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(54) Title: QUANTITATIVE DNA-BASED IMAGING AND SUPER-RESOLUTION IMAGING

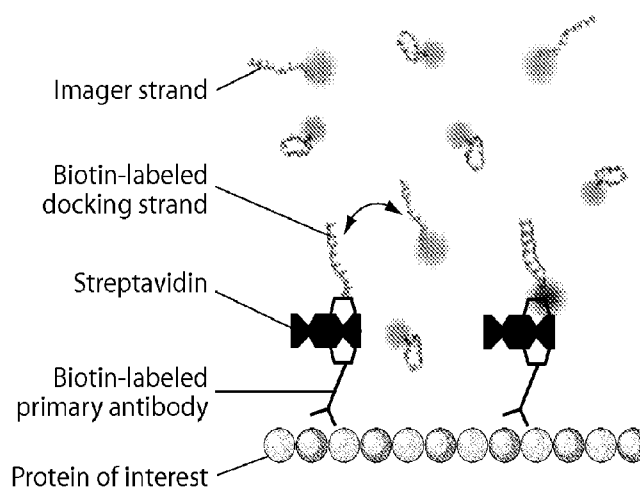


Fig. 2

(57) Abstract: The present disclosure provides, *inter alia*, methods and compositions (e.g., conjugates) for imaging, at high spatial resolution, targets of interest.



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QUANTITATIVE DNA-BASED IMAGING AND SUPER-RESOLUTION IMAGING

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 61/934,759, filed February 1, 2014, U.S. provisional application number 61/884,126, filed September 29, 2013, and U.S. provisional application number 61/859,891, filed July 30, 2013, each of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The present disclosure relates generally to the field of detection and quantification of targets.

BACKGROUND OF THE INVENTION

Far-field fluorescence microscopy has seen major advances since the advent of methods that circumvent the classical diffraction limit, *e.g.*, super-resolution microscopy (refs. 1, 2). Most implementations switch molecules between fluorescent ON- and OFF-states to allow consecutive localization of individual molecules. Switching is traditionally obtained in one of two ways: “targeted” switching actively confines the fluorescence excitation to an area smaller than the diffraction of light (*e.g.*, stimulated emission depletion microscopy, or STED (ref. 3)), whereas “stochastic” switching uses photoswitchable proteins (photoactivated localization microscopy, or PALM (ref. 4)) or photoswitchable organic dyes (stochastic optical reconstruction microscopy, or STORM (ref. 1)). Although these methods offer enhanced spatial resolution, they tend to require either expensive instrumentation or highly specialized experimental conditions, and thus have not yet been developed into common biological laboratory techniques.

SUMMARY OF THE INVENTION

The present disclosure provides, *inter alia*, methods, compositions (*e.g.*, conjugates) and kits for imaging, at high or low spatial resolution, targets (*e.g.*, biomolecules) of interest in, for example, a cellular environment. The methods, compositions and kits of the present disclosure take advantage of repetitive, transient binding of short, labeled (*e.g.*, fluorescently labeled) oligonucleotides (*e.g.*, DNA oligonucleotides), or “imager” strands, to complementary “docking” strands, which are attached to targets of interest, in some embodiments, through an intermediate molecule such as an antibody such as a primary or a

secondary antibody, to obtain stochastic switching between fluorescent ON- and OFF-states (FIGs. 1A and 1B). In the unbound state, only background fluorescence from partially quenched (ref. 8) imager strands is observed (depicted by dimmer fluorescence of unbound imager strands in FIG. 1A). This is considered an “OFF” state. Upon binding and
5 immobilization of an imager strand, fluorescence emission is detected using, for example, total internal reflection (TIR) or highly inclined and laminated optical sheet (HILO) microscopy (ref. 9). This is considered an “ON” state. In general, the methods, compositions and kits as provided herein increase the imaging resolution and thus the sensitivity of detection. In some aspects, they also increase the specificity as well as the number of
10 utilizable fluorophores available for detecting targets of interest including but not limited to, *e.g.*, naturally-occurring biomolecules.

By linking a short docking strand to a binding partner (*e.g.*, a protein-binding moiety or a nucleic acid-binding moiety, whether primary or secondary), such as an antibody including a primary and a secondary antibody, different species of targets (*e.g.*, biomolecules,
15 optionally in a cellular environment) can be labeled and subsequently detected by introducing fluorescently-labeled imager strands that are complementary to and bind to the docking strands through transient Watson-Crick interactions. Unlike existing detection methods, the methods of the present disclosure are not limited by the number of spectrally distinct fluorophores available for detecting distinct targets (*e.g.*, biomolecules). Rather, the
20 programmability of nucleic acid (*e.g.*, DNA and/or RNA) molecules and sequential time-lapsed imaging are used herein to provide images of up to hundreds of distinct species of targets using, in some embodiments, only a single optimized fluorophore. Further, these different species of targets (*e.g.*, biomolecules) can be quantified using predictable kinetics of binding of single fluorescently-labeled imager strands to their complementary target docking
25 strands.

In some instances, the methods can be used to generate super-resolution images, significantly even without the need for a super-resolution microscope. It should be understood that while the methods, compositions and kits as provided herein may be described for use in super-resolution imaging, they may also be used, in some embodiments,
30 for imaging that does not require super-resolution. Thus, in some embodiments, the methods, compositions and kits of the present disclosure may be used for imaging, generally.

In some aspects, provided herein is a protein-nucleic acid conjugate, comprising a protein linked to a docking strand that is capable of transiently binding to a complementary labeled imager strand. In some aspects, provided herein is a protein-nucleic acid conjugate,

comprising a protein linked to a docking strand that is transiently bound to a complementary labeled imager strand. Imager strands, in some embodiments, are labeled with a detectable label. The detectable label may be, for example, a fluorescent label or other detectable label, *e.g.*, gold nanoparticle. While various aspects and embodiments herein refer to fluorescently-labeled imager strands, it should be understood that such fluorescent labels, in many instances, can be interchanged with other detectable labels. Thus, in some embodiments, a fluorescently-labeled imager strand (which may be detected by, for example, fluorescent microscopy) may be interchanged with an imager strand labeled with, for example, gold nanoparticles (which may be detected by, for example, dark field microscopy). It is also to be understood that the docking strands may be capable of transiently binding a plurality of complementary labeled strands (*e.g.*, the docking strand may comprise a plurality of binding sites for complementary labeled strands).

In some embodiments, a method may be carried out involving a plurality of docking strand and imager strand pairs. Such a method can be used to detect a plurality of targets (*e.g.*, with each docking strand-imager strand pair corresponding to one target). The docking strand-imager strand pairs in the plurality must share an approximately equal probability of hybridizing under a single environment or condition (as defined for example by temperature, salt concentration, strand molarity, *etc.*), such that if there is an observed difference between the level of binding (and thus the detection) of a population of imager strands, an end user can conclude that such difference is a function of the amount of docking strand and thus ultimately the amount of target. In some embodiments, the docking and imager strands are typically selected such that their bound states have a thermal stability in the range of about ± 0.5 kcal/mol. With this range of thermal stability, it is possible to select at least 200 orthogonal (*e.g.*, different) sequences to be used in these multiplexing methods.

In some embodiments, a protein is an antibody such as a primary antibody or a secondary antibody, an antigen-binding antibody fragment, or a peptide aptamer.

In some embodiments, a protein is linked to the docking strand through an intermediate linker. In some embodiments, the intermediate linker comprises biotin and streptavidin.

In some embodiments, an antibody is a monoclonal antibody.

In some embodiments, a complementary fluorescently-labeled imager strand comprises at least one fluorophore.

In some embodiments, a complementary labeled, optionally fluorescently labeled, imager strand is about 4 to about 30 nucleotides, or about 8 to about 10 nucleotides, in length. In some embodiments, a complementary labeled imager strand is longer than 30 nucleotides.

In this and other aspects and embodiments described herein, the docking strand may
5 comprise a plurality of domains, each complementary to a labeled imager strand. The domains may be identical in sequence (and thus will bind to the identical imager strands) or they may be of different sequence (and thus may bind to imager strands that are not identically labeled). Such domains may also be referred to herein as binding sites for imager strands.

10 In some embodiments, a docking strand includes at least two or at least three domains, each respectively complementary to a labeled imager strand.

In some aspects, provided herein is a target bound to at least one protein-nucleic acid conjugate.

In some embodiments, the target is a protein. In some embodiments, the target is a
15 nucleic acid (*e.g.*, DNA or RNA).

In some aspects, provided herein is a plurality of protein-nucleic acid conjugates. In some embodiments, the plurality comprises at least two subsets of the protein-nucleic acid conjugates, and the protein-nucleic acid conjugates of each subset bind to different targets.

In some aspects, provided herein is a composition or kit comprising a plurality of
20 protein-nucleic acid conjugates, optionally wherein at least one of the protein-nucleic acid conjugates is bound to at least one target.

In some aspects, provided herein is a composition or kit comprising at least one protein-nucleic acid conjugate that comprises a protein linked to a docking strand, optionally wherein the at least one protein-nucleic acid conjugate is bound to a target, and at least one
25 complementary labeled, optionally fluorescently labeled, imager strand that is transiently bound to (or is capable of transiently binding to) the at least one protein-nucleic acid conjugate.

In some embodiments, a composition or kit comprises at least two complementary labeled, optionally fluorescently labeled, imager strands, wherein the at least two
30 complementary labeled imager strands are identical. In some embodiments, the composition or kit comprises at least two complementary labeled imager strands, wherein the at least two complementary labeled imager strands are different.

In some embodiments, the number of complementary labeled, optionally fluorescently labeled, imager strands is less than, greater than or equal to the number of protein-nucleic acid conjugates.

In some embodiments, a composition or kit comprises at least 2, 3, 4, 5, 6, 7, 9 or 10 different complementary labeled, optionally fluorescently labeled, imager strands. In some embodiments, the composition or kit comprises at least 50 or at least 100 different complementary fluorescently-labeled imager strands.

In some aspects, provided herein is a composition or a kit comprising a (*e.g.*, one or more) docking strand and an (*e.g.*, one or more) imager strand. The docking strand may be modified to include an affinity label, thereby facilitating its subsequent attachment to one or more binding partners, such as an antibodies. For example, the docking strand may be biotinylated or it may be attached to avidin or streptavidin. Other affinity labels can be used instead. The imager strands may be labeled, such as fluorescently labeled. The imager strands may be a plurality of identical imager strands (*e.g.*, with respect to sequence and label) or they may be a plurality of different imager strands (*e.g.*, with respect to sequence and label). The composition or kit may further comprise a target-specific binding partner, such as an antibody. It is to be understood that the components may be bound to each other or they may be unbound, including physically separated from each other, in such compositions and kits. These and other compositions and kits may further comprise one or more buffers including oxygen scavengers.

In some aspects, provided herein is a composition or kit comprising an antibody-nucleic acid conjugate, wherein the antibody is a “secondary antibody” having specificity for an antibody, typically specificity for a particular isotype or an Fc domain of an antibody from a particular species (*e.g.*, a mouse antibody that is specific for a human IgG1 antibody). The nucleic acid in the conjugate is a docking strand, as described herein. The composition or kit may further comprise one or more imager strands (or one or more subsets or populations of imager strands), as described herein. These and other compositions and kits may further comprise one or more buffers including oxygen scavengers.

In some aspects, the present disclosure provides an antibody-DNA conjugate, comprising a monoclonal antibody linked to a docking strand that is bound to a complementary labeled, optionally fluorescently labeled, imager strand, wherein the antibody and the docking strand are each biotinylated and linked to each other through an avidin or streptavidin linker or a biotin-streptavidin linker.

In some aspects, provided herein is an aptamer-nucleic acid conjugate, comprising a nucleic acid aptamer linked to a docking strand that is transiently bound to a complementary labeled, optionally fluorescently labeled, imager strand.

5 In some aspects, provided herein is a method of detecting a target in a sample, the method comprising contacting a sample with (a) at least one protein-nucleic acid conjugate that comprises a protein linked to a docking strand and (b) at least one fluorescently-labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one protein-nucleic acid conjugate, and determining whether the at least one protein-nucleic acid conjugate binds to the target in the sample. In some embodiments, the
10 determining step comprises imaging transient binding of the at least one fluorescently-labeled imager strand to the docking strand of the at least one protein-nucleic acid conjugate.

In some aspects, provided herein is a method of detecting a target in a sample, the method comprising contacting a sample with (a) at least one protein-nucleic acid conjugate that comprises a protein linked to a docking strand and (b) at least one fluorescently-labeled
15 imager strand that is complementary to and transiently binds to the docking strand of the at least one protein-nucleic acid conjugate, and imaging transient binding, optionally using time-lapsed imaging, of the at least one fluorescently-labeled imager strand to the docking strand of the at least one protein-nucleic acid conjugate.

In some embodiments, a protein of the protein-nucleic acid conjugate is an antibody, an antigen-binding antibody fragment, or a peptide aptamer. In some embodiments, an
20 antibody is a monoclonal antibody.

In some embodiments, a protein of the protein-nucleic acid conjugate is linked to the docking strand through an intermediate linker. In some embodiments, an intermediate linker comprises biotin and/or streptavidin.

25 In some embodiments, a complementary fluorescently-labeled imager strand comprises at least one fluorophore.

In some embodiments, a complementary labeled, optionally fluorescently labeled, imager strand is about 4 to about 10 nucleotides, or about 8 to about 10 nucleotides in length.

In some embodiments, a sample is a cell or cell lysate.

30 In some embodiments, a target is a protein. In some embodiments, a target is a nucleic acid (*e.g.*, DNA or RNA).

In some embodiments, a target is obtained from a cell or cell lysate.

