the positions of one or more fiducial markers is tracked over time to determine the extent and direction of (x, y) drift in each movie frame. In some embodiments, each frame N of the movie is analyzed to determine the (x, y) offset that provides maximal correlation of pixel intensity values of frame N with the intensity values of frame 1 (the first frame of the movie).

Some embodiments comprise generating a differential intensity map movie. For example, some embodiments comprise subtracting the intensity value of each pixel P in each frame N of the movie from the corresponding intensity value of the same pixel P in the next frame N+1 of the movie. The result of these subtractions is a time series of differential intensity maps comprising one fewer frame than the original movie. Some embodiments comprise subtracting the intensity value of each pixel P in each frame N of the movie from the corresponding intensity value of the same pixel P in frame N+2, N+3, N+4, N+n of the movie to produce a time series of differential intensity maps comprising 2, 3, 4, or n fewer frames than the original movie.

Some embodiments comprise recording one or more of the position, intensity, and/or frame number of each intensity maximum (e.g., corresponding to query probe binding events) within each frame of the differential intensity map. In some embodiments, the position is determined by a transformation of the data comprising, e.g., a two-dimensional Gaussian fitting, a centroid fitting, or other methods that are used to determine the position of a particle, e.g., in some embodiments with an error of 1 pixel or less.

Some embodiments comprise recording one or more of the position, intensity (e.g., absolute value of the intensity), and/or frame number of each intensity minimum (e.g., corresponding to query probe dissociation events) within each frame of the differential intensity map. In some embodiments, the position is determined by a transformation of

the data comprising, e.g., a two-dimensional Gaussian fitting, a centroid fitting, or other methods that are used to determine the position of a particle, e.g., in some embodiments with an error of 1 pixel or less.

Some embodiments optionally comprise applying the drift correction calculated in step to the series of (x, y) positions determined from the differential intensity map (e.g., some embodiments comprise correcting the series of (x, y) positions determined from the differential intensity map for lateral drift, e.g., as described above).

Some embodiments comprise combining (x, y) positions of intensity maxima and/or intensity minima. Some embodiments further comprise performing clustering analysis (e.g., hierarchical clustering) on the (x, y) positions of intensity maxima and/or intensity minima to identify regions of high density of query probe binding and dissociation events. In some embodiments, the clustering analysis produces clusters wherein each cluster contains 1 or more binding and/or dissociation event(s) that are detected within a limited region of the sensor.

In some embodiments, optionally, if multiple intensity maxima (or multiple intensity minima) are identified in consecutive frames of the differential intensity map within each cluster, the multiple intensity maxima (or multiple intensity minima) are assumed to correspond to the same binding event (or dissociation event) and are combined by averaging their (x, y) positions of the multiple intensity maxima (or multiple intensity minima) and summing the intensity values of the multiple intensity maxima (or multiple intensity minima).

Some embodiments comprise calculating one or more statistical measures for the events within each cluster, including but not limited to, the number of query probe binding and/or dissociation events; one or more of the mean, median, maximum, minimum, range, and standard deviation of the number of frames between a given binding event and the next dissociation event; one or more of the mean, median, maximum, minimum, range, and standard deviation of the number of frames between a given dissociation event and the next binding event; one or more of the mean, median, maximum, minimum, range, and standard deviation of the (x, y) position of query probe binding and dissociation events; and/or one or more of the mean, median, maximum, minimum, range, and standard deviation of the signal intensity change associated with query probe binding and dissociation events.

Some embodiments comprise comparing the statistics measured as described above for each cluster of query probe binding events to statistics measured using a standard reference material (e.g., a positive control). Some embodiments comprise

comparing the statistics measured as described above for each cluster of query probe binding events to statistics measured using a negative control (e.g., a comprising no analyte, a substance closely related to the analyte, an analyte comprising a modification and/or not comprising a modification, etc.). In some embodiments, comparing the statistics measured as described above for each cluster of query probe binding events to statistics measured using a standard reference material and/or a negative control is used to determine whether the cluster of query probe binding events is probable to have originated from query probe binding to a single molecule of the target analyte.

Some embodiments comprise calculating the number of clusters in the dataset that represent query probe binding to the target analyte. In some embodiments, calculating the number of clusters in the dataset that represent query probe binding to the target analyte comprises using one or more of the statistical tests described above. In some embodiments, calculating the number of clusters in the dataset that represent query probe binding to the target analyte provides a measure of the number of analytes (e.g., the apparent number of analytes) present in the region of the imaging surface that was assayed by the method. In some embodiments, calculating the number of clusters in the dataset that represent query probe binding to the target analyte provides a measure of the concentration of analyte, provides an indication that the analyte is present or absent in the sample, and/or provides an indication of the state (e.g., modified, not modified) of the analyte in the sample.

Some embodiments optionally comprise comparing the apparent number, concentration, state, presence, or absence of analyte as described above to a previously determined value of apparent number, concentration, state, presence, or absence of analyte for a known analyte concentration. Some embodiments comprise use of a standard curve (e.g., generated with one or more compositions comprising a standard reference material of the target analyte having known concentrations) to determine the concentration of the target analyte in the sample.

In some embodiments, steps of the described methods are implemented in software code, e.g., a series of procedural steps instructing a computer and/or a microprocessor to produce and/or transform data as described above. In some embodiments, software instructions are encoded in a programming language such as, e.g., BASIC, C, C++, Java, MATLAB, Mathematica, Perl, Python, or R.

In some embodiments, one or more steps or components of the super-resolution identification, detection, quantification, and/or characterization of analytes are provided in individual software objects connected in a modular system. In some embodiments, the

software objects are extensible and portable. In some embodiments, the objects comprise data structures and operations that transform the object data. In some embodiments, the objects are used by manipulating their data and invoking their methods. Accordingly, embodiments provide software objects that imitate, model, or provide concrete entities, e.g., for numbers, shapes, data structures, that are manipulable. In some embodiments, software objects are operational in a computer or in a microprocessor. In some embodiments, software objects are stored on a computer readable medium.

In some embodiments, a step of a method described herein is provided as an object method. In some embodiments, data and/or a data structure described herein is provided as an object data structure.

Some embodiments provide an object-oriented pipeline for processing a time series of sensor array data, e.g., comprising one or more software objects, to produce a drift-corrected dataset and/or a differential intensity map movie; to identify one or more of the position, intensity, and/or frame number of one or more intensity maxima and/or intensity minima; to transform data, e.g., using a two-dimensional Gaussian fitting, a centroid fitting, or other methods that are used to determine the position of a particle; to correct the series of (x, y) positions determined from the differential intensity map for lateral drift; to combine (x, y) positions of intensity maxima and/or intensity minima; to sum the intensity values of the multiple intensity maxima (or multiple intensity minima); to calculate one or more statistical measures for the events within each cluster; to compare the statistics measured as described above for each cluster of query probe binding events to statistics measured using a standard reference material and/or to a negative control; to calculate the number of clusters in the dataset that represent query probe binding to the target analyte, e.g., to produce a measure of the number of analytes (e.g., the apparent number of analytes) present in the region of the imaging surface that was assayed by the method, a measure of the concentration of analyte, an indication that the analyte is present or absent in the sample, and/or an indication of the state of the analyte in the sample; and/or to compare the apparent number, concentration, state, presence, or absence of analyte as described above to a previously determined value of apparent number, concentration, state, presence, or absence of analyte for a known analyte concentration.

Embodiments comprise use of code that produces and manipulates software objects, e.g., as encoded using a language such as but not limited to Java, C++, C#,

Python, PHP, Ruby, Perl, Object Pascal, Objective-C, Swift, Scala, Common Lisp, and Smalltalk.

Systems

Embodiments of the technology relate to systems for detecting analytes. For example, in some embodiments, the technology provides a system for quantifying one or more target analytes, wherein the system comprises a surface-bound capture probe or a surface-bound moiety that stably binds the target analyte. In some embodiments, the surface-bound capture probe or the surface-bound moiety stably binds the analyte via a binding site, a epitope, or a recognition site (e.g., a first binding site, a first epitope, or a first recognition site). In some embodiments, systems further comprise a query probe that binds the target analyte with a low affinity at a second binding site, a second epitope, or a second recognition site. In some embodiments, the query probe is freely diffusible in the bulk solution contacting the surface of the system. Furthermore, some system embodiments comprise a detection component that records a signal from the interaction of the query probe with the target analyte. For example, in some embodiments the detection component records the change in the signal as a function of time produced from the interaction of the query probe with the target analyte. In some embodiments, the detection component records the spatial position (e.g., as an x, y coordinate pair) and intensity of binding and dissociation events of the query probe to and from said target analyte. In some embodiments, the detection component records the spatial position (e.g., as an x, y coordinate pair) and the beginning and/or ending time of binding and dissociation events of the query probe to and from said target analyte. In some embodiments, the detection component records the spatial position (e.g., as an x, y coordinate pair) and the length of time of binding and dissociation events of the query probe to and from said target analyte.

System embodiments comprise analytical processes (e.g., embodied in a set of instructions, e.g., encoded in software, that direct a microprocessor to perform the analytical processes) to identify an individual molecule of the target analyte. In some embodiments, analytical processes use the spatial position data and timing (e.g., start, end, or length of time) of repeated binding and dissociation events to said target analyte as input data.

Embodiments of systems are not limited in the analyte that is detected. For example, in some embodiments the analyte is polypeptide, e.g., a protein or a peptide. In

some embodiments, the target analyte is a nucleic acid. In some embodiments, the target analyte is a small molecule.

In some embodiments, the interaction between the target analyte and the query probe is distinguishably influenced by a covalent modification of the target analyte. For example, in some embodiments, the analyte is a polypeptide comprising a post-translational modification, e.g., a protein or a peptide comprising a post-translational modification. In some embodiments, a post-translational modification of a polypeptide affects the transient binding of a query probe with the analyte, e.g., the query probe signal is a function of the presence or absence of the post-translational modification on the polypeptide. For example, in some embodiments, the analyte is a nucleic acid comprising an epigenetic modification, e.g., a nucleic acid comprising a methylated base. In some embodiments, a modification of a nucleic acid affects the transient binding of a query probe with the analyte, e.g., the query probe signal is a function of the presence or absence of the modification on the nucleic acid.

In some embodiments, the transient interaction between the post-translational modification and the query probe is mediated by a chemical affinity tag, e.g., a chemical affinity tag comprising a nucleic acid.

In some embodiments, the query probe is a nucleic acid or an aptamer.

In some embodiments, the query probe is a low-affinity antibody, antibody fragment, or nanobody.

In some embodiments, the query probe is a DNA-binding protein, RNA-binding protein, or a DNA-binding ribonucleoprotein complex.

In some embodiments, the analyte is a nucleic acid comprising a covalent modification to a nucleobase, a ribose, or a deoxyribose moiety of the target analyte.

In some embodiments, the capture probe is a high-affinity antibody, antibody fragment, or nanobody. In some embodiments, the capture probe is a nucleic acid. In some embodiments, capture is mediated by a covalent bond cross-linking the target analyte to the surface. In some embodiments, the target analyte is subjected to thermal denaturation in the presence of a carrier prior to surface immobilization. In some embodiments, the analyte is subjected to chemical denaturation in the presence of a carrier prior to surface immobilization, e.g., the analyte is denatured with a denaturant such as urea, formamide, guanidinium chloride, high ionic strength, low ionic strength, high pH, low pH, or sodium dodecyl sulfate (SDS).

Some system embodiments of the technology comprise components for the detection and quantification of a target analyte. Systems according to the technology

comprise, e.g., a solid support (e.g., a microscope slide, a coverslip, an avidin (e.g., streptavidin)-conjugated microscope slide or coverslip, a solid support comprising a zero mode waveguide array, or the like), and a query probe as described herein.