In some aspects, provided herein is a method of detecting at least one or at least two targets in a sample, the method comprising contacting a sample with (a) at least two protein-

nucleic acid conjugates, each comprising a protein linked to a docking strand, and (b) at least two labeled (optionally spectrally distinct, or fluorescently labeled, or spectrally distinct and fluorescently labeled) imager strands that are complementary to and transiently bind to respective docking strands of the at least one, or at least, two different protein-nucleic acid conjugates and determining whether the at least two protein-nucleic acid conjugates bind to at least two targets in the sample. In some embodiments, the determining step comprises, in the following order, imaging transient binding of one of the at least two labeled imager strands to a docking strand of one of the at least two protein-nucleic acid conjugates to produce a first image (*e.g.*, of a fluorescent signal), and imaging transient binding of another of the at least two labeled imager strands to a docking strand of another of the at least two protein-nucleic acid conjugates to produce at least one other image (*e.g.*, of a fluorescent signal). In some embodiments, the method further comprises combining the first image and the at least one other image to produce a composite image of signal (*e.g.*, fluorescent signal), wherein the signal of the composite image is representative of the at least two targets.

In some embodiments, a protein of the protein-nucleic acid conjugate is an antibody, an antigen-binding antibody fragment, or a peptide aptamer. In some embodiments, an antibody is a monoclonal antibody.

In some embodiments, a protein of the protein-nucleic acid conjugate is linked to the docking strand through an intermediate linker. In some embodiments, an intermediate linker comprises biotin and streptavidin.

In some embodiments, each of the at least two spectrally distinct, fluorescently-labeled imager strands comprises at least one fluorophore.

In some embodiments, each of the at least two labeled, optionally spectrally distinct, fluorescently labeled, imager strands is about 4 to about 10 nucleotides, or about 8 to about 10 nucleotides in length.

In some embodiments, a sample is a cell or cell lysate.

In some embodiments, at least two targets are proteins. In some embodiments, at least two targets are nucleic acids (*e.g.*, DNA or RNA).

In some embodiments, at least two targets are obtained from a cell or cell lysate.

In some aspects, provided herein is a method of detecting at least one, or at least two, protein targets in a sample, comprising (a) contacting a sample with at least two protein-nucleic acid conjugates, each comprising a protein linked to a docking strand and (b) sequentially contacting the sample with at least two labeled (*e.g.*, optionally spectrally indistinct, or fluorescently labeled, or spectrally distinct and fluorescently labeled) imager

strands that are complementary to and transiently bind to respective docking strands of the at least two protein-nucleic acid conjugates, and determining whether the at least one or at least two protein-nucleic acid conjugates bind to at least two targets in the sample. In some embodiments, a method comprises, in the following ordered steps, contacting the sample with
5 a first protein-nucleic acid conjugate and at least one other protein-nucleic acid conjugate, contacting the sample with a first labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently binds to the docking strand of the first protein-nucleic acid conjugate, imaging the sample to obtain a first image, optionally using time-lapsed imaging, removing the first labeled imager strand, contacting the sample with at least one
10 other labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other protein-nucleic acid conjugate, and imaging the sample to obtain at least one other image, optionally using time-lapsed imaging.

In some embodiments, a method comprises, in the following ordered steps, contacting a sample with a first protein-nucleic acid conjugate, contacting the sample with a first
15 labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently binds to the docking strand of the first protein-nucleic acid conjugate, imaging the sample to obtain a first image, optionally using time-lapsed imaging, removing the first labeled imager strand, contacting the sample with at least one other protein-nucleic acid conjugate, contacting the sample with at least one other labeled, optionally fluorescently
20 labeled, imager strand that is complementary to and transiently binds to the docking strand of the at least one other protein-nucleic acid conjugate, and imaging the sample to obtain at least one other image, optionally using time-lapsed imaging.

In some embodiments, a method further comprises determining whether the first protein-DNA conjugate binds to a first target and/or whether the at least one other protein-DNA conjugate binds to at least one other target.
25

In some embodiments, a method further comprises assigning a pseudo-color to the signal (*e.g.*, fluorescent signal) in a first image, and assigning at least one other pseudo-color to the fluorescent signal in the at least one other image.

In some embodiments, a method further comprises combining a first image and the at
30 least one other image to produce a composite image of the pseudo-colored signals, wherein the pseudo-colored signals of the composite image are representative of the at least two targets.

In some embodiments, the protein of the protein-nucleic acid conjugate(s) is an antibody, an antigen-binding antibody fragment, or a peptide aptamer. In some embodiments, the antibody is a monoclonal antibody.

5 In some embodiments, the protein of the protein-nucleic acid conjugate(s) is linked to the docking strand through an intermediate linker. In some embodiments, the intermediate linker comprises biotin and/or streptavidin.

In some embodiments, each of the fluorescently-labeled imager strands comprises at least one fluorophore.

10 In some embodiments, each of the fluorescently-labeled imager strands is about 4 to about 30 nucleotides, or about 8 to about 10 nucleotides in length.

In some embodiments, a sample is a cell or cell lysate.

In some embodiments, a target(s) is a protein. In some embodiments, a target(s) is a nucleic acid (*e.g.*, DNA or RNA).

In some embodiments, a target(s) is obtained from a cell or cell lysate.

15 In some aspects, provided herein is a method of detecting a target, optionally a naturally-occurring biomolecule, comprising contacting a sample containing at least one target, optionally a naturally-occurring biomolecule, with (a) at least one BP-NA conjugate, optionally each BP-NA conjugate comprising a protein or nucleic acid linked to a docking strand, and (b) at least one labeled, optionally fluorescently labeled, imager strand that is
20 complementary to and transiently binds the docking strand of the at least one BP-NA conjugate, and determining whether the at least one BP-NA conjugate binds to at least one target, optionally a naturally-occurring biomolecule, in the sample. In this and other aspects or embodiments described herein, it is to be understood that the method may be carried out using a sample that is suspected of containing at least one target or a sample that an end-user
25 desires to analyze for the presence of the at least one target without any prior knowledge of the sample respecting its likelihood of containing the target.

In some embodiments, the determining step comprises imaging transient binding of the at least one labeled, optionally fluorescently labeled, imager strand to the docking strand of the at least one BP-NA conjugate.

30 In some embodiments, a sample is a cell or cell lysate.

In some embodiments, an at least one target, optionally a naturally-occurring biomolecule, is obtained from a cell or cell lysate.

In some embodiments, a protein is an antibody, an antigen-binding antibody fragment, or a peptide aptamer. In some embodiments, an antibody is a monoclonal antibody.

In some embodiments, a protein is linked to the docking strand through an intermediate linker. In some embodiments, an intermediate linker comprises biotin and/or streptavidin.

In some embodiments, a nucleic acid is a nucleic acid aptamer.

5 In some embodiments, a fluorescently-labeled imager strand comprises at least one fluorophore.

In some embodiments, an imager strand, optionally a fluorescently-labeled imager strand, is about 4 to about 30, or about 8 to about 10 nucleotides in length.

10 In some aspects, provided herein is a method of detecting a target, optionally a naturally-occurring biomolecule, comprising contacting a sample containing at least two targets, optionally naturally-occurring biomolecules, with (a) at least two different BP-NA conjugates, optionally each BP-NA conjugate comprising a protein or nucleic acid linked to a DNA docking strand, and (b) at least two labeled (optionally spectrally indistinct, or fluorescently labeled, or spectrally distinct and fluorescently labeled) imager strands that are
15 complementary to and transiently bind to respective docking strands of the at least two BP-NA conjugates, and determining whether the at least two BP-NA conjugates bind to at least one or at least two naturally-occurring biomolecules in the sample.

In some embodiments, a method comprises, in the following ordered steps, contacting the sample with a first BP-NA conjugate and at least one other BP-NA conjugate, contacting
20 the sample with a first labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently binds to the docking strand of the first BP-NA conjugate, imaging the sample to obtain a first image, optionally using time-lapsed imaging, removing the first labeled imager strand, contacting the sample with at least one other labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently binds
25 to the docking strand of the at least one other BP-NA conjugate, and imaging the sample to obtain at least one other image, optionally using time-lapsed imaging.

In some embodiments, a method comprises, in the following ordered steps, contacting the sample with a first BP-NA conjugate, contacting the sample with a first labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently
30 binds to the docking strand of the first BP-NA conjugate, imaging the sample to obtain a first image, optionally using time-lapsed imaging, removing the first labeled imager strand, contacting the sample with at least one other BP-NA conjugate, contacting the sample with at least one other labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently binds to the docking strand of the at least one other BP-NA conjugate, and

imaging the sample to obtain a at least one other image, optionally using time-lapsed imaging.

In some embodiments, a method further comprises determining whether the first protein DNA conjugate binds to a first target, optionally a naturally-occurring biomolecule, and/or whether the at least one other protein-DNA conjugate binds to at least one other target, optionally a naturally-occurring biomolecule.

In some embodiments, a method further comprises assigning a pseudo-color to the signal (*e.g.*, fluorescent signal) in a first image, and assigning at least one other pseudo-color to the signal (*e.g.*, fluorescent signal) in at least one other image.

In some embodiments, a method further comprises combining a first image and at least one other image to produce a composite image of pseudo-colored signals, wherein the pseudo-colored signals of the composite image are representative of at least one, or at least two, targets (*e.g.*, naturally-occurring biomolecules).

In some aspects, provided herein is a method of determining the number of targets in a test sample, comprising obtaining a sample that comprises targets transiently bound directly or indirectly to labeled, optionally fluorescently labeled, imager strands, obtaining a time-lapsed image, optionally a time-lapsed diffraction-limited fluorescence image, of the sample, performing spot detection (*e.g.*, fluorescence spot detection) and localization (*e.g.*, through the use of Gaussian fitting) on the diffraction-limited image to obtain a high-resolution image of the sample, calibrating $k_{on} \cdot c_{imager}$, optionally using a control sample with a known number of targets, wherein k_{on} is a second order association constant, and c_{imager} is concentration of labeled (*e.g.*, fluorescently labeled) imager strands in the test sample, determining variable τ_d , optionally by fitting the fluorescence OFF-time distribution to a cumulative distribution function, and determining the number of test targets in the sample based on the equation,

$$number\ of\ test\ targets = (k_{on} \cdot c_{imager} \cdot \tau_d)^{-1}.$$

In some aspects, provided herein is a method of determining a relative amount of targets in a test sample, comprising obtaining a sample that comprises targets transiently bound directly or indirectly to labeled imager strands, obtaining a time-lapsed image of the sample, performing spot detection and localization on the image to obtain a high-resolution image of the sample, determining variable τ_d , and determining the relative amount of two or more test targets in the sample based on τ_d .

In some embodiments, test targets are protein targets.

In some embodiments, protein targets are bound to protein-nucleic acid conjugates that comprise a protein linked to a docking strand, and the labeled (*e.g.*, fluorescently labeled)

imager strands are complementary to and transiently bind to respective docking strands of the protein-nucleic acid conjugates.

In some embodiments, the protein of the protein-nucleic acid conjugate is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

5 In some embodiments, test targets are single-stranded nucleic acids.

In some embodiments, single-stranded nucleic acids are DNA or RNA.

In some embodiments, each of the fluorescently-labeled imager strands comprises at least one fluorophore.

10 In some embodiments, each of the labeled, optionally fluorescently labeled, imager strands is about 4 to about 30 nucleotides, or about 8 to about 10 nucleotides in length.

In some embodiments, a time-lapsed fluorescence image is obtained over a period of about 25 minutes.

In some embodiments, the number of test targets is determined with an accuracy of greater than 90%.

15 In some aspects, provided herein is a single-stranded DNA probe comprising a target binding domain of about 20 nucleotides in length linked, optionally at its 3' end, to a docking domain of at least one, at least two, or at least three subdomains, wherein the at least one, at least two, or at least three subdomains are respectively complementary to at least one, at least two, or at least three labeled, optionally fluorescently labeled, imager strands of about 4 to
20 about 30, or about 8 to 10 nucleotides in length, and wherein the target binding domain is bound to a complementary domain of a single-stranded mRNA target strand.

In some embodiments, at least one of the at least one, at least two, or at least three subdomains is transiently bound to at least one labeled, optionally fluorescently labeled, imager strand.

25 In some aspects, provided herein is a method of performing drift correction for a plurality of images, wherein each of the plurality of images comprises a frame of a time sequence of images, wherein the time sequence of images captures a plurality of transient events, the method comprising determining a time trace for each of a plurality of drift markers identified in the plurality of images, wherein a time trace for each drift marker
30 corresponds to movement of an object in the image over the time sequence of images, determining, with at least one computer processor, a first drift correction from at least one of the plurality of drift markers based, at least in part, on the time traces for the plurality of drift markers, determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images, wherein each

drift template in the plurality of drift templates describes a geometrical relationship between the plurality of geometrically-addressable marker sites of transient events in the drift template, determining a second drift correction based, at least in part, on the time traces for the plurality of geometrically-addressable marker sites from the plurality of drift templates, correcting the plurality of images based, at least in part, on the first drift correction and the second drift correction, and outputting a final image based on the corrected plurality of images.

In some embodiments, a method further comprises identifying a plurality of localizations in each of the plurality of images, creating a two-dimensional histogram of the plurality of localizations, and identifying locations of the plurality of drift markers based, at least in part, on the two-dimensional histogram, wherein determining the time traces for each of the plurality of drift markers comprises determining the time traces based, at least in part, on the locations of the plurality of drift markers.

In some embodiments, identifying a plurality of localizations comprises identifying a plurality of spots on each of the plurality of images, and determining a fitted center position of each of the plurality of spots using a local Gaussian fitting algorithm, wherein each of the plurality of localizations comprises the spot identified on an image and its associated fitted center position.

In some embodiments, each of the plurality of localizations further comprises a detected photon count corresponding to the localization.

In some embodiments, creating the two-dimensional histogram of the plurality of localizations comprises binning all localizations into a two-dimensional grid and using a total number of localizations in each bin as a histogram count.

In some embodiments, creating the two-dimensional histogram of the plurality of localizations comprises binning all localizations into a two-dimensional grid and using a total number of photon count of the plurality of localizations in each bin as a histogram count.

In some embodiments, identifying locations of the plurality of drift markers based, at least in part, on the two-dimensional histogram comprises at least one of the following: binarizing the two-dimensional histogram using one or more selection criteria, wherein the one or more selection criteria include a lower-bound threshold of a histogram value or an upper-bound threshold of a histogram value; partitioning the binarized image into partitions and filtering the partitions based on one or more selection criteria, wherein the one or more selection criteria include one or more of a lower-bound threshold of an area of a partition area, an upper-bound threshold of the area, a lower-bound or an upper-bound of a longest or

shortest linear dimension of a partition longest, and a lower-bound or an upper-bound of an eccentricity of a partition; and expanding and shrinking the binarized image using one or more binary image operations, wherein the one or more binary image operations include one or more of the following: dilate, erode, bridge, close, open, fill, clean, top-hat, bottom-hat, thicken, thin, and more.

In some embodiments, determining a first drift correction based, at least in part, on the time traces for the plurality of drift markers comprises: determining a relative time trace for each of the plurality of drift markers, wherein the relative time trace is determined by comparing the time trace for the drift marker with the average position of the same trace; and determining a combined time trace based on the relative time traces for each of the plurality of drift markers, wherein determining the first drift correction based, at least in part, on the time traces for the plurality of drift markers comprises determining the first drift correction based, at least in part, on the relative time traces for each of the plurality of drift markers.

In some embodiments, determining the first drift correction based, at least in part, on the relative time traces for each of the plurality of drift markers comprises performing a weighted average of the relative time traces for each of the plurality of drift markers.

In some embodiments, performing the weighted average comprises: determining a quality score for each of the relative time traces, wherein the quality score is determined based, at least in part, on a measure of variability over time associated with the time trace and/or a measure of localization uncertainty of individual localizations within the time trace.

In some embodiments, the measure of variability over time comprises a standard deviation of the time trace over time.

In some embodiments, the measure of localization uncertainty of individual localizations comprises, at least in part, an estimate of uncertainty from a Gaussian fitting or a comparison with other simultaneous localizations, wherein the other simultaneous localizations are from within a same image and from other time traces from the plurality of drift markers, wherein the comparison comprises a mean and standard deviation of all simultaneous localizations.

In some embodiments, the method further comprises determining that a first drift marker of the plurality of drift markers is not present in at least one frame of the time sequence of images, and linearly interpolating the time trace for the first drift marker for the at least one frame to produce a smoothed time trace for the first drift marker.

In some embodiments, determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the

plurality of images comprises: identifying a plurality of localizations in each of the plurality of images; creating a two-dimensional histogram of the plurality of localizations; and identifying the plurality of drift templates based, at least in part, on the two-dimensional histogram, wherein identifying the plurality of drift templates comprises evaluating the two-dimensional histogram using an lower-bound and/or an upper-bound threshold in a histogram count.

In some embodiments, determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images comprises determining a time trace for each of a plurality of geometrically-addressable marker sites within each of the plurality of drift templates, and wherein determining the second drift correction comprises determining the second drift correction based, at least in part, on the time traces for each of the plurality of marker sites within each of the plurality of drift templates.

In some embodiments, determining the second drift correction based, at least in part, on the time traces for each of the plurality of geometrically-addressable marker sites from each of the plurality of drift templates comprises: identifying a plurality of geometrically-addressable marker sites within each of the plurality of drift templates; and determining a relative time trace for each of a plurality of geometrically-addressable drift markers for each of the plurality of drift templates, wherein determining the second drift correction based, at least in part, on the time traces for the plurality of drift templates comprises determining the second drift correction based, at least in part, on the relative time traces for each of the plurality of drift markers within each of the plurality of drift templates.

In some embodiments, identifying a plurality of geometrically addressable marker sites from each of the plurality of drift templates comprises determining a plurality of marker sites based on, at least in part, a two-dimensional histogram of the plurality of localizations in the corresponding drift template, and/or one or more selection criteria, wherein the one or more selection criteria include one or more of a total number of localizations, a surface density of localizations, and standard deviation of localizations.

In some embodiments, determining the second drift correction based, at least in part, on the relative time traces for each of the plurality of drift markers within each of the plurality of drift templates comprises performing a weighted average of the relative time traces for each of the plurality of drift markers within each of the drift templates.

In some embodiments, performing the weighted average comprises:

determining a quality score for each of the relative time traces, wherein the quality score is determined based, at least in part, on a measure of variability over time associated with the time trace and/or a localization uncertainty within the time trace.

5 In some embodiments, the measure of variability over time comprises a standard deviation of the time trace over time.

In some embodiments, the measure of localization uncertainty of individual localizations comprises an estimate of uncertainty from a Gaussian fitting or a comparison with other simultaneous localizations, wherein the other simultaneous localizations are from within a same image and from the other time traces from the plurality of marker sites from the plurality of drift templates, wherein the comparison comprises a mean and standard
10 deviation of all simultaneous localizations.

In some embodiments, correcting the plurality of images based, at least in part, on the first drift correction and the second drift correction comprises correcting the plurality images using the first drift correction to produce a first corrected plurality of images, and wherein
15 determining a time trace for each of a plurality of drift templates identified from the plurality of images comprises determining a time trace for each of the plurality of drift templates identified from the first corrected plurality of images.

In some embodiments, a method further comprises smoothing the first drift correction prior to correcting the plurality of images using the first drift correction.

20 In some embodiments, smoothing the first drift correction comprises processing the first drift correction using local regression with a window determined by a characteristic drift time scale of the first drift correction.

In some embodiments, a method further comprises smoothing the second drift correction prior to correcting the plurality of images using the second drift correction.

25 In some embodiments, smoothing the second drift correction comprises processing the second drift correction using local regression with a window determined by a characteristic drift time scale of the second drift correction.

In some embodiments, a method further comprises selecting a single drift marker of the plurality of drift markers; and determining a third drift correction based, at least in part,
30 on the selected single drift marker, wherein correcting the plurality of images comprises correcting the plurality of images based, at least in part, on the third drift correction.

In some embodiments, correcting the plurality of images based, at least in part, on the third drift correction is performed prior to correcting the plurality of images based, at least in part on the first drift correction and the second drift correction.

In some embodiments, a method further comprises identifying locations of a first plurality of points in a first image of the plurality of frames; identifying locations of a second plurality of points in a second image of the plurality of images, wherein the second image corresponds to a neighboring frame of the first image in the time sequence of images; and
5 determining a fourth drift correction based, at least in part, on differences between the locations of the first plurality of points and the second plurality of points, wherein correcting the plurality of images comprises correcting the plurality of images based, at least in part, on the fourth drift correction.

10 In some embodiments, the second image corresponds to a frame immediately following the frame corresponding to the first image in the time sequence of images.

In some embodiments, determining the fourth drift correction based, at least in part, on differences between the locations of the first plurality of points and the second plurality of points comprises: creating a histogram of distances between the locations of the first plurality of points and the second plurality of points; determining based, at least in part, on the
15 histogram, pairs of points between the first image and the second image that correspond to the same transient event; and determining a location offset between each of the determined pairs of points, wherein determining the fourth drift correction is based on a vector average of the location offsets for each of the determined pairs of points.

20 In some embodiments, the plurality of images correspond to DNA-based images and wherein the plurality of transient events are binding events between an imaging strand and a DNA docking strand.

In some embodiments, the imaging strand is a fluorescent imaging probe configured to fluoresce when associated with the DNA docking strand.

25 In some embodiments, at least one of the drift markers is a DNA based nanostructure.

In some embodiments, the DNA based nanostructure is a DNA origami nanostructure with docking strands.

In some embodiments, at least one of the drift templates is a DNA based nanostructure.

30 In some embodiments, the DNA based nanostructure is a DNA origami nanostructure with docking strands.

In some embodiments, at least one of the drift templates is a three-dimensional drift template.

In some embodiments, the three-dimensional drift template is a tetrahedron.

In some embodiments, at least one of the drift templates includes multiple colors corresponding to different types of transient events.

In some embodiments, the different types of transient events include a first binding event of a first imaging strand with a first type of DNA docking strand and a second binding
5 event of a second imaging strand with a second type of DNA docking strand.

In some embodiments, outputting the final image comprises displaying the final image on a display.

In some embodiments, outputting the final image comprises sending the final image to a computer via at least one network.

10 In some embodiments, outputting the final image comprises storing the final image on at least one storage device.

In some aspects, provided herein is a non-transitory computer readable medium encoded with a plurality of instructions that, when executed by at least one computer processor, performs a method of performing drift correction for a plurality of images,
15 wherein each of the plurality of images comprises a frame of a time sequence of images, wherein the time sequence of images captures a plurality of transient events, the method comprising: determining a time trace for each of a plurality of drift markers identified in the plurality of images, wherein a time trace for each drift marker corresponds to movement of an object in the image over the time sequence of images; determining a first drift correction
20 from at least one of the plurality of drift markers based, at least in part, on the time traces for the plurality of drift markers; determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images, wherein each drift template in the plurality of drift templates describes a geometrical relationship between the plurality of geometrically-addressable marker sites of
25 transient events in the drift template; determining a second drift correction based, at least in part, on the time traces for the plurality of geometrically-addressable marker sites from the plurality of drift templates; correcting the plurality of images based, at least in part, on the first drift correction and the second drift correction; and outputting a final image based on the corrected plurality of images.

30 In some aspects, provided herein is a computer, comprising: an input interface configured to receive a plurality of images, wherein each of the plurality of images comprises a frame of a time sequence of images, wherein the time sequence of images captures a plurality of transient events; at least one processor programmed to: determine a time trace for each of a plurality of drift markers identified in the plurality of images, wherein a time trace

for each drift marker corresponds to movement of an object in the image over the time sequence of images; determine a first drift correction from at least one of the plurality of drift markers based, at least in part, on the time traces for the plurality of drift markers; determine a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images, wherein each drift template in the plurality of drift templates describes a geometrical relationship between the plurality of geometrically-addressable marker sites of transient events in the drift template; determine a second drift correction based, at least in part, on the time traces for the plurality of geometrically-addressable marker sites from the plurality of drift templates; correct the plurality of images based, at least in part, on the first drift correction and the second drift correction; and determine a final image based on the corrected plurality of images; and output interface configured to output the final image.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows microtubule-like DNA origami polymers “labeled” with single-stranded DNA docking strands on a pair of opposite faces (colored in dark gray) spaced approximately 16 nanometers (nm) apart. Complementary fluorescently-labeled imager strands transiently bind from solution to the docking strands. Biotinylated DNA strands (present on the bottom two center helices) are used to bind the microtubule-like DNA structures to glass surfaces for fluorescence imaging. FIG. 1B shows a graph demonstrating that transient binding of fluorescently-labeled imager strands to the docking strands produces fluorescence “blinking” (fluorescence intensity vs. time trace). This blinking is used to consecutively localize points below the diffraction limit. FIG. 1C shows a transmission electron microscope (TEM) image of DNA origami polymers with a measured width of 16 ± 1 nm (mean \pm stdv) [scale bar: 40 nm]. FIG. 1D shows super-resolution fluorescence images obtained using Cy3b-labeled imager strands (15,000 frames, 5 Hz frame rate). Two distinct lines are visible [scale bars: 40 nm]. FIG. 1E shows a cross-sectional histogram of highlighted areas <i> and <ii> in FIG. 1D (arrows denote histogram direction), which show that the designed distance of approximately 16 nm is clearly resolved (full width at half maximum (FWHM) of each distribution is observed to be approximately 9 nm).

FIG. 2 shows an example of a biomolecule labeling scheme of the present disclosure where a protein (*e.g.*, protein target) is labeled with antibody-DNA conjugates of the present disclosure and complementary fluorescently-labeled imager strands. The antibodies are

linked to the docking strands through a linker that contains biotin and streptavidin (*e.g.*, biotin-streptavidin-biotin linker).

FIG. 3A shows a super-resolution image of a microtubule network inside a fixed HeLa cell using an antibody-DNA conjugate and Atto655-labeled imager strands (10,000 frames, 10 Hz frame rate) [scale bar: 5 μm]. FIG. 3B shows a high magnification image of the highlighted area in FIG. 3A [scale bar: 1 μm]. FIG. 3C shows a diffraction-limited representation of the same area in FIG. 3B. Arrows highlight positions where the increase in resolution of the image is clearly visible. Adjacent microtubules with an apparent width of approximately 46 nm at position *i* in FIG. 3B are separated by approximately 79 nm [scale bar: 1 μm]. FIG. 3D shows a dual-color super-resolution image (15,000 frames, 10 Hz frame rate) of microtubules and mitochondria inside a fixed HeLa cell obtained using antibody-DNA conjugates, Cy3b-labeled imager strands for microtubules (linear-like structures) and Atto655-labeled imager strands for mitochondria (patch-like structures) [scale bar: 5 μm]. FIG. 3E shows a high magnification image of the highlighted area in FIG. 3D [scale bar: 1 μm]. FIG. 3F shows a diffraction-limited image of the same area shown in FIG. 3E [scale bar: 1 μm].

FIG. 4A shows one embodiment of the present disclosure using spectrally indistinct imager strands (*e.g.*, each labeled with the same color fluorophore). In step [1], three different species of docking strands (*a, b, c*) label a surface. Such labeling may occur using the docking strand alone or linked to a protein-binding (*e.g.*, antibody) or a nucleic acid-binding molecule that binds to the surface/biomolecule of interest. In step [2], multiple copies of the imager strand *a** are introduced (where *a** has a sequence complementary to *a*), and points labeled with docking strands *a* are imaged. In step [3], copies of the imager strand *a** are flushed away, and imager strand *b** is introduced to image the *b* labeled points. Images are obtained, and imager strands *b** are washed away. In step [4], *c* labeled points are imaged in the same manner. In step [5], images from [2–4] are assigned pseudo-colors and combined to create the final image. Although pseudo-colors may be used in the final rendering of the image, all imager strands are actually labeled with the same color dye (*e.g.*, fluorophore). FIGs. 4B(1)–4B(3) show that reducing the image density simultaneously increases the achievable resolution by up to a factor of $2\sqrt{2}\ln 2 \approx 2.35$. Here, the resolution, previously defined as the FWHM of the reconstructed localizations, can be understood as the standard deviation of the localization as in localization microscopy with sparse points. FIG. 4B(1) shows seven points in a linear geometry spaced at 10 nm (top). Simulated super-

resolution data with approximately 14 nm resolution (center). The points cannot be resolved. Cross-sectional histogram data shows a broad peak (bottom). FIGs. 4B(2) and 4B(3) show that imaging every other site allows the localization of individual spots. These localizations can then be combined to form the final image of the full pattern. FIG. 4C shows an image of a DNA origami structure that displays docking strands spaced at 10 nm intervals.