Some system embodiments comprise a detection component that is a fluorescence microscope comprising an illumination configuration to excite bound query probes (e.g., a prism-type total internal reflection fluorescence (TIRF) microscope, an objective-type TIRF microscope, a near-TIRF or HiLo microscope, a confocal laser scanning microscope, a zero-mode waveguide, and/or an illumination configuration capable of parallel monitoring of a large area of the slide or coverslip (> 100 μm^2) while restricting illumination to a small region of space near the surface). Some embodiments comprise a fluorescence detector, e.g., a detector comprising an intensified charge coupled device (ICCD), an electron-multiplying charge coupled device (EM-CCD), a complementary metal-oxide-semiconductor (CMOS), a photomultiplier tube (PMT), an avalanche photodiode (APD), and/or another detector capable of detecting fluorescence emission from single chromophores. Some particular embodiments comprise a component configured for lens-free imaging, e.g., a lens-free microscope, e.g., a detection and/or imaging component for directly imaging on a detector (e.g., a CMOS) without using a lens.

Some embodiments comprise a computer and software encoding instructions for the computer to perform, e.g., to control data acquisition and/or analytical processes for processing data.

Some embodiments comprise optics, such as lenses, mirrors, dichroic mirrors, optical filters, etc., e.g., to detect fluorescence selectively within a specific range of wavelengths or multiple ranges of wavelengths.

For example, in some embodiments, computer-based analysis software is used to translate the raw data generated by the detection assay (e.g., the presence, absence, or amount of one or more analytes, e.g., as a function time and/or position (e.g., x, y coordinates) on the surface) into data of predictive value for a clinician. The clinician can access the predictive data using any suitable means.

Some system embodiments comprise a computer system upon which embodiments of the present technology may be implemented. In various embodiments, a computer system includes a bus or other communication mechanism for communicating information and a processor coupled with the bus for processing information. In various embodiments, the computer system includes a memory, which can be a random access memory (RAM) or other dynamic storage device, coupled to the bus, and instructions to

be executed by the processor. Memory also can be used for storing temporary variables or other intermediate information during execution of instructions to be executed by the processor. In various embodiments, the computer system can further include a read only memory (ROM) or other static storage device coupled to the bus for storing static information and instructions for the processor. A storage device, such as a magnetic disk or optical disk, can be provided and coupled to the bus for storing information and instructions.

In various embodiments, the computer system is coupled via the bus to a display, such as a cathode ray tube (CRT) or a liquid crystal display (LCD), for displaying information to a computer user. An input device, including alphanumeric and other keys, can be coupled to the bus for communicating information and command selections to the processor. Another type of user input device is a cursor control, such as a mouse, a trackball, or cursor direction keys for communicating direction information and command selections to the processor and for controlling cursor movement on the display. This input device typically has two degrees of freedom in two axes, a first axis (e.g., x) and a second axis (e.g., y), that allows the device to specify positions in a plane.

A computer system can perform embodiments of the present technology. Consistent with certain implementations of the present technology, results can be provided by the computer system in response to the processor executing one or more sequences of one or more instructions contained in the memory. Such instructions can be read into the memory from another computer-readable medium, such as a storage device. Execution of the sequences of instructions contained in the memory can cause the processor to perform the methods described herein. Alternatively, hard-wired circuitry can be used in place of or in combination with software instructions to implement the present teachings. Thus, implementations of the present technology are not limited to any specific combination of hardware circuitry and software.

The term “computer-readable medium” as used herein refers to any media that participates in providing instructions to the processor for execution. Such a medium can take many forms, including but not limited to, non-volatile media, volatile media, and transmission media. Examples of non-volatile media can include, but are not limited to, optical or magnetic disks, such as a storage device. Examples of volatile media can include, but are not limited to, dynamic memory. Examples of transmission media can include, but are not limited to, coaxial cables, copper wire, and fiber optics, including the wires that comprise the bus.

Common forms of computer-readable media include, for example, a floppy disk, a flexible disk, hard disk, magnetic tape, or any other magnetic medium, a CD-ROM, any other optical medium, punch cards, paper tape, any other physical medium with patterns of holes, a RAM, PROM, and EPROM, a FLASH-EPROM, any other memory chip or cartridge, or any other tangible medium from which a computer can read.

Various forms of computer readable media can be involved in carrying one or more sequences of one or more instructions to the processor for execution. For example, the instructions can initially be carried on the magnetic disk of a remote computer. The remote computer can load the instructions into its dynamic memory and send the instructions over a network connection (e.g., a LAN, a WAN, the internet, a telephone line). A local computer system can receive the data and transmit it to the bus. The bus can carry the data to the memory, from which the processor retrieves and executes the instructions. The instructions received by the memory may optionally be stored on a storage device either before or after execution by the processor.

In accordance with various embodiments, instructions configured to be executed by a processor to perform a method are stored on a computer-readable medium. The computer-readable medium can be a device that stores digital information. For example, a computer-readable medium includes a compact disc read-only memory (CD-ROM) as is known in the art for storing software. The computer-readable medium is accessed by a processor suitable for executing instructions configured to be executed.

In accordance with such a computer system, some embodiments of the technology provided herein further comprise functionalities for collecting, storing, and/or analyzing data (e.g., presence, absence, concentration of an analyte). For example, some embodiments contemplate a system that comprises a processor, a memory, and/or a database for, e.g., storing and executing instructions, analyzing fluorescence, image data, performing calculations using the data, transforming the data, and storing the data. It some embodiments, an algorithm applies a statistical model (e.g., a Poisson model or hidden Markov model) to the data.

Many diagnostics involve determining the presence of, or a nucleotide sequence of, one or more nucleic acids.

In some embodiments, an equation comprising variables representing the presence, absence, concentration, amount, or sequence properties of one or more analytes produces a value that finds use in making a diagnosis or assessing the presence or qualities of an analyte. As such, in some embodiments this value is presented by a device, e.g., by an indicator related to the result (e.g., an LED, an icon on a display, a

sound, or the like). In some embodiments, a device stores the value, transmits the value, or uses the value for additional calculations. In some embodiments, an equation comprises variables representing the presence, absence, concentration, amount, or properties of one or more analytes.