FIG. 5A shows one embodiment of the present disclosure using DNA origami structures with different species of docking strands at designated positions resembling numbers 0–3 (0, 1, 2 and 3). For each round, the respective imager strand sequence is added to an imaging chamber, image acquisition is carried out and the imager strands are washed out. In each imaging round the designed number is imaged, showing a very sequence-specific interaction with no crosstalk between different rounds [Scale bar: 50 nm]. Note that all imager strands are labeled with the same color dye, though each structure (*e.g.*, 0-3 (0, 1, 2 and 3)) is rendered a distinct color (*e.g.*, purple, yellow, blue, or red; color rendering not shown). FIGs. 5B(i)-(v) show another embodiment of the present disclosure using DNA origami structures with different species of docking strands at designated positions resembling numbers 0-9 (0, 1, 2, 3, 4, 5, 6, 7, 8 and 9). FIG. 5B(i) shows an exchange-PAINT schematic showing sequential imaging of multiple targets using imager strands labeled with the same fluorophore. FIG. 5B(ii) shows a schematic of a DNA origami (70 100 nm) displaying docking strands that resemble digit 4. FIG. 5B(iii) shows a combined overview image of all ten Exchange-PAINT cycles, demonstrating specific interaction with the respective target with no crosstalk between imaging cycles. Scale bar: 250 nm. FIG. 5B(iv) shows a four-“color” image of digits 0 to 3 that are all present on the same DNA origami (10,000 frames each, 5 Hz frame rate; schematic at the bottom). Scale bar: 25 nm. FIG. 5B(v) shows pseudocolor images of ten different origami structures, each rendered a distinct color (*e.g.*, orange, green, blue, purple, pink, etc.; color rendering not shown), displaying digits 0 to 9 in one sample with high resolution (FWHM of bar-like features < 10 nm) and specificity. Image obtained using only one fluorophore (Cy3b) through ten imaging-washing cycles (imaging: 7,500 frames per cycle, 5 Hz frame rate; washing: 1-2 minutes per cycle). Scale bar: 25 nm.

FIG. 6A shows an experimental schematic for one embodiment of the present disclosure using fixed HeLa cells, where in one round, docking strands are bound to a target, labeled imager strands are then added, an image is acquired, and the imager strands are washed away. Each round is repeated with docking strands specific for different targets along with different labeled imager strands. The docking strands may be used alone or linked

to a protein-binding (*e.g.*, antibody) or a nucleic acid-binding molecule that binds to the target of interest. FIG. 6B shows two rounds of a method of the present disclosure using Cy3b-labeled imager strands in fixed HeLa cells. Here, microtubules (green pseudo-color; color rendering not shown) were labeled with docking sequence *a* and mitochondria (magenta pseudo-color; color rendering not shown) with orthogonal docking sequence *b*. FIG. 6C shows two rounds of a method of the present disclosure using ATTO655-labeled imager strands performed in fixed HeLa cells similar to FIG. 6B. [Scale bars: 5 μ m]. Note that all imager strands are labeled with the same color dye.

FIG. 7A shows that fluorescently-labeled imager strands transiently bind from solution to complementary docking strands on a structure or molecule of interest. The transient binding produces an apparent blinking as shown in the binding vs. time trace with characteristic fluorescence ON- and OFF-times (τ_b and τ_d , respectively). The detected binding frequency of imager strands from solution is linearly dependent on the number of available docking strands in a given image area (*i.e.*, the more docking strands, the higher the binding frequency). The time in-between binding events, *i.e.*, the fluorescence OFF-time (τ_d), is inversely proportional to the number of docking strands. FIG. 7B shows that the average fluorescence OFF-time (τ_d) can be determined by calculating a cumulative distribution function (CDF) for the OFF-time distribution. Given a known association constant k_{on} and imager strand concentration *c*, the number of binding sites can be calculated by: number of binding sites = $(\tau_d \cdot c \cdot k_{on})^{-1}$. FIG. 7C shows a super-resolution image of DNA origami structures designed to display 13 binding sites as a proof-of-concept platform. The incorporation efficiency for docking sites is not 100% leading to a distribution of actually incorporated sites (FIG. 7D(1)). The structures serve as an ideal test system, as the number of displayed docking sites can be determined visually by counting the number of spots (direct counting) and comparing it with the corresponding number of sites calculated using the proposed binding kinetic analysis. FIG. 7D(1) shows the binding site distribution for 377 origami structures obtained by direct visual counting. FIG. 7D(2) shows the binding site distribution for the same structures obtained by binding kinetic analysis (kinetics) of the present disclosure. FIG. 7D(3) shows the “offset” between direct and kinetic counting: the counting “error” or uncertainty for the method of the present disclosure is less than 7% (determined by the coefficient of variation of the Gaussian distribution).

FIG. 8A shows mRNA molecules of interest in fixed *Escherichia coli* cells tagged using docking strands in a FISH-like hybridization scheme. FIG. 8B shows a readout scheme used to determine the binding frequency for each imaging color. The intensity vs. time

profile of each single mRNA location yields a specific transient binding pattern (blinking) per color. The frequency of binding events depends on the number of binding sites allowing the use of the binding frequency to distinguish between different integer numbers of binding sites. FIG. 8C shows an *in vitro* proof-of-principle experiment on DNA origami structures displaying 3, 9, 22 and 44 binding sites for each of the red, green, and blue imager strands, respectively (color rendering not shown). The different binding levels are clearly distinguishable for each color, suggesting 4 possible “frequency levels” per color, yielding up to 124 different possible combinations for barcoding, *e.g.*, mRNA molecules inside cells. The barcoding space can be increased by using the fluorescence ON-time as an additional coding entity.

FIG. 9 shows a graph demonstrating that the fluorescence ON-time (related to the dissociation rate k_{off}) can be tuned independently of the fluorescence OFF-time (related to the association rate k_{on}). Extending the imaging/docking duplex from 9 to 10 nucleotides (nt) by adding a single CG base pair, the kinetic OFF-rate is reduced by almost one order of magnitude (8).

FIG. 10A shows a barcode probe that is roughly 50 nucleotide (nt) in length and can tag a biomolecule using a 21 nt target detection domain t^* followed by an approximately 30 nt long “barcode” region with a combination of 8, 9, or 10 nt long binding domain for red, green, or blue imager strands. Here, 8, 9 or 10 nt long docking strands are displayed for three colors with a k_{off} of 10, 1 and 0.1 per second, respectively (color rendering not shown). FIG. 10B shows characteristic intensity vs. time traces with increased fluorescence ON-times τ_b for the 9 nt interaction domain compared to the 8 nt interaction domain. FIG. 10C shows stochastic simulations demonstrating that k_{off} values of 10, 1 and 0.1 per second can be distinguished.

FIG. 11A shows that in the traditional method of detection, where a single fluorophore is stably attached to the imaging surface (*see* FIG. 11B(1)), a limited number of photons per “switching” event is emitted (top panel), that extraction of all photons from “replenishable” imager strands (*see* FIG. 11B(2)) leads to higher localization accuracy per switching event (middle panel), and that a DNA metafluorophore (*see* FIG. 11B(3) and FIG. 11B(4)) yields a significantly larger number of photons per switching event than the single fluorophore in FIG. 11B(2) (bottom). FIGs. 11B(1)-11B(3) show schematics of current imaging methods and methods of the present disclosure. FIG. 11B(1) shows a traditional detection method (*e.g.*, in STORM), which uses a fluorophore stably attached to the imaging surface. FIG. 11B(2) shows one embodiment of a detection method of the present disclosure

with fluorophores transiently binding to the imaging surface. FIG. 11B(3) shows a bright metafluorophore with 8 fluorophores in a compact DNA nanostructure. FIG. 11B(4) shows a conditional metafluorophore decorated with both quenchers (dark dots) and fluorophores (stars) that only fluoresces when transiently bound to the surface. FIG. 11C shows a DNA origami structure with docking sites arranged in a 4×3 grid, spacing 20 nm. Single sites are optically localized with an accuracy of approximately 3 nm, currently the highest demonstrated resolution [scale bar: 50 nm]. FIG. 11D shows a 280 nm × 240 nm DNA nano-rectangle (single-stranded tile structure (15), 10x larger area than origami) displaying 2000 single-stranded docking strands (dots) with 7 or 5 nm spacing used as a test platform for ultra-resolution imaging [scale bar: 100 nm].

FIG. 12A(i) illustrates schematics showing the principle of each stage of drift correction. In each image, black markers and lines indicate source data, and gray values and curves indicate the calculated drift correction. FIG. 12A(ii) shows a schematic drawing of the major type of drift markers (*e.g.*, DNA drift markers) used in each stage. FIG. 12B(i) illustrates an example structure showing the imaging quality after each stage of correction, and FIG. 12B(ii) shows a zoomed image of the corresponding green rectangle in FIG. 12B(i) at each stage. The scale bars shown in FIGS. 12B(i) and 12B(ii) correspond to 50 nm. FIG. 12C(i) illustrates an example drift trace after each stage of correction, and FIG. 12C(ii) shows a zoomed image of the corresponding rectangle in FIG. 12C(i) at each stage. The scale bars in FIG. 12C(i) correspond to x: 500 nm, t: 500 s, and the scale bars in FIG. 12C(ii) correspond to x: 10nm, t: 10 s.

FIG. 13 illustrates a process for performing drift correction in accordance with some embodiments.

FIG. 14 illustrates a process for performing drift correction corresponding to stage 230 of FIG 13.

FIG. 15 illustrates a process for performing drift correction corresponding to stage 240 of FIG 13.

FIG. 16 illustrates 3D tetrahedrons used as templates for 3D drift correction. The four corners are labeled with docking sites. FIG. 16A shows that the four corners are clearly resolved. FIG. 16B illustrates the X-Z projection of the structures with a height of ~85 nm.

FIG. 17 shows an illustrative implementation of computer system 600 that may be used in connection with any of the embodiments of the present disclosure described herein.

FIGs. 18A-18C illustrate an alternative representation of stages in a drift correction process in accordance with some embodiments of the present disclosure. A super-resolved

image of a 10 nm-spaced regular grid on a single-molecule DNA origami nanostructure is shown. The DNA origami structure was designed to be a 5 x 8 square lattice of 10 nm spacing both vertically and horizontally. FIG. 18A shows a scatter plot of collected and filtered localizations, represented by crosses. FIG. 18B shows a binned 2-D histogram view of the above structure. FIG. 18C shows a 1-D histogram by projecting all localizations in the rectangle in FIGs. 18A and 18B onto the x-axis, and least square fitting with 8 Gaussian components. The fitted Gaussian peaks all have standard deviation in the range of 1.5-2.4 nm, allowing for 3.5-5.6 nm resolution in principle; and spacing between neighboring peaks in the range of 9.8-11.0 nm, consistent with the DNA origami design. A few (5 in this case) spots are missing in the structure because of imperfect incorporation of staples in the assembly reaction, but not missed during the super-resolution imaging.

FIGs. 19A and 19B show that RNA aptamers modulate the fluorescence of GFP-like fluorophores. FIG 19A shows structures of HBI (green), in the context of GFP, and DMHBI. FIG. 19B shows that the 13-2 RNA aptamer enhances the fluorescence of DMHBI by stabilizing a particular molecular arrangement favorable for fluorescence emission. Solutions containing DMHBI, 13-2 RNA, DMHBI with 13-2 RNA, or DMHBI with total HeLa cell RNA were photographed under illumination with 365 nm of light. The image is a montage obtained under identical image-acquisition conditions. (Image from Paige *et al.*, ref. 19)

FIGs. 20A and 20B show single-molecule fluorescence characterization of the DFHBI binding kinetics. FIG. 20A shows a 5'-extended Spinach (green) is immobilized on a BSA/Biotin-coated glass substrate using a biotinylated DNA capture sequence (labeled with a red dye, *e.g.* Alexa647; color rendering not shown). FIG. 20B shows a bulk fluorescence measurement of the Spinach-DFHBI before (bottom line) and after (top line) addition of the aptamer shows that the DFHBI binding activity is well maintained after the addition an extension to Spinach required for immobilizing to the glass surface in FIG. 20A.

FIGs. 21A and 21B show benchmarking Spinach-PAINT performance. FIG. 20A shows a six-helix DNA origami structure used for placing two Spinach molecules in a defined distance. FIG. 20B shows a simulated representation of a super-resolved reconstruction using DNA-PAINT to localize the DNA structure (P) and Spinach-PAINT to localize the Spinach molecules in three different distances.

FIG. 22 shows Spinach-based sensors. The allosteric variant of the Spinach-based sensor (left) comprises the Spinach domain (black), the transducer module (medium gray), and the recognition module (light gray). In the absence of the target molecule, the transducer module is in a primarily unstructured state, which prevents the stabilization of the Spinach

structure needed for activation of DFHBI. Upon binding of the target molecule, the transducer module forms a duplex, leading to structural rigidification of the Spinach module, and activation of DFHBI fluorescence (ref. 24).

FIGs. 23A and 23B show examples of *in vitro* and *in situ* Exchange-PAINT chambers.

DESCRIPTION OF THE INVENTION

The present disclosure provides, *inter alia*, methods, compositions and kits for multiplexed imaging, for example, in a cellular environment using nucleic acid-based imaging probes (*e.g.*, DNA-based imaging probes). The methods, compositions and kits for multiplexed fluorescence imaging are not limited by the degree of resolution attained. Thus, the methods, compositions and kits as provided herein may be used for imaging, generally.

In some aspects, the present disclosure further provides, *inter alia*, methods, compositions and kits for multiplexed super-resolution fluorescence imaging, for example, in a cellular environment using nucleic acid-based imaging probes (*e.g.*, DNA-based imaging probes). As used herein, “super-resolution” imaging refers to the process of combining a set of low resolution images of the same area to obtain a single image of higher resolution. Many aspects of the present disclosure may be used to “switch” targets (*e.g.*, biomolecules) of interest between fluorescent ON- and OFF-states to permit consecutive, or in some instances simultaneous, localization of individual targets. A fluorescent “ON” state is a state in which fluorescence is emitted. A fluorescent “OFF” state is a state in which fluorescence is not emitted. Switching between the two states is achieved, in some embodiments, with diffusing molecules that are labeled with a detectable label (*e.g.*, fluorescent molecules) that interact transiently with the targets using an intermediate moiety that comprises the detectable label (*e.g.*, fluorescent molecule(s)) and binds to the target. The methods, compositions and kits of the present disclosure are useful, in some aspects, for detecting, identifying and quantifying target targets of interest.

Binding partner-nucleic acid conjugates (“BP-NA conjugates”) of the present disclosure transiently bind to complementary labeled, optionally fluorescently labeled, imager strands. As used herein, “binding partner-nucleic acid conjugate,” or “BP-NA conjugate,” refers to a molecule linked (*e.g.*, through an N-Hydroxysuccinimide (NHS) linker) to a single-stranded nucleic acid (*e.g.*, DNA) docking strand. The binding partner of the conjugate may be any moiety (*e.g.*, antibody or aptamer) that has an affinity for (*e.g.*, binds to) a target, such as a biomolecule (*e.g.*, protein or nucleic acid), of interest. In some

embodiments, the binding partner is a protein. BP-NA-conjugates that comprise a protein (or peptide) linked to a docking strand may be referred to herein as “protein-nucleic acid conjugates,” or “protein-NA conjugates.” Examples of proteins for use in the conjugates of the present disclosure include, without limitation, antibodies (*e.g.*, monoclonal monobodies),
5 antigen-binding antibody fragments (*e.g.*, Fab fragments), receptors, peptides and peptide aptamers. Other binding partners may be used in accordance with the present disclosure. For example, binding partners that bind to targets through electrostatic (*e.g.*, electrostatic particles), hydrophobic or magnetic (*e.g.*, magnetic particles) interactions are contemplated herein.

10 As used herein, “antibody” includes full-length antibodies and any antigen binding fragment (*e.g.*, “antigen-binding portion”) or single chain thereof. The term “antibody” includes, without limitation, a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or
15 modified forms thereof (*e.g.*, humanized, chimeric).

As used herein, “antigen-binding portion” of an antibody, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. The antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term “antigen-binding
20 portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_H , V_L , C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_H and V_L domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, *Nature* 341:544 546, 1989), which
25 consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR) or (vii) a combination of two or more isolated CDRs, which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_H and V_L , are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_H and V_L regions
30 pair to form monovalent molecules (known as single chain Fv (scFv); *see, e.g.*, Bird *et al. Science* 242:423 426, 1988; and Huston *et al. Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988). Such single chain antibodies are also encompassed within the term “antigen-binding portion” of an antibody. These antibody fragments are obtained using conventional

techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

As used herein, “receptors” refer to cellular-derived molecules (*e.g.*, proteins) that bind to ligands such as, for example, peptides or small molecules (*e.g.*, low molecular weight (<900 Daltons) organic or inorganic compounds).

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

In some embodiments, the molecule of the BP-NA conjugate is a nucleic acid such as, for example, a nucleic acid aptamer. As used herein, “nucleic acid aptamer” refers to a small RNA or DNA molecules that can form secondary and tertiary structures capable of specifically binding proteins or other cellular targets (*see, e.g.*, Ni X, *et al. Curr Med Chem.* 18(27): 4206–4214, 2011). Thus, in some embodiments, the BP-NA conjugate may be an aptamer-nucleic acid conjugate.

As used herein a “docking strand” refers to a single-stranded nucleic acid (*e.g.*, DNA) that is about 5 nucleotides to about 50 nucleotides in length (or is 5 nucleotides to 50 nucleotides in length). In some embodiments, a docking strand is about 4 to about 60, about 6 nucleotides to about 40 nucleotides, about 7 nucleotides to about 30 nucleotides, about 8 to about 20 nucleotides, or about 9 nucleotides to about 15 nucleotides in length. In some embodiments, a docking strand is (or is about) 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, or more nucleotides in length.

A docking strand may have one domain or more than one domain (*i.e.*, a plurality of domains), each domain complementary to a respective imager strand. As used herein, a “docking strand domain” refers to a nucleotide sequence of the docking strand that is complementary to a nucleotide sequence of an imager strand. A docking strand, for example, may contain one, two three, or more domains, each domain complementary to an imager strand. Each complementary imager strand can contain a distinct label (*e.g.*, a red fluorophore, a blue fluorophore, or a green fluorophore), or all complementary imager strands can contain the same label (*e.g.*, red fluorophores). For example, for a three-domain docking strand, the strand may contain a first domain complementary to an imager strand labeled with a red fluorophore, a second domain complementary to an imager strand labeled with a blue fluorophore, and a third domain complementary to an imager strand labeled with a green

fluorophore. Alternatively, each of three docking domains may be complementary to imager strands labeled with a red fluorophore. In some embodiments, a docking strand has at least 2, at least 3, at least 4, at least 5, or more domains, each respectively complementary to an imager strand. In some embodiments, a docking strand has 1 to 5, 1 to 10, 1 to 15, 1 to 20, 1 to 25, 1 to 50, or 1 to 100 domains, each respectively complementary to an imager strand.

As used herein, an “imager strand” is a single-stranded nucleic acid (*e.g.*, DNA) that is about 4 to about 30 nucleotides, about 5 to about 18 nucleotides, about 6 to about 15 nucleotides, about 7 to about 12 nucleotides, or about 8 to 10 nucleotides in length and is fluorescently-labeled. In some embodiments, the imager strand may be (or may be about) 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length. An imager strand of the present disclosure is complementary to and transiently binds to a docking strand. Two nucleic acids or nucleic acid domains are “complementary” to one another if they base-pair, or bind, with each other to form a double-stranded nucleic acid molecule via Watson-Crick interactions. As used herein, “binding” refers to an association between at least two molecules due to, for example, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions. An imager strand is considered to “transiently bind” to a docking strand if it binds to a complementary region of a docking strand and then disassociates (unbinds) from the docking strand within a short period of time, for example, at room temperature. In some embodiments, an imager strand remains bound to a docking strand for about 0.1 to about 10, or about 0.1 to about 5 seconds. For example, an imager strand may remain bound to a docking strand for about 0.1, about 1, about 5 or about 10 seconds.

Imager strands of the present disclosure may be labeled with a detectable label (*e.g.*, a fluorescent label, and thus are considered “fluorescently labeled”). For example, in some embodiments, an imager strand may comprise at least one (*i.e.*, one or more) fluorophore. Examples of fluorophores for use in accordance with the present disclosure include, without limitation, xanthene derivatives (*e.g.*, fluorescein, rhodamine, Oregon green, eosin and Texas red), cyanine derivatives (*e.g.*, cyanine, indocarbocyanine, oxacarbocyanine, thiocarbocyanine and merocyanine), naphthalene derivatives (*e.g.*, dansyl and prodan derivatives), coumarin derivatives, oxadiazole derivatives (*e.g.*, pyridyloxazole, nitrobenzoxadiazole and benzoxadiazole), pyrene derivatives (*e.g.*, cascade blue), oxazine derivatives (*e.g.*, Nile red, Nile blue, cresyl violet and oxazine 170), acridine derivatives (*e.g.*, proflavin, acridine orange and acridine yellow), arylmethine derivatives (*e.g.*, auramine, crystal violet and malachite green), and tetrapyrrole derivatives (*e.g.*, porphyrin,

phthalocyanine and bilirubin). Other detectable labels may be used in accordance with the present disclosure, such as, for example, gold nanoparticles or other detectable particles or moieties.

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

The BP-NA conjugates (*e.g.*, protein-nucleic acid conjugates) of the present disclosure may, in some embodiments, comprise an intermediate linker that links (*e.g.*, covalently or non-covalently) the molecule to a docking strand. The intermediate linker may comprise biotin and/or streptavidin. For example, in some embodiments, an antibody and a docking strand may each be biotinylated (*i.e.*, linked to at least one biotin molecule) and linked to each other through biotin binding to an intermediate streptavidin molecule, as shown in FIG. 2. Other intermediate linkers may be used in accordance with the present disclosure. In some embodiments, such as those where the molecule is a nucleic acid, an intermediate linker may not be required. For example, the docking strand of a BP-NA conjugate may be an extension (*e.g.*, 5' or 3' extension) of a nucleic acid molecule such as, for example, a nucleic acid aptamer.

Pluralities of BP-NA conjugates (*e.g.*, protein-nucleic acid conjugates) and imager strands are provided herein. A plurality may be a population of the same species or distinct species. A plurality of BP-NA conjugates of the same species may comprise conjugates that all bind to the same target (*e.g.*, biomolecule) (*e.g.*, the same epitope or region/domain). Conversely, a plurality of BP-NA conjugates of distinct species may comprise conjugates, or subsets of conjugates, each conjugate or subset of conjugates binding to a distinct epitope on the same target or to a distinct target. A plurality of imager strands of the same species may comprise imager strands with the same nucleotide sequence and the same fluorescent label (*e.g.*, Cy2, Cy3 or Cy4). Conversely, a plurality of imager strands of distinct species may

comprise imager strands with distinct nucleotide sequences (*e.g.*, DNA sequences) and distinct fluorescent labels (*e.g.*, Cy2, Cy3 or Cy4) or with distinct nucleotide sequences and the same fluorescent (*e.g.*, all Cy2). The number of distinct species in a given plurality of BP-NA conjugates is limited by the number of binding partners (*e.g.*, antibodies) and the number of docking strands of different nucleotide sequence (and thus complementary imager strands). In some embodiments, a plurality of BP-NA conjugates (*e.g.*, protein-nucleic acid conjugates) comprises at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4 , 50000, 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} BP-NA conjugates. Likewise, in some embodiments, a plurality of fluorescently-labeled imager strands comprises at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4 , 50000, 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} fluorescently-labeled imager strands. In some embodiments, a plurality may contain 1 to about 200 or more distinct species of BP-NA conjugates and/or imager strands. For example, a plurality may contain at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200 or more distinct species. In some embodiments, a plurality may contain less than about 5 to about 200 distinct species of BP-NA conjugates and/or imager strands. For example, a plurality may contain less than 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175 or 200 distinct species.

The present disclosure also contemplates docking strands that can bind directly to a target. For example, as shown in FIG. 8A, a docking strand may contain, in addition to imager-binding domain(s) (*e.g.*, one, two, three, or more, with the same or distinct fluorophores), a target domain that is complementary to and binds to a target, such as, for example, mRNA or other nucleic acid.

25 *Methods*

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

containing the target(s). In any one of the aspects and embodiments provided herein, a sample may contain or may be suspected of containing one or more target(s).

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

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

Methods provided herein may comprise, in some embodiments, contacting a sample with (a) at least one BP-NA conjugate (*e.g.*, protein-nucleic acid conjugate) that comprises a binding partner linked to a docking strand and (b) at least one labeled, optionally fluorescently labeled, imager strand that is complementary to and transiently binds to the docking strand of the at least one BP-NA conjugate, and then determining whether the at least one BP-NA conjugate binds to at least one target (such as a biomolecule target) in the sample. In some embodiments, the determining step comprises imaging (*e.g.*, with time-lapsed fluorescent microscopy techniques) transient binding of the at least one labeled, optionally fluorescently labeled, imager strand to the docking strand of the at least one BP-NA conjugate.

Other methods provided herein may comprise, in some embodiments, contacting a sample with (a) at least two BP-NA conjugates, each comprising a binding partner linked to a docking strand, and (b) at least two labeled, optionally spectrally distinct, fluorescently labeled, imager strands that are complementary to and transiently bind to respective docking strands of the at least two different BP-NA conjugates, and then determining whether the at least two BP-NA conjugates bind to at least one, or at least two, targets (such as biomolecule targets) in the sample. Binding of the BP-NA conjugates to respective targets can be determined by imaging transient binding of one of the at least two labeled, optionally spectrally distinct, fluorescently labeled, imager strands to a docking strand of one of the at least two BP-NA conjugates to produce a first image, and then imaging transient binding of another of the at least two labeled, optionally spectrally distinct, fluorescently labeled, imager strands to a docking strand of another of the at least two BP-NA conjugates to produce a second image. In some embodiments, the methods further comprise combining the first image and the second image to produce a composite image of signals (*e.g.*, fluorescent signals), wherein the signals (*e.g.*, fluorescent signals) of the composite image are representative of the at least two targets. As used herein, a “composite image” refers to a single image produced by combining (*e.g.*, overlaying) multiple images of the same (or

substantially similar) area. A composite image may also be referred to as a super-resolution image, as described elsewhere herein.

FIG. 3 demonstrates one embodiment of the present disclosure in which two distinct species of BP-NA conjugates (*e.g.*, antibody-nucleic acid conjugates) are used to label biomolecules in a fixed HeLa cell sample. One species of antibody- nucleic acid conjugate comprises an antibody that recognizes and binds to an epitope on mitochondria. The mitochondrial specific antibody is linked to a docking strand with a sequence complementary to a Cy3b-labeled imager strand. The other species of antibody-nucleic acid conjugate comprises an antibody that recognizes and binds to an epitope on microtubules. The microtubule specific antibody is linked to a docking strand with a sequence complementary to an ATTO655-labeled imager strand. Two spectrally distinct species of imager strands are then introduced at the same time: one species is labeled with Cy3b and is complementary to the docking strand that is linked to the mitochondrial specific antibody, and the other species is labeled with ATTO655 and is complementary to the docking strand that is linked to the microtubule specific antibody. While both the Cy3b-labeled imager strand and the ATTO655-labeled imager strand are present at the same time in solution with the sample, imaging is carried out sequentially in Cy3b and ATTO655 channels.

Yet other methods provided herein may comprise, in some embodiments, contacting a sample with (a) at least two BP-NA conjugates, each comprising a protein linked to a docking strand and (b) at least two spectrally indistinct (*e.g.*, labeled with the same fluorophore) fluorescently-labeled imager strands that are complementary to and transiently bind to respective docking strands of the at least two BP-NA conjugates, and then determining whether the at least two BP-NA conjugates bind to at least two targets (*e.g.*, biomolecule targets) in the sample. In some embodiments, the methods comprise, in the following ordered steps, contacting the sample with a first BP-NA conjugate and at least one other BP-NA conjugate, contacting the sample with a first fluorescently-labeled imager strand that is complementary to and transiently binds to the docking strand of the first BP-NA conjugate, determining whether the first BP-NA conjugate binds to a first target, removing the first fluorescently-labeled imager strand, contacting the sample with at least one other fluorescently-labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other BP-NA conjugate, and determining whether the at least one other BP-NA conjugate binds to at least one other target.