Thus, in some embodiments, the present technology provides the further benefit that a clinician, who is not likely to be trained in analytical assays, need not understand the raw data. The data are presented directly to the clinician in its most useful form. The clinician is then able to utilize the information to optimize the care of a subject. The present technology contemplates any method capable of receiving, processing, and transmitting the information to and from laboratories conducting the assays, information providers, medical personal, and/or subjects. For example, in some embodiments of the present technology, a sample is obtained from a subject and submitted to a profiling service (e.g., a clinical lab at a medical facility, genomic profiling business, etc.), located in any part of the world (e.g., in a country different than the country where the subject resides or where the information is ultimately used) to generate raw data. Where the sample comprises a tissue or other biological sample, the subject may visit a medical center to have the sample obtained and sent to the profiling center or subjects may collect the sample themselves and directly send it to a profiling center. Where the sample comprises previously determined biological information, the information may be directly sent to the profiling service by the subject (e.g., an information card containing the information may be scanned by a computer and the data transmitted to a computer of the profiling center using electronic communication systems). Once received by the profiling service, the sample is processed and a profile is produced that is specific for the diagnostic or prognostic information desired for the subject. The profile data are then prepared in a format suitable for interpretation by a treating clinician. For example, rather than providing raw expression data, the prepared format may represent a diagnosis or risk assessment for the subject, along with recommendations for particular treatment options. The data may be displayed to the clinician by any suitable method. For example, in some embodiments, the profiling service generates a report that can be printed for the clinician (e.g., at the point of care) or displayed to the clinician on a computer monitor. In some embodiments, the information is first analyzed at the point of care or at a regional facility. The raw data are then sent to a central processing facility for further analysis and/or to convert the raw data to information useful for a clinician or patient. The central processing facility provides the advantage of privacy (all data are stored in a central facility with uniform

security protocols), speed, and uniformity of data analysis. The central processing facility can then control the fate of the data following treatment of the subject. For example, using an electronic communication system, the central facility can provide data to the clinician, the subject, or researchers. In some embodiments, the subject is able to access the data using the electronic communication system. The subject may chose further intervention or counseling based on the results. In some embodiments, the data are used for research use. For example, the data may be used to further optimize the inclusion or elimination of markers as useful indicators of a particular condition associated with the disease.

Samples

In some embodiments, analytes are isolated from a biological sample. Analytes can be obtained from any material (e.g., cellular material (live or dead), extracellular material, viral material, environmental samples (e.g., metagenomic samples), synthetic material (e.g., amplicons such as provided by PCR or other amplification technologies)), obtained from an animal, plant, bacterium, archaeon, fungus, or any other organism. Biological samples for use in the present technology include viral particles or preparations thereof. Analytes can be obtained directly from an organism or from a biological sample obtained from an organism, e.g., from blood, urine, cerebrospinal fluid, seminal fluid, saliva, sputum, stool, hair, sweat, tears, skin, and tissue. Exemplary samples include, but are not limited to, whole blood, lymphatic fluid, serum, plasma, buccal cells, sweat, tears, saliva, sputum, hair, skin, biopsy, cerebrospinal fluid (CSF), amniotic fluid, seminal fluid, vaginal excretions, serous fluid, synovial fluid, pericardial fluid, peritoneal fluid, pleural fluid, transudates, exudates, cystic fluid, bile, urine, gastric fluids, intestinal fluids, fecal samples, and swabs, aspirates (e.g., bone marrow, fine needle, etc.), washes (e.g., oral, nasopharyngeal, bronchial, bronchialalveolar, optic, rectal, intestinal, vaginal, epidermal, etc.), breath condensate, and/or other specimens.

Any tissue or body fluid specimen may be used as a source of analytes for use in the technology, including forensic specimens, archived specimens, preserved specimens, and/or specimens stored for long periods of time, e.g., fresh-frozen, methanol/acetic acid fixed, or formalin-fixed paraffin embedded (FFPE) specimens and samples. Analytes can also be isolated from cultured cells, such as a primary cell culture or a cell line. The cells or tissues from which analytes are obtained can be infected with a virus or other intracellular pathogen. A sample can also be total RNA extracted from a biological

specimen, a cDNA library, viral, or genomic DNA. A sample may also be isolated DNA from a non-cellular origin, e.g. amplified/isolated DNA that has been stored in a freezer.

Analytes (e.g., nucleic acid molecules, polypeptides, lipids) can be obtained, e.g., by extraction from a biological sample, e.g., by a variety of techniques such as those described by Maniatis, et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. (see, e.g., pp. 280–281).

In some embodiments, the technology provides for the size selection of analytes, e.g., to provide a defined size range of molecules including the target analytes.

Uses

Various embodiments relate to the detection of a wide range of analytes. For example, in some embodiments the technology finds use in detecting a nucleic acid (e.g., a DNA or RNA). In some embodiments, the technology finds use in detecting a nucleic acid comprising a particular target sequence. In some embodiments, the technology finds use in detecting a nucleic acid comprising a particular mutation (e.g., a single nucleotide polymorphism, an insertion, a deletion, a missense mutation, a nonsense mutation, a genetic rearrangement, a gene fusion, etc.). In some embodiments, the technology finds use in detection a polypeptide (e.g., a protein, a peptide). In some embodiments, the technology finds use in detecting a polypeptide encoded by a nucleic acid comprising a mutation (e.g., a polypeptide comprising a substitution, a truncated polypeptide, a mutant or variant polypeptide).