Alternatively, in other embodiments, methods comprise, in the following ordered steps, contacting the sample with a first BP-NA conjugate, contacting the sample with a first

fluorescently-labeled imager strand that is complementary to and transiently binds to the docking strand of the first BP-NA conjugate, determining whether the first BP-NA conjugate binds to a first target (*e.g.*, biomolecule), removing the first fluorescently-labeled imager strand, contacting the sample with at least one other BP-NA conjugate, contacting the sample with at least one other fluorescently-labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other BP-NA conjugate, and determining whether the at least one other BP-NA conjugate binds to at least one other target.

In some embodiments, the first determining step comprises imaging transient binding of the first fluorescently-labeled imager strand to the docking strand of the first BP-NA conjugate to produce a first image, and the second determining step comprises imaging transient binding of the at least one other fluorescently-labeled imager strand to the docking strand of the at least one other BP-NA conjugate to produce a second image. In some embodiments, the methods further comprise assigning a pseudo-color to the fluorescent signal in the first image, and assigning at least one other pseudo-color to the fluorescent signal in the second image. Further still, in some embodiments, the methods comprise combining the first image and the second image to produce a composite image of the pseudo-colored signals, wherein the pseudo-colored signals of the composite image are representative of the at least two targets (*e.g.*, biomolecule targets). As illustrated in FIG. 4A, step [1], three distinct species of docking strands (*a, b, c*) label the surface of a grid (chosen for illustrative purposes). In step [2], multiple copies of the imager strand *a** are introduced, and points labeled with docking strands *a* are imaged. In step [3], copies of the imager strand *a** are flushed away, and imager strand *b** is introduced to image the *b* labeled points. In step [4], *c* labeled points are imaged in the same manner. In step [5], images from steps [2–4] are assigned artificial pseudo-colors (*e.g.*, using a software program) and combined to create the final composite image. All imager strands may be labeled with the same fluorophore – that is, the imager strands are spectrally indistinct. In some embodiments, the docking strands are linked to binding partners (*e.g.*, proteins such as antibodies, or nucleic acids such as DNA or nucleic acid aptamers).

An advantage of the methods of the present disclosure is that partitioning and sequential imaging can be used to obtain multiplexed super-resolved images of up to hundreds of different species using only a single optimized fluorescent dye. Using these methods, the number of distinct nucleotide sequences (*e.g.*, DNA sequences), as opposed to the number of spectrally distinct dyes, limits the multiplexing capability. In some methods of the present disclosure, for example, those that use an imager strand with a length of 9

nucleotides, there are several hundred species within tight bounds for binding kinetics that may be used for a single sample, representing a tremendous increase in multiplexing compared to direct “traditional” imaging approaches.

FIG. 5A illustrates another embodiment of the present disclosure using spectrally indistinct imager strands. A single DNA nanostructure displays four distinct species of docking strands (optionally linked to protein binding partners or nucleic acid binding partners) designed to resemble the digits from 0 to 3, respectively. Imaging is performed sequentially using a simple flow chamber setup, first flushing in fluorescently-labeled imager strands complementary to the docking strands of the number 0, and then exchanging the solution for fluorescently-labeled imager strands with a sequence complementary to docking strands of the number 1, and so forth. The resulting images have been pseudo-colored to represent the respective imaging cycles. As used herein, an “imaging cycle,” or “imaging round” refers to the process of introducing fluorescently-labeled imager strands complementary to docking strands under conditions that allow the imager strand to bind to the docking strand, even if such binding is transient, and obtaining an image (or imaging an area).

Aspects of the present disclosure contemplate multiplex detection using multi-domain docking strands (*e.g.*, docking strands with more than one domain), as described above. For illustrative purposes, the following embodiments are described in terms of a docking strand binding to a target, *e.g.*, without an intermediate binding partner. It should be understood, however, that multi-domain docking strands may be linked to a binding partner (*e.g.*, of a BP-NA conjugate, as provided herein).

In some embodiments, methods comprise contacting one or more target(s) with one or more docking strands, each containing two or more binding domains. In other embodiments, methods comprise contacting one or more target(s) with two or more docking strands, each containing one binding domain. The docking strand domains may have orthogonal sequences. In the examples that follow, all three targets (protein #1-#3) are present in the sample.

Detection Based on Spectral Resolution. An exemplary multiplexed target detection method follows. A sample contains, or is suspected of containing, three target species – protein #1, protein #2, and protein #3. Three docking strands are designed such that: the first, containing imager binding domain A, binds to protein #1; the second, containing imager binding domain B, binds to protein #2; and the third, containing imager binding domains A and B, binds to protein #3. Complementary imager strand A' binds to imager binding

domain A of a docking strand and is labeled with a blue fluorophore, and imager strand B' binds to imager binding domain B of a docking strand and is labeled with a red fluorophore. The sample is first contacted with the docking strands, and subsequently contacted with the imager strands A' and B'. The sample is then imaged. The sample containing the docking strands and the imager strands is first imaged under conditions that detect the blue fluorophore. Imaging the blue fluorophore detects protein #1 and protein #3, each bound by an imager strand labeled with the blue fluorophore. Imaging the red fluorophore detects protein #2 and protein #3, each bound by an imager strand labeled with the red fluorophore. Overlapping images of the red and blue fluorophores detects protein #3 only, the only protein bound by an imager strand labeled with a red fluorophore and an imager strand labeled with a blue fluorophore. Thus, the identification and location of proteins #1-#3 are identified by the overlay of images respectively detecting the red and blue fluorophores.

Detection Based on Exchange of Imager Strands. Another exemplary multiplexed target detection method follows: A sample contains, or is suspected of containing, three target species – protein #1, protein #2, and protein #3. Three docking strands are designed such that: the first, containing imager binding domain A, binds to protein #1; the second, containing imager binding domain B, binds to protein #2; and the third, containing imager binding domains A and B, binds to protein #3. Complementary imager strand A' binds to imager binding domain A of a docking strand and is labeled with a blue fluorophore, and imager strand B' binds to imager binding domain B of a docking strand and is also labeled with a blue fluorophore. The sample is first contacted with the docking strands, and subsequently contacted with imager strands A'. The sample is then imaged under conditions that detect the blue fluorophore. Imaging the blue fluorophore detects protein #1 and protein #3, each bound by imager strand A' labeled with the blue fluorophore. The sample is then washed to remove imager strands A'. Next, the sample is contacted with imager strands B'. The sample is then imaged again under conditions that detect the blue fluorophore. Imaging the blue fluorophore now detects protein #2 and protein #3, each bound by imager strand B' labeled with the blue fluorophore. Overlapping images of the blue fluorophores (*e.g.*, resulting in a stronger signal relative to non-overlapping fluorophores) detects protein #3 only, the only protein bound by two imager strands labeled with a blue fluorophore. Thus, the identification and location of proteins #1-#3 are identified by the overlay of images detecting the blue fluorophores and is based on signal intensity.

Detection Based on a Combination of Spectral and Exchange Detection. Yet another exemplary multiplexed target detection method follows: A sample contains, or is suspected

of containing, fifteen target species. The docking strands are designed such that each target species binds to a docking strand, each docking strand containing a single distinct domain or a distinct combination of domains A-D (*e.g.*, A, or A and B (*i.e.*, A/B), or A/C, or A/D, or A/B/C, or A/B/D, or A/C/D, or A/B/C/D, or B, or B/C, or B/D, or B/C/D, or C, or C/D, or D).

5 The imager strands are divided into two sets: the first set (set #1) contains imager strand A' labeled by a red fluorophore and imager strand B' labeled by a blue fluorophore; the second set contains imager strand C' labeled with a red fluorophore and imager strand D' labeled with a blue fluorophore. The sample is first contacted with the docking strands, and subsequently contacted with imager strand set #1. The sample is then imaged under
10 conditions that detect blue and red fluorophores. Targets bound by imager strands A' will be detected red and targets bound by imager strands B' will be detected blue. Thus, all target species with docking domains A and B will be detected in a first image or first set of images. The sample is then washed to remove imager strand set #1. Next, the sample is contacted with imager strand set #2. The sample is then imaged again under conditions that detect blue
15 and red fluorophores. Targets bound by imager strands C' will be detected red and targets bound by imager strands D' will be detected blue. Thus, all target species with docking domains C and D will be detected in a second image or second set of images. By combining all images collected, each of the 15 target species can be identified using only four imager strands and two fluorophores. It should be understood that more than four imager strands can
20 be used as well as more than two fluorophores, depending on, for example, the number of targets.

Detection Based on Duration of Transient Binding. In some embodiments, the disclosure contemplates contacting target species with different docking strands domain sequence and different lengths of those sequences. The length of a docking strand imager
25 binding domain affects the duration of transient binding to an imager strand. Docking strands with longer binding domains bind to respectively complementary imager strands for longer durations relative to shorter binding domains. In the following exemplary embodiment, a sample contains, or is suspected of containing, four target species. Four docking strands are designed such that: the first, containing binding domain A of 10 nucleotides in length (A10),
30 binds to protein #1; the second, containing imager binding domain A10 and imager binding domain B of 8 nucleotides in length (B8), binds to protein #2; the third, containing imager binding domain A of 8 nucleotides in length (A8) and imager binding domain B of 10 nucleotides in length (B10), binds to protein #3; and four, containing imager strand binding domain B10. Imager strand A' is 10 nucleotides in length, binds to both A8 and A10, and is

labeled with a blue fluorophore. Imager strand B' is 10 nucleotides in length, binds to both B8 and B10, and is labeled with a red fluorophore. The sample is first contacted with the docking strands, and subsequently contacted with imager strands A' and B'. The sample is then imaged under conditions that detect the blue fluorophore. Imaging the blue fluorophore detects protein #3 and protein #4 with a longer bound time (i.e., time of binding between imager strand and docking strand) and protein #2 with a shorter bound time. Imaging the red fluorophore detects protein #1 and protein #2 with a longer bound time and protein #3 with a shorter bound time. Overlapping images of the blue and red fluorophores detects each of the four protein targets.

The present disclosure also contemplates combining multiplexed detection based on spectral resolution and duration, exchange and duration, and spectral resolution, exchange and duration.

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

A "target" is any moiety that one wishes to observe or quantitate and for which a binding partner exists. A target, in some embodiments, may be non-naturally occurring. The target, in some embodiments, may be a biomolecule. As used herein, a "biomolecule" is any molecule that is produced by a living organism, including large macromolecules such as proteins, polysaccharides, lipids and nucleic acids (*e.g.*, DNA and RNA such as mRNA), as well as small molecules such as primary metabolites, secondary metabolites, and natural products. Examples of biomolecules include, without limitation, DNA, RNA, cDNA, or the DNA product of RNA subjected to reverse transcription, A23187 (Calcimycin, Calcium Ionophore), Abamectine, Abietic acid, Acetic acid, Acetylcholine, Actin, Actinomycin D, Adenosine, Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Adenosine triphosphate (ATP), Adenylate cyclase, Adonitol, Adrenaline, epinephrine, Adrenocorticotrophic hormone (ACTH), Aequorin, Aflatoxin, Agar, Alamethicin, Alanine, Albumins, Aldosterone, Aleurone, Alpha-amanitin, Allantoin, Allethrin, α -Amanatin, Amino acid, Amylase, Anabolic steroid, Anethole, Angiotensinogen, Anisomycin, Antidiuretic

hormone (ADH), Arabinose, Arginine, Ascomycin, Ascorbic acid (vitamin C), Asparagine, Aspartic acid, Asymmetric dimethylarginine, Atrial-natriuretic peptide (ANP), Auxin, Avidin, Azadirachtin A – C₃₅H₄₄O₁₆, Bacteriocin, Beauvericin, Bicuculline, Bilirubin, Biopolymer, Biotin (Vitamin H), Brefeldin A, Brassinolide, Brucine, Cadaverine, Caffeine, 5 Calciferol (Vitamin D), Calcitonin, Calmodulin, Calmodulin, Calreticulin, Camphor - (C₁₀H₁₆O), Cannabinol, Capsaicin, Carbohydrazide, Carbohydrate, Carnitine, Carrageenan, Casein, Caspase, Cellulase, Cellulose - (C₆H₁₀O₅), Cerulenin, Cetrimonium bromide (Cetrimide) - C₁₉H₄₂BrN, Chelerythrine, Chromomycin A₃, Chaparonin, Chitin, α -Chloralose, Chlorophyll, Cholecystokinin (CCK), Cholesterol, Choline, Chondroitin sulfate, 10 Cinnamaldehyde, Citral, Citric acid, Citrinin, Citronellal, Citronellol, Citrulline, Cobalamin (vitamin B₁₂), Coenzyme, Coenzyme Q, Colchicine, Collagen, Coniine, Corticosteroid, Corticosterone, Corticotropin-releasing hormone (CRH), Cortisol, Creatine, Creatine kinase, Crystallin, α -Cyclodextrin, Cyclodextrin glycosyltransferase, Cyclopamine, Cyclopiazonic acid, Cysteine, Cystine, Cytidine, Cytochalasin, Cytochalasin E, Cytochrome, Cytochrome C, 15 Cytochrome c oxidase, Cytochrome c peroxidase, Cytokine, Cytosine – C₄H₅N₃O, Deoxycholic acid, DON (DeoxyNivalenol), Deoxyribofuranose, Deoxyribose, Deoxyribose nucleic acid (DNA), Dextran, Dextrin, DNA, Dopamine, Enzyme, Ephedrine, Epinephrine – C₉H₁₃NO₃, Erucic acid – CH₃(CH₂)₇CH=CH(CH₂)₁₁COOH, Erythritol, Erythropoietin (EPO), Estradiol, Eugenol, Fatty acid, Fibrin, Fibronectin, Folic acid (Vitamin M), Follicle 20 stimulating hormone (FSH), Formaldehyde, Formic acid, Formnoci, Fructose, Fumonisin B₁, Gamma globulin, Galactose, Gamma globulin, Gamma-aminobutyric acid, Gamma-butyrolactone, Gamma-hydroxybutyrate (GHB), Gastrin, Gelatin, Geraniol, Globulin, Glucagon, Glucosamine, Glucose – C₆H₁₂O₆, Glucose oxidase, Gluten, Glutamic acid, Glutamine, Glutathione, Gluten, Glycerin (glycerol), Glycine, Glycogen, Glycolic acid, 25 Glycoprotein, Gonadotropin-releasing hormone (GnRH), Granzyme, Green fluorescent protein, Growth hormone, Growth hormone-releasing hormone (GHRH), GTPase, Guanine, Guanosine, Guanosine triphosphate (+GTP), Haptoglobin, Hematoxylin, Heme, Hemerythrin, Hemocyanin, Hemoglobin, Hemoprotein, Heparan sulfate, High density lipoprotein, HDL, Histamine, Histidine, Histone, Histone methyltransferase, HLA antigen, Homocysteine, 30 Hormone, human chorionic gonadotropin (hCG), Human growth hormone, Hyaluronate, Hyaluronidase, Hydrogen peroxide, 5-Hydroxymethylcytosine, Hydroxyproline, 5-Hydroxytryptamine, Indigo dye, Indole, Inosine, Inositol, Insulin, Insulin-like growth factor, Integral membrane protein, Integrase, Integrin, Intein, Interferon, Inulin, Ionomycin, Ionone, Isoleucine, Iron-sulfur cluster, K252a, K252b, KT5720, KT5823, Keratin, Kinase, Lactase,