In some embodiments, the technology finds use in detecting post-translational modifications to polypeptides (e.g., phosphorylation, methylation, acetylation, glycosylation (e.g., O-linked glycosylation, N-linked glycosylation, ubiquitination, attachment of a functional group (e.g., myristoylation, palmitoylation, isoprenylation, prenylation, farnesylation, geranylation, geranylgeranylation, glypiation, glycosylphosphatidylinositol (GPI) anchor formation), hydroxylation, biotinylation, pegylation, oxidation, SUMOylation, disulfide bridge formation, disulfide bridge cleavage, proteolytic cleavage, amidation, sulfation, pyrrolidone carboxylic acid formation. In some embodiments, the technology finds use in the detection of the loss of these features, e.g., dephosporylation, demethylation, deacetylation, deglycosylation, deamidation, dehydroxylation, deubiquitination, etc. In some embodiments, the technology finds use in detecting epigenetic modifications to DNA or RNA (e.g., methylation (e.g., methylation of CpG sites), hydroxymethylation). In some embodiments, the technology finds use in detecting the loss of these features, e.g.,

demethylation of DNA or RNA, etc. In some embodiments, the technology finds use in detecting alterations in chromatin structure, nucleosome structure, histone modification, etc., and in detecting damage to nucleic acids.

In some embodiments, the technology finds use as a molecular diagnostic assay, e.g., to assay samples having small specimen volumes (e.g., a droplet of blood, e.g., for mail-in service). In some embodiments, the technology provides for the early detection of cancer or infectious disease using sensitive detection of very low-abundance analyte biomarkers. In some embodiments, the technology finds use in molecular diagnostics to assay epigenetic modifications of protein biomarkers (e.g., post-translational modifications).

In some embodiments, the technology finds use in characterizing multimolecular complexes (e.g., characterizing one or more components of a multimolecular complex), e.g., a multiprotein complex, a nucleic acid/protein complex, a molecular machine, an organelle (e.g., a cell-free mitochondrion, e.g., in plasma), cell, virus particle, organism, tissue, or any macromolecular structure or entity that can be captured and is amenable to analysis by the technology described herein. For example, in some embodiments a multimolecular complex is isolated and the technology finds use in characterizing, identifying, quantifying, and/or detecting one or more molecules (analytes) associated with the multimolecular complex. In some embodiments an extracellular vesicle is isolated and the technology finds use in characterizing, identifying, quantifying, and/or detecting one or more molecules (analytes) associated with the vesicle. In some embodiments, the technology finds use in characterizing, identifying, quantifying, and/or detecting a protein (e.g., a surface protein) and/or an analytes present inside the vesicle, e.g., a protein, nucleic acid, or other analyte described herein. In some embodiments, the vesicle is fixed and permeabilized prior to analysis.

Examples

Example 1 – Identifying surface-immobilized targets via repetitive query probe binding.

During the development of embodiments of the technology described herein, experiments were conducted in which a target analyte (e.g., a molecule such as a protein, a peptide, a nucleic acid, etc.) was stably immobilized to a surface. Then, a low-affinity probe was added and repetitive binding of the low-affinity query probe to the target analyte was detected and recorded to generate a dataset of query probe signal vs. spatial position on the surface (see Figure 1).

During the experiments, data were collected comprising a time series of query probe signal (association (binding of query probe to analyte) and dissociation events) versus position on the surface. These data were analyzed suing a method of frame-by-frame subtraction to generate differential intensity profiles showing the query probe binding or dissociation events within each frame. The differential intensity profiles produced by this treatment of the data yielded intensity minima and maxima that were better resolved than the intensity maxima within the initial signal versus position map. After determining the position (e.g., using x, y coordinates) of each event, all events are clustered according to spatial position and the kinetics of events within each cluster are subjected to statistical analysis to determine whether the cluster of events originates from a given target analyte (Figure 1).

Example 2 – Comparison with other imaging methods

During the development of embodiments of the technology described herein, experiments were conducted to compare data collected using the technology described herein with other imaging technologies. In particular, DNA analytes were immobilized to a surface and imaged using the super-resolution imaging and event clustering as described herein (see, e.g., Example 1). As shown in Figure 2, the present technology provides an improvement in the dynamic range of measurement of a DNA target analyte present in the picomolar range when using the presently described super-resolution imaging and event clustering from differential intensity maps (filled squares) relative to a diffraction-limited imaging method (e.g., as described in US20160046988) (empty circles). The localization of each individual binding or dissociation event, followed by clustering and kinetic analysis of the binding or dissociation events, permits the resolution of multiple closely spaced target analytes on a surface.

Example 3 – Specificity of the assay

During the development of the technology described herein, experiments were conducted to assess the specificity of the super-resolution imaging and event clustering technologies as described herein. In particular, the specificity of detection of a nucleic acid comprising a mutant sequence was tested by comparing the number of molecules detected in the presence of either (1) only mutant sequence at a known concentration, or (2) only wild-type sequence at a 10,000-fold higher concentration. In the presence of the mutant DNA sequence, several (e.g., approximately 399) clusters of binding events (Figure 3A, dark black spots) within an imaging field of view exhibited kinetics

characteristic of query probe binding to the mutant sequence, while nonspecific binding events (Figure 3A, light gray circles) are removed by statistical analysis. In contrast, when the same experiment was performed in the absence of mutant and in the presence of a 10,000-fold higher concentration of wild-type DNA sequence, all query probe binding events are removed by statistical analysis of local clusters of events, resulting in no false positive detection events (Figure 3B).

All publications and patents mentioned in the above specification are herein incorporated by reference in their entirety for all purposes. Various modifications and variations of the described compositions, methods, and uses of the technology will be apparent to those skilled in the art without departing from the scope and spirit of the technology as described. Although the technology has been described in connection with specific exemplary embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the following claims.

CLAIMS

WE CLAIM:

1. A method of characterizing an analyte in a sample, the method comprising:
   (a) recording a time-dependent signal of query probe events for analytes immobilized to a surface as a function of (x, y) position on the surface;
   (b) clustering events into local clusters by (x, y) position; and
   (c) calculating a kinetic parameter for each event cluster to characterize the analyte.

2. The method of claim 1 wherein the surface is a solid support.

3. The method of claim 1 wherein the surface is diffusible.

4. The method of claim 1 wherein recording a time-dependent signal of query probe events comprises measuring the signal for an analyte with single-molecule sensitivity.

5. The method of claim 1 further comprising calculating a differential intensity map comprising the time-dependent signal intensity changes at the surface as a function of (x, y) position.

6. The method of claim 1 wherein (x, y) position is determined with sub-pixel accuracy.

7. The method of claim 1 wherein clustered events represent binding events for a single analyte molecule.

8. The method of claim 1 wherein characterizing the analyte comprises indicating the presence, absence, concentration, or number of the analyte in the sample.

9. The method of claim 1 wherein the analyte comprises a polypeptide.

10. The method of claim 9 wherein the method indicates the presence or absence of a post-translational modification on the polypeptide.

11. The method of claim 10 wherein the post-translational modification mediates a transient association of the query probe with the polypeptide.

12. The method of claim 10 wherein a chemical affinity tag mediates a transient association between the post-translational modification and the query probe.

13. The method of claim 12 wherein the chemical affinity tag is a nucleic acid.

14. The method of claim 1 wherein the analyte is a nucleic acid.

15. The method of claim 1 wherein a transient association of the query probe with the analyte is distinguishably affected by a covalent modification of the analyte.

16. The method of claim 1 wherein the query probe is a nucleic acid or aptamer.

17. The method of claim 1 wherein the query probe is a low-affinity antibody, an antibody fragment, or a nanobody.

18. The method of claim 1 wherein the query probe is a DNA-binding protein, an RNA-binding protein, or a DNA-binding ribonucleoprotein complex.

19. The method of claim 5 wherein the (x, y) position of each query probe event is determined by processing the differential intensity profile using centroid determination, least-squares fitting to a Gaussian function, least-squares fitting to an airy disk function, least-squares fitting to a polynomial function, or maximum likelihood estimation.

20. The method of claim 1 wherein the analyte is subjected to thermal or chemical denaturation in the presence of a carrier prior to surface immobilization.

21. A system for quantifying an analyte in a sample, the system comprising:

(a) a functionality to stably immobilize an analyte to a surface;

(b) a freely diffusing query probe that binds to the target analyte with a low affinity; and

(c) a detection system that records query probe events and the spatial position of query probe events for analytes.

22. The system of claim 21 further comprising analytical procedures to identify an individual molecular copy of the target analyte according to the spatial position and timing of repeated binding and dissociation events to said target analyte.

23. The system of claim 21 wherein the query probe is a nucleic acid or aptamer.

24. The system of claim 21 wherein the query probe is a low-affinity antibody, an antibody fragment, or a nanobody.

25. The system of claim 24 wherein the query probe is a DNA-binding protein, an RNA-binding protein, or a DNA-binding ribonucleoprotein complex.

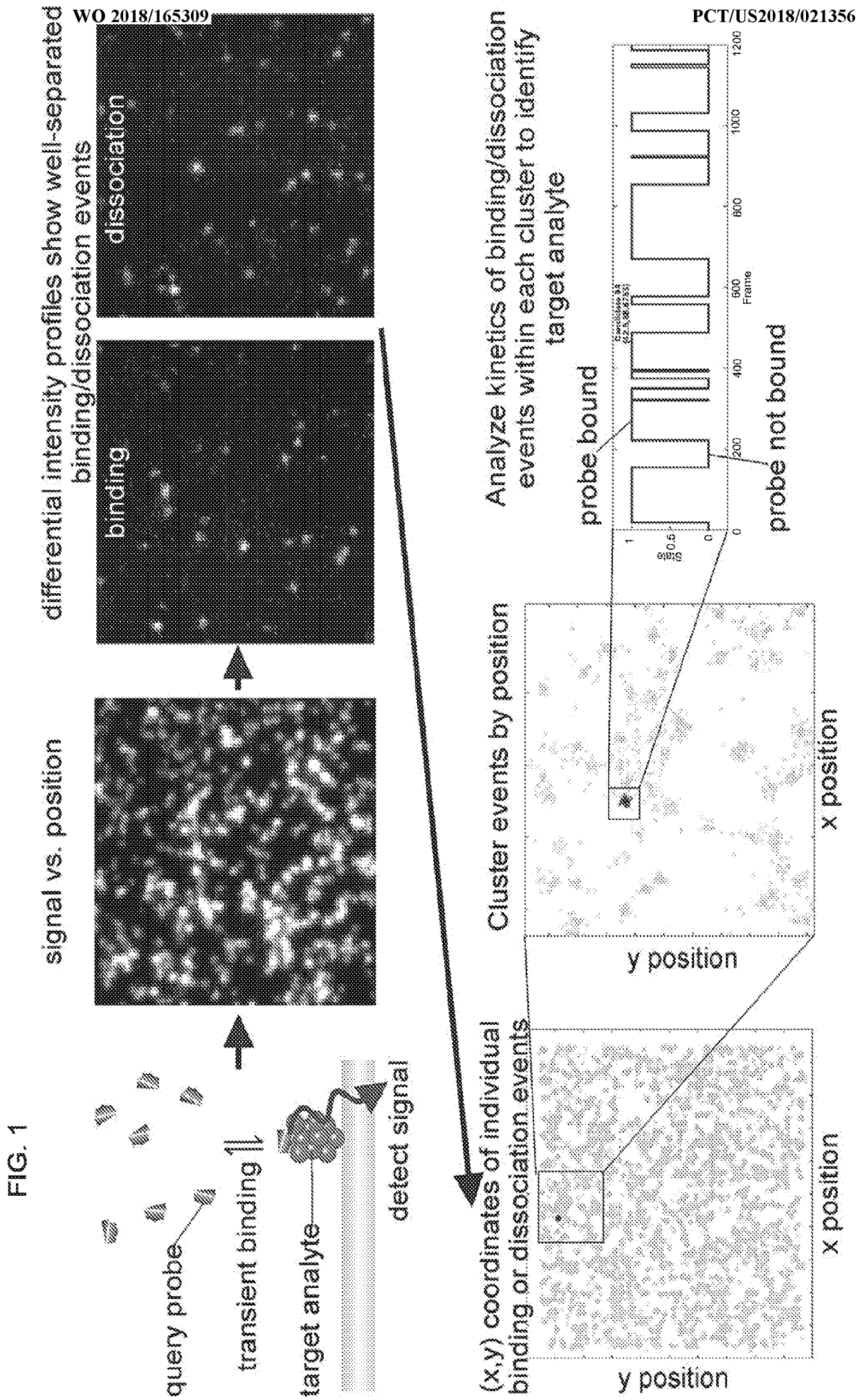
26. The system of claim 24 wherein the functionality to stably immobilize an analyte to a surface comprise a surface-bound capture probe that stably binds the target analyte.