Lactic acid, Lactose, Lanolin, Lauric acid, Leptin, Leptomycin B, Leucine, Lignin,
 Limonene, Linalool, Linoleic acid, Linolenic acid, Lipase, Lipid, Lipid anchored protein,
 Lipoamide, Lipoprotein, Low density lipoprotein, LDL, Luteinizing hormone (LH),
 Lycopene, Lysine, Lysozyme, Malic acid, Maltose, Melatonin, Membrane protein,
 5 Metalloprotein, Metallothionein, Methionine, Mimosine, Mithramycin A, Mitomycin C,
 Monomer, Mycophenolic acid, Myoglobin, Myosin, Natural phenols, Nucleic Acid,
 Ochratoxin A, Oestrogens, Oligopeptide, Oligomycin, Orcin, Orexin, Ornithine, Oxalic acid,
 Oxidase, Oxytocin, p53, PABA, Paclitaxel, Palmitic acid, Pantothenic acid (vitamin B5),
 parathyroid hormone (PTH), Paraprotein, Pardaxin, Parthenolide, Patulin, Paxilline, Penicillic
 10 acid, Penicillin, Penitrem A, Peptidase, Pepsin, Peptide, Perimycin, Peripheral membrane
 protein, Perosamine, Phenethylamine, Phenylalanine, Phosphagen, phosphatase,
 Phospholipid, Phenylalanine, Phytic acid, Plant hormones, Polypeptide, Polyphenols,
 Polysaccharides, Porphyrin, Prion, Progesterone, Prolactin (PRL), Proline, Propionic acid,
 Protamine, Protease, Protein, Proteinoid, Putrescine, Pyrethrin, Pyridoxine or pyridoxamine
 15 (Vitamin B6), Pyrrollysine, Pyruvic acid, Quinone, Radicicol, Raffinose, Renin, Retinene,
 Retinol (Vitamin A), Rhodopsin (visual purple), Riboflavin (vitamin B2), Ribofuranose,
 Ribose, Ribozyme, Ricin, RNA - Ribonucleic acid, RuBisCO, Safrrole, Salicylaldehyde,
 Salicylic acid, Salvinorin-A – C₂₃H₂₈O₈, Saponin, Secretin, Selenocysteine,
 Selenomethionine, Selenoprotein, Serine, Serine kinase, Serotonin, Skatole, Signal
 20 recognition particle, Somatostatin, Sorbic acid, Squalene, Staurosporin, Stearic acid,
 Sterigmatocystin, Sterol, Strychnine, Sucrose (sugar), Sugars (in general), superoxide, T2
 Toxin, Tannic acid, Tannin, Tartaric acid, Taurine, Tetrodotoxin, Thaumatin, Topoisomerase,
 Tyrosine kinase, Taurine, Testosterone, Tetrahydrocannabinol (THC), Tetrodotoxin,
 Thapsigargin, Thaumatin, Thiamine (vitamin B1) – C₁₂H₁₇ClN₄OS•HCl, Threonine,
 25 Thrombopoietin, Thymidine, Thymine, Triacsin C, Thyroid-stimulating hormone (TSH),
 Thyrotropin-releasing hormone (TRH), Thyroxine (T4), Tocopherol (Vitamin E),
 Topoisomerase, Triiodothyronine (T3), Transmembrane receptor, Trichostatin A, Trophic
 hormone, Trypsin, Tryptophan, Tubulin, Tunicamycin, Tyrosine, Ubiquitin, Uracil, Urea,
 Urease, Uric acid – C₅H₄N₄O₃, Uridine, Valine, Valinomycin, Vanabins, Vasopressin,
 30 Verruculogen, Vitamins (in general), Vitamin A (retinol), Vitamin B, Vitamin B1 (thiamine),
 Vitamin B2 (riboflavin), Vitamin B3 (niacin or nicotinic acid), Vitamin B4 (adenine),
 Vitamin B5 (pantothenic acid), Vitamin B6 (pyridoxine or pyridoxamine), Vitamin B12
 (cobalamin), Vitamin C (ascorbic acid), Vitamin D (calciferol), Vitamin E (tocopherol),

Vitamin F, Vitamin H (biotin), Vitamin K (naphthoquinone), Vitamin M (folic acid), Wortmannin and Xylose.

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

In some embodiments, a target may be a nucleic acid target such as, for example, nucleic acids of a cellular environment. As used herein with respect to targets, docking strands, and imager strands, a “nucleic acid” refers to a polymeric form of nucleotides of any length, such as deoxyribonucleotides or ribonucleotides, or analogs thereof. For example, a nucleic acid may be a DNA, RNA or the DNA product of RNA subjected to reverse transcription. Non-limiting examples of nucleic acids include coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, isolated DNA of any sequence,

isolated RNA of any sequence, nucleic acid probes, and primers. Other examples of nucleic acids include, without limitation, cDNA, aptamers, peptide nucleic acids (“PNA”), 2'-5' DNA (a synthetic material with a shortened backbone that has a base-spacing that matches the A conformation of DNA; 2'-5' DNA will not normally hybridize with DNA in the B form, but it will hybridize readily with RNA), locked nucleic acids (“LNA”), and nucleic acids with modified backbones (*e.g.*, base- or sugar-modified forms of naturally-occurring nucleic acids). A nucleic acid may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs (“analogous” forms of purines and pyrimidines are well known in the art). If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. A nucleic acid may be a single-stranded, double-stranded, partially single-stranded, or partially double-stranded DNA or RNA.

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

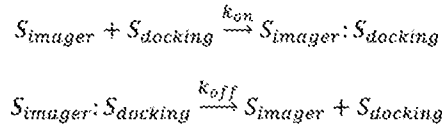
The nucleic acid docking strands and imager strands described herein can be any one of the nucleic acids described above (*e.g.*, DNA, RNA, modified nucleic acids, nucleic acid analogues, naturally-occurring nucleic acids, synthetic nucleic acids).

Quantitative Imaging

The present disclosure also provides methods for quantitating fluorescent moieties or emitters in a dense cluster that cannot be spatially resolved using prior art imaging techniques. Prior to the invention, no systematic model existed that describes the kinetics of photoswitching of fluorescent signals.

Stochastic super-resolution imaging using transient binding of short oligonucleotides (*e.g.*, imager strands) to their targets offers a unique possibility to quantitatively count integer numbers of labeled molecules in a diffraction-limited area. “Switching” molecules from a fluorescent OFF- to an ON-state in the method of the present disclosure is facilitated by single-molecule nucleic acid (*e.g.*, DNA) hybridization events, which are governed by a very

predictable kinetic model with a second order association rate k_{on} and a first order dissociation rate k_{off} :



The kinetic parameters k_{on} and k_{off} are now directly linked to fluorescent ON- and OFF-times (τ_b and τ_d , respectively) depicted in FIG. 7A. The fluorescence ON-time τ_b is determined by the dissociation rate k_{off} : $\tau_b = 1/k_{off}$, and the fluorescence OFF-time τ_d is determined by the association rate k_{on} , the concentration of imager strands in solution c_{imager} , and the number of observed binding sites bs :

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

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

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

- Accordingly, the quantification of a fluorescence image may be done automatically using binding kinetics analysis software. In brief, a typical image is recorded in a time-lapsed fashion (*e.g.*, 15000 frames with a frame rate of 10 Hz). Fluorescence spot detection and fitting (*e.g.*, Gaussian fitting, Centroid fitting, or Bessel fitting) is performed on the diffraction-limited image, and thus a super-resolved image is obtained. In the next step, a calibration marker is selected (*e.g.*, a DNA origami structure with a defined number of spots as in FIG. 7C). The software automatically calculates the fluorescence dark time τ_d by fitting the OFF-time distribution to a cumulative distribution function. Using the equations described above, the product of $k_{on} \cdot c_{imager}$ can be calculated. This product is used to calculate the number of docking sites, and thus targets in the imaged area.

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

Thus, in some embodiments, the methods of the present disclosure comprise providing a sample that comprises targets transiently bound directly or indirectly to fluorescently-labeled imager strands, obtaining a time-lapsed diffraction-limited fluorescence

image of the sample, performing fluorescence spot detection and fitting (*e.g.*, Gaussian fitting, Centroid fitting, or Bessel fitting) on the diffraction-limited image to obtain a high-resolution image of the sample, calibrating $k_{on} \cdot C_{imager}$ using a sample with a known number of targets, wherein k_{on} is a second order association constant, and C_{imager} is the concentration of fluorescently-labeled imager strands in the sample, including unbound imager strands, determining variable τ_d by fitting the fluorescence OFF-time distribution to a cumulative distribution function, and determining the number of targets in the sample based on the equation, $number\ of\ targets = (k_{on} \cdot C_{imager} \cdot \tau_d)^{-1}$.

Some aspects of the present disclosure relate to fitting functions. A “fitting function,” as used herein, refers to a mathematical function used to fit the intensity profile of molecules. Examples of fitting functions for use as provided herein include, without limitation, Gaussian fitting, Centroid fitting, and Bessel fitting. It should be understood that while many aspects and embodiments of the present disclosure refer to Gaussian fitting, other fitting functions may be used instead of, or in addition to, Gaussian fitting.

Compositions

Provided herein are compositions that comprise at least one or at least two (*e.g.*, a plurality) BP-NA conjugate(s) (*e.g.*, protein-nucleic acid conjugate(s)) of the present disclosure. The BP-NA conjugates may be bound to a target of interest (*e.g.*, biomolecule) and/or transiently bound to a complementary fluorescently-labeled imager strand. A composition may comprise a plurality of the same species or distinct species of BP-NA conjugates. In some embodiments, a composition may comprise at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4 , 50000, 10^5 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} BP-NA conjugates. In some embodiments, a composition may comprise at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4 , 50000, 10^5 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} complementary fluorescently-labeled imager strands. In some embodiments, a composition may contain 1 to about 200 or more distinct species of BP-NA conjugates and/or imager strands. For example, a composition may contain at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200 or more distinct species. In some embodiments, a composition may contain less than about 5 to about 200 distinct species of BP-NA conjugates and/or imager strands. For example, a composition may contain less than 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175 or 200 distinct species.

It should be understood that the number of complementary fluorescently-labeled imager strands imager stands in a composition may be less than, equal to or greater than the number of BP-NA conjugates in the composition.

5 *Kits*

The present disclosure further provides kits comprising one or more components as provided herein. The kits may comprise, for example, a BP-NA conjugate and/or a fluorescently-labeled imager strands. The kits may also comprise components for producing a BP-NA conjugate or for labeling an imager strand. For example, the kits may comprise a
10 binding partner (*e.g.*, antibody), docking strands and intermediate linkers such as, for example, biotin and streptavidin molecules, and/or imager strands. The kits can be used for any purpose apparent to those of skill in the art, including, those described above.

The kits may include other reagents as well, for example, buffers for performing hybridization reactions. The kit may also include instructions for using the components of
15 the kit, and/or for making and/or using the BP-NA conjugates and/or labeled imager strands.

In some embodiments, a kit comprises at least one docking strand and at least one labeled imager strand that is capable of transiently binding to a docking strand. The docking strands may or may not be conjugated to a binding partner. In some embodiments, the docking strands are conjugated to “generic” non-target-specific affinity molecule (*e.g.*, biotin
20 or streptavidin), which may be used to link a docking strand to binding partner chosen by an end user. In some embodiments, the affinity molecule is a secondary antibody. Thus, in some embodiments, a kit comprises at least one docking strand, at least one affinity molecule such as a secondary antibody, and at least one imager strand.