27. The system of claim 26 wherein the capture probe is a high-affinity antibody, an antibody fragment, or a nanobody.

28. The system of claim 24 wherein the functionality to stably immobilize an analyte to a surface is a covalent bond cross-linking the target analyte to the surface.

29. The system of claim 24 wherein the analyte is subjected to thermal or chemical denaturation in the presence of a carrier prior to surface immobilization.

30. The method of claim 1 or the system of claim 21 wherein the analyte is a component of a macromolecular complex.


query probe
transient binding
target analyte
detect signal
signal vs. position
differential intensity profiles show well-separated binding/dissociation events
binding
dissociation
(x,y) coordinates of individual binding or dissociation events
Cluster events by position
x position
y position
x position
y position
Analyze kinetics of binding/dissociation events within each cluster to identify target analyte
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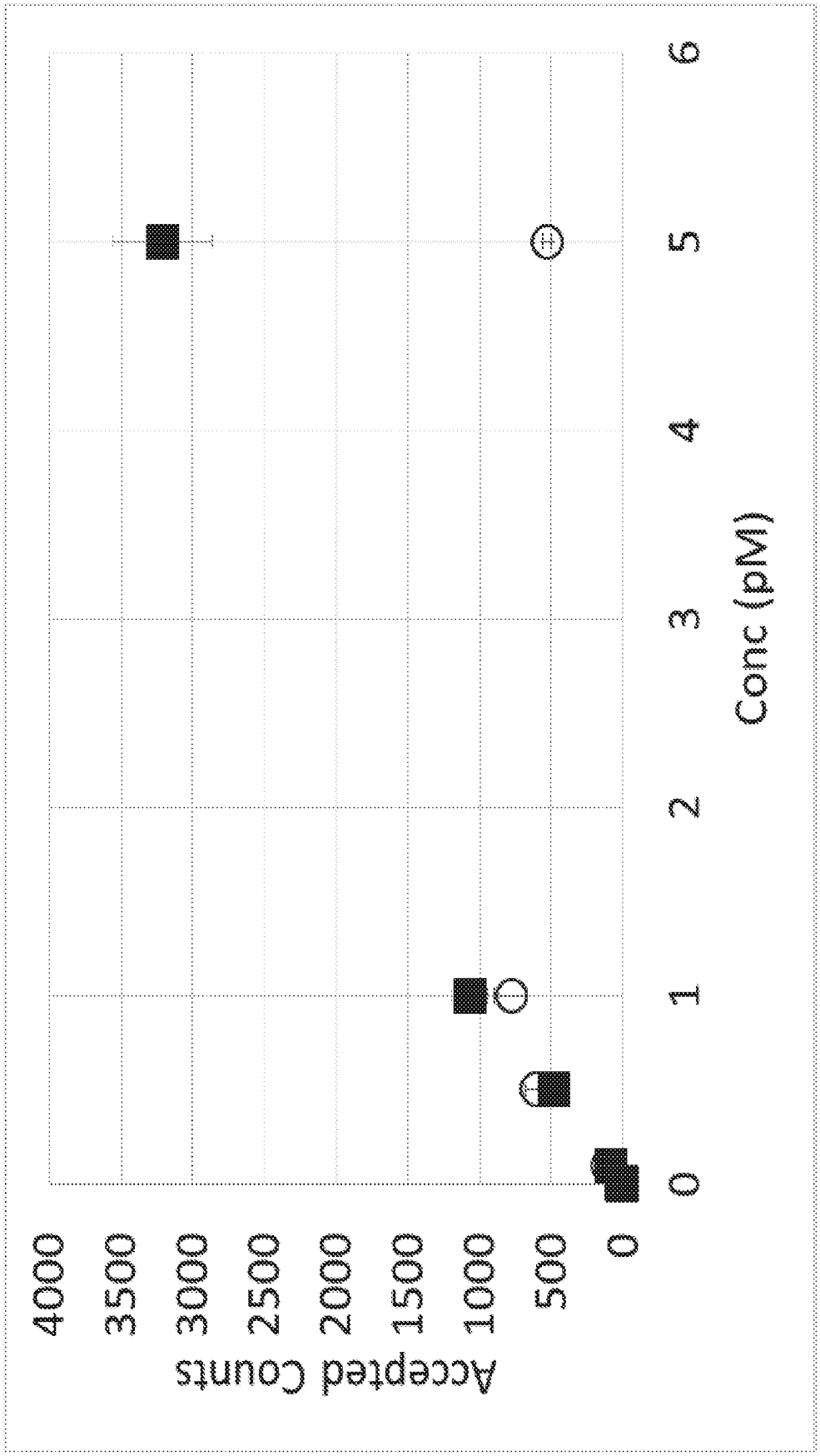
probe not bound
State
Frame
0
200
400
600
800
1000
1200
0
0.5
1

FIG. 1


Accepted Counts
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1
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3
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Conc (pM)