In some embodiments, a kit comprises (a) at least one docking strand linked to a
25 binding partner such as a protein (*e.g.*, a protein that binds to a target) and (b) at least one (*e.g.*, at least 2, at least 3, at least 4, at least 5, at least 10, at least 100) labeled imager strand that is capable of transiently binding (*e.g.*, transiently binds) to a docking strand. A docking strand may comprise, for example, at least two domains or at least three domain, wherein each domain binds to a respective complementary labeled imager strand. The number of
30 labeled imager strands may be, for example, less than, greater than or equal to the number of docking strands. The binding partner may be a protein such as, for example, an antibody (*e.g.*, monoclonal antibody), an antigen-binding antibody fragment, or a peptide aptamer. In some embodiments, a kit comprises at least two different binding partners (*e.g.*, proteins), each specific for a different target. A binding partner (*e.g.*, protein), in some embodiments, is

linked to a docking strand through an intermediate linker such as, for example, a linker that includes biotin and streptavidin (*e.g.*, a biotin-streptavidin-biotin linker). In some embodiments, a docking strand is modified to contain an affinity molecule that can be used to link the docking strand to a binding partner. In some embodiments, the affinity molecule is a secondary antibody. An imager strand, in some embodiments, is labeled with at least one fluorescent label (*e.g.*, at least one fluorophore). In some embodiments, the length of an imager strand is 4 to 30 nucleotides, or longer. For example, the length of an imager strand may be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides. In some embodiments, the length of an imager strand is 8 to 10 nucleotides. In some embodiments, a kit comprises at least two imager strands, each different from one another. In some embodiments, the thermal stability of a docking strand transiently bound to its complementary labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

In some embodiments, a kit comprises (a) at least one docking strand linked to a monoclonal antibody or an antigen binding fragment thereof (*e.g.*, a monoclonal antibody or an antigen binding fragment thereof that binds to a target) and (b) at least one (*e.g.*, at least 2, at least 3, at least 4, at least 5, at least 10, at least 100) labeled imager strand that is capable of transiently binding (*e.g.*, transiently binds) to a docking strand. A docking strand may comprise, for example, at least two domains, wherein each domain binds to a respectively complementary labeled imager strand. The number of labeled imager strands may be, for example, less than, greater than or equal to the number of docking strands. In some embodiments, a kit comprises at least two different monoclonal antibodies or antigen binding fragments thereof, each specific for a different target. A monoclonal antibody or an antigen binding fragment thereof, in some embodiments, is linked to a docking strand through an intermediate linker that includes biotin and streptavidin (*e.g.*, a biotin-streptavidin-biotin linker). An imager strand, in some embodiments, is labeled with at least one fluorescent label (*e.g.*, at least one fluorophore). In some embodiments, the length of an imager strand is 4 to 30 nucleotides, or longer. For example, the length of an imager strand may be 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides. In some embodiments, the length of an imager strand is 8 to 10 nucleotides. In some embodiments, a kit comprises at least two imager strands, each different from one another. In some embodiments, the thermal stability of a docking strand transiently bound to a

complementary labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

Applications

5 The BP-NA conjugates (*e.g.*, protein-nucleic acid conjugates or antibody-nucleic acid conjugates) of the present disclosure can be used, *inter alia*, in any assay in which existing target detection technologies are used.

 Typically assays include detection assays including diagnostic assays, prognostic assays, patient monitoring assays, screening assays, biowarfare assays, forensic analysis
10 assays, prenatal genomic diagnostic assays and the like. The assay may be an *in vitro* assay or an *in vivo* assay. The present disclosure provides the advantage that many different targets can be analyzed at one time from a single sample using the methods of the present disclosure, even where such targets are spatially not resolvable (and thus spatially indistinct) using prior art imaging methods. This allows, for example, for several diagnostic tests to be performed
15 on one sample.

 The BP-NA conjugates can also be used to simply observe an area or region.

 The methods of the present disclosure may be applied to the analysis of samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease. For example, a blood sample can be assayed
20 according to any of the methods described herein to determine the presence and/or quantity of markers of a cancerous cell type in the sample, thereby diagnosing or staging the cancer.

 Alternatively, the methods described herein can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample. Thus,
25 the targets detected using the methods, compositions and kits of the present disclosure may be either patient markers (such as a cancer marker) or markers of infection with a foreign agent, such as bacterial or viral markers.

 The quantitative imaging methods of the present disclosure may be used, for example, to quantify targets (*e.g.*, target biomolecules) whose abundance is indicative of a biological
30 state or disease condition (*e.g.*, blood markers that are upregulated or downregulated as a result of a disease state).

 Further, the methods, compositions and kits of the present disclosure may be used to provide prognostic information that assists in determining a course of treatment for a patient. For example, the amount of a particular marker for a tumor can be accurately quantified from

even a small sample from a patient. For certain diseases like breast cancer, overexpression of certain proteins, such as Her2-neu, indicate a more aggressive course of treatment will be needed.

The methods of the present disclosure may also be used for determining the effect of a perturbation, including chemical compounds, mutations, temperature changes, growth hormones, growth factors, disease, or a change in culture conditions, on various targets, thereby identifying targets whose presence, absence or levels are indicative of a particular biological states. In some embodiments, the present disclosure is used to elucidate and discover components and pathways of disease states. For example, the comparison of quantities of targets present in a disease tissue with "normal" tissue allows the elucidation of important targets involved in the disease, thereby identifying targets for the discovery/screening of new drug candidates that can be used to treat disease.

The sample being analyzed may be a biological sample, such as blood, sputum, lymph, mucous, stool, urine and the like. The sample may be an environmental sample such as a water sample, an air sample, a food sample and the like. The assay may be carried out with one or more components of the binding reaction immobilized. Thus, the targets or the BP-NA conjugates may be immobilized. The assay may be carried out with one or more components of the binding reaction non-immobilized. The assays may involve detection of a number of targets in a sample, essentially at the same time, in view of the multiplexing potential offered by the BP-NA conjugates and fluorescently-labeled imager strands of the present disclosure. As an example, an assay may be used to detect a particular cell type (*e.g.*, based on a specific cell surface receptor) and a particular genetic mutation in that particular cell type. In this way, an end user may be able to determine how many cells of a particular type carry the mutation of interest, as an example.

Devices

Also provided herein are fluidic chamber devices for liquid handling, as shown in FIGs. 23A and 23B. In some embodiments, the device is a polymer-based (*e.g.*, polydimethylsiloxane (PDMS)) device comprising first and second channels, each connected at one end to a sample chamber. This configuration permits one or more fluid(s) to be sequentially administered to the sample at a controlled rate. For example, a syringe may be used to administer a first fluid to the sample through a first channel of the device. The syringe may then be used to administer a second fluid, which passes through the first channel of the device into the sample chamber, thereby forcing the first fluid out of the sample

chamber, passing through a second channel and into, for example, a reservoir connected to the second channel (FIG. 23A). In some embodiments, the device is positioned on a glass slide to permit viewing from a microscope objective positioned below the device.

5 *Super Resolution Imaging*

Super-resolution imaging with increased spatial resolution (referred to herein as “ultra-resolution” imaging) may be achieved, in some embodiments, by increasing the number of photons per localization event using one of two strategies, depicted in FIG. 11A. In the first strategy, the maximum number of photons from single, replenishable fluorophores is extracted. High laser excitation power, in combination with fluorophore stabilization buffers (16,17) may be used to “bleach” transiently bound fluorophores at docking sites (or sites of docking strands), thus extracting the maximal number of photons per binding event and dye (FIG. 11A). The repetitive binding of imager strands permits “photobleaching” of every bound strand, thus making maximal use of emitted photons and resulting in a significant increase in localization accuracy over traditional imaging techniques. In the second strategy, bright metafluorophores are used. A fluorescent DNA nanostructure, or “metafluorophore,” may be constructed by decorating a compact DNA nanostructure with many fluorophores (Fig. 11B3). The sum of the individual dye emissions from a metafluorophore are interpreted as originating from the same point source, and thus, using the metafluorophore in place of a standard fluorophore (*e.g.*, Cy3) further improves the localization precision. A more advanced version of the metafluorophore with active background suppression is depicted in FIG. 11B4. Here, the clam-shell-like structure acts as a conditional fluorophore that only fluoresces when it is bound to the docking strand.

The present disclosure also provides algorithms used for spot detection, fitting and drift correction, as described below.

Software Algorithm for Drift Correction

Some embodiments are directed to methods and apparatus for correcting drift in images recorded in a time sequence. A non-limiting application of the techniques for performing drift correction, discussed in further detail below, is to correct for drift in molecular scale DNA-based imaging described herein involving transiently binding between docking strands and imaging strands. However, it should be appreciated that the techniques described herein may alternatively be used to correct for drift in other imaging applications where one or more transient imaging events are recorded during a time sequence of images,

and embodiments related to drift correction are not limited to molecular scale DNA-based imaging.

In some embodiments, a DNA nanostructure can be used as a drift marker. Any suitable DNA nanostructure (*see, e.g.*, (Rothemund US-2007/0117109 A1), single-stranded tiles (Yin et al., “Programming DNA Tube Circumferences,” *Science* (2008): 321: 824-826), DNA hairpins (Yin et al. US-2009/0011956 A1; Yin et al., “Programming biomolecular self-assembly pathways,” *Nature* (2008) 451:318-323) may be used, and may be made using, *e.g.*, DNA origami techniques. Drift correction using DNA nanostructure-based drift markers in combination with advanced analysis and post-processing techniques has the advantage of high precision correction, compatibility with long time imaging and simplicity of implementation. Conventional nucleic acid-based imaging techniques incorporating drift markers based on fluorescent beads suffer from the limited length of imaging time before the beads are bleached; whereas bright field imaging requires specialized equipment, *e.g.* dual-field camera view.

Drift correction techniques in accordance with some embodiments described herein may include a plurality of stages, where each of the stages uses a different technique to perform drift correction. In some embodiments, the output from one stage is provided as input to a subsequent stage for additional drift correction processing. In a first stage, a coarse drift correction is performed by comparing localizations from neighboring frames. In a second stage, a single drift marker is selected and its time trace is used as a different coarse correction. In a third stage, a group of drift markers is selected, either automatically or with user input, and their time traces are then combined to compute a more precise drift correction. In a fourth stage, localizations are pooled from template-based drift markers displaying spots in a defined and spatially resolvable geometry (*e.g.*, 4x3 grid points). In a fifth stage, a smoothing of the drift correction is performed to further reduce noise and enabling the resolution of the final image to approach molecular-scale resolution.

Any number and/or combination of these five stages may be performed in accordance with the techniques described herein. For example, in some embodiments, an amount of drift in the time sequence of images may be characterized using a quality measure, and based, at least in part, on the quality measure, one or more of the stages may be eliminated. In other embodiments, all five stages may be performed, as embodiments are not limited in this respect. In yet other embodiments, additional drift correction stages used in combination with at least one of the stages described herein may also be used.

In the following description of techniques for performing drift correction, the term FWHM (Full Width at Half Maximum) is used as the mathematical surrogate for "resolution." The approximation that $\text{FWHM} \approx \sigma * 2.35$ for a Gaussian distribution, where σ is the standard deviation, is also used. The techniques described herein for performing drift correction relate to processing a time sequence of images. The recorded image stack is referred to herein as a "movie" and each of the individual images as a "frame." Each frame of the movie is operated on with a spot finding algorithm, and then a local Gaussian fitting algorithm may be used for each identified spot; the spot and its fitted center position localization are interchangeably referred to herein as a "localization." The frames of the movie capture one or more transient events that are present in some frames but not others. In the illustrative application of the techniques where the frames of the movie related to nucleic acid-based imaging as discussed above, the hybridization of an imager strand to a docking strand until their disassociation is referred to herein as a binding "event"; thus an event could have, and typically will consist of, several localizations in a series of neighboring frames. Although binding events are discussed in further detail below as one illustrative transient event that may be analyzed using the techniques described herein for performing drift correction, it should be appreciated that other types of transient events imaged in a time sequence may alternatively be used. Within a certain area of the field of view, the collection of all localizations throughout the entire movie is collectively referred to as the "time trace," which reflects the movement of an observed structure, and is used for drift correction in several different ways, as described in more detail below.

Overview of drift correction techniques

FIG. 12 illustrates a schematic overview of five stages of a drift correction procedure that may be performed in accordance with the techniques described herein. The stages are illustrated as being performed consecutively in an order from an unprocessed image to a final image that has been processed using the techniques of each of the five stages. Each of these stages will be discussed in more detail below. Briefly, FIG. 12A(i) illustrates schematics showing the principle of each stage of drift correction. In each image, black markers and lines indicate source data, and red values and curves indicate the calculated drift correction. FIG. 12A(ii) shows a schematic drawing of the major type of drift markers (*e.g.*, DNA drift markers) used in each stage. FIG. 12B(i) illustrates an example structure showing the imaging quality after each stage or correction, and FIG. 12B(ii) shows a zoomed image of the corresponding green rectangle in FIG. 12B(i) at each stage. The scale bars shown in FIGS.

12B(i) and 12B(ii) correspond to 50 nm. FIG. 12C(i) illustrates an example drift trace after each stage of correction, and FIG. 12C(ii) shows a zoomed image of the corresponding green rectangle in FIG. 12C(i) at each stage. The scale bars in FIG. 12C(i) correspond to x: 500 nm, t: 500 s, and the scale bars in FIG. 12C(ii) correspond to x: 10nm, t: 10 s. FIG. 18

5 illustrates an alternate representation of stages in a drift correction process in accordance with some embodiments.

In some embodiments, an image resolution output from the first stage may be on the order of 1 μ m.

10 In some embodiments, an image resolution output from the second stage may be on the order of 200 nm.

In some embodiments, an image resolution output from the third stage may be on the order of 20 nm.

In some embodiments, an image resolution output from the fourth stage may be on the order of 5 nm.

15 In some embodiments, an image resolution output from the fourth stage may be on the order of less than 5 nm.

Imaging quality and limit of achievable resolution

20 The finest possible quality of a drift-corrected image is limited by the quality of individual localizations, which is determined by the various conditions used during an imaging session (*e.g.*, a microscopy imaging session). To quantitatively assess and effectively compare between the quality of different imaging conditions, a quantity called Distance between Neighboring Frame Localizations (DNFL) is defined as the mean separation between localizations detected from consecutive image frames, which originated from the same transient event (*e.g.*, a binding event).

25 The procedure for calculating the DNFL for an image is outlined as follows. For each pair of NF (Neighboring Frames, *e.g.* frame #1 and #2), all localizations from both frames are pooled and the distance between every pair of localizations from different frames (*e.g.* one localization from frame #1 versus another from frame #2) is calculated, assuming no drift between the frames. The resulting distances from all NF pairs are pooled to provide a bimodal distribution. The first mode of the bimodal distribution is broad, high in amplitude, and spans the width of the field of view. The second mode of the bimodal distribution is sharp, low in amplitude, and close to zero, and corresponds to localizations from the same binding event. The maximum of the second mode may be determined and a local Gaussian

fitting algorithm may be performed around the maximum to determine the center of the peak. This value may be considered the DNFL of a certain image. Without combining localizations from consecutive frames, the DNFL value sets the limit of the finest possible resolution that can be achieved from a certain image, with a mathematical relation between the best

5 achievable resolution and DNFL being: best achievable