FIG. 2

FIG. 3A

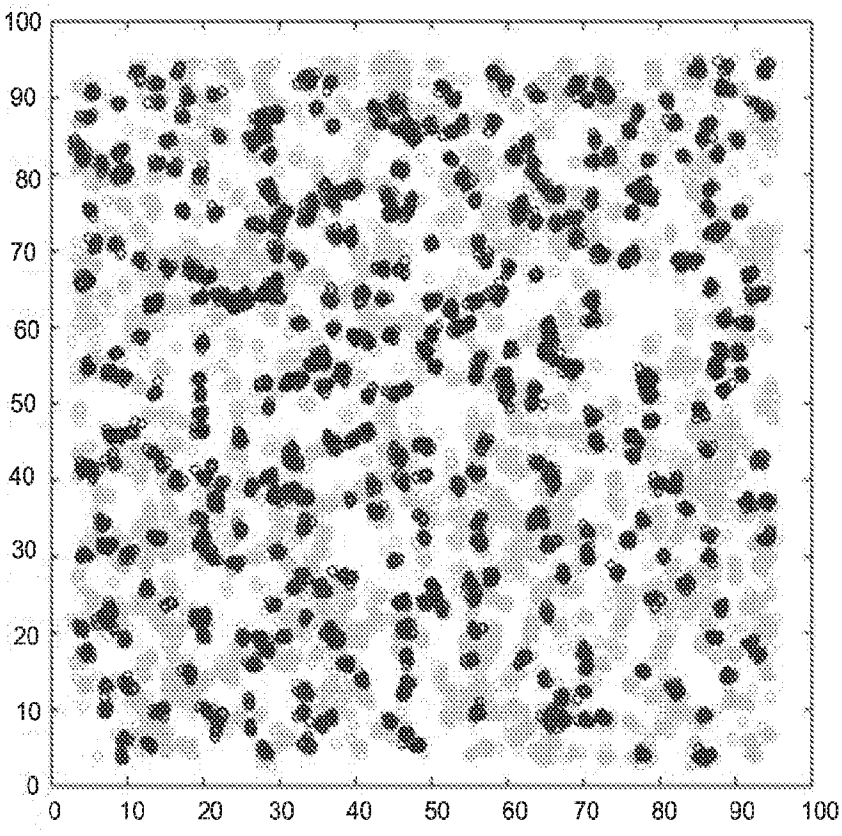
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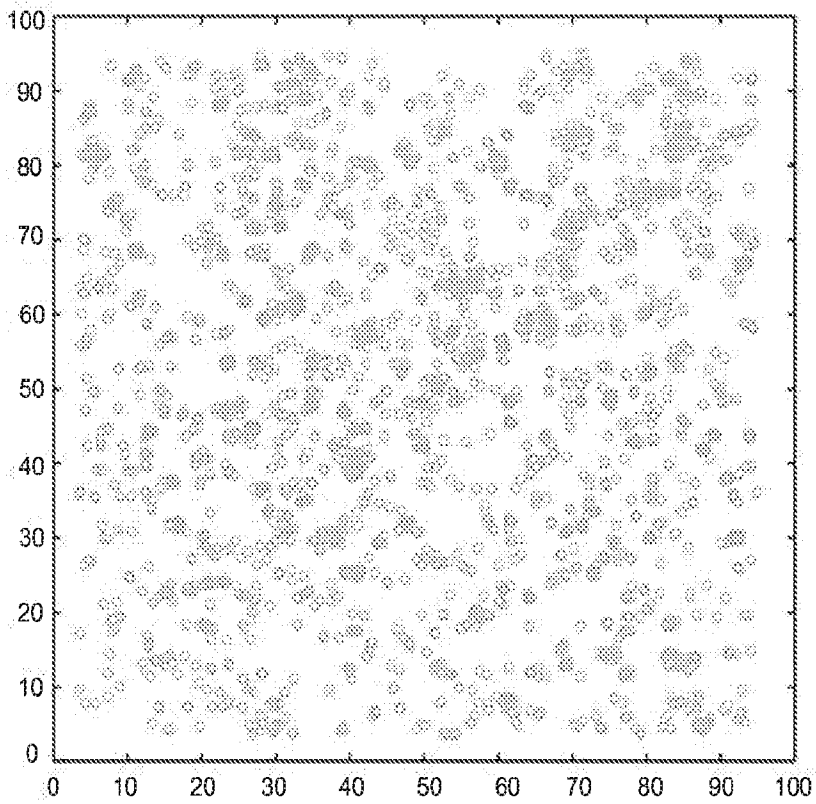
FIG. 3B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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/021356

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 16/00; C12N 15/115; C12Q 1/68; C12Q 1/6816; G01N 21/29; G01N 21/64 (2018.01)

CPC - C12Q 1/68; C12Q 1/6816; C12Q 1/6825; C12Q 1/6834; C12Q 1/6876; C12Q 2525/204; C12Q 2525/205; C12Q 2563/107; C12Q 2565/601; G01N 21/64; G01N 21/6458; G01N 2458/10; G02B 21/0032; G06F 19/24; G06T 7/0016 (2018.02)

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

USPC - 435/6.11; 435/6.19; 436/172; 436/501; 436/517; 506/9; 536/22.1; 536/24.3; 702/19; 702/23; 702/27 (keyword delimited)

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.
X	US 2016/0161472 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 09 June 2016 (09.06.2016) entire document	1, 2, 4-9, 14-16, 19, 21-23, 30
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Y		3, 10-13, 17, 18, 20, 24-29
Y	US 2005/0244863 A1 (MIR) 03 November 2005 (03.11.2005) entire document	3
Y	US 2016/0312272 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 27 October 2016 (27.10.2016) entire document	10-13
Y	US 2016/0046988 A1 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 18 February 2016 (18.02.2016) entire document	17, 18, 24-29
Y	US 2016/0169903 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 16 June 2016 (16.06.2016) entire document	20, 29
P, A	COHEN et al. "Digital Direct Detection of MicroRNAs Using Single Molecule Arrays," Nucleic Acids Research, 20 June 2017 (20.06.2017), Vol. 45, No. 14, e137, Pgs.1-9. entire document	1-30
P, X	WANG et al. "A Dynamic Sandwich Assay on Magnetic Beads for Selective Detection of Single-Nucleotide Mutations at Room Temperature," Biosensors and Bioelectronics, 15 August 2017 (15.08.2017), Vol. 94, Pgs. 305-311. entire document	1-30

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* 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
27 April 2018	25 MAY 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/021356

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JUNGMANN et al. "Quantitative Super-Resolution Imaging with qPAINT Using Transient Binding Analysis," Nature Methods, 28 March 2016 (28.03.2016), Vol. 13, Pgs. 439-442. entire document	1-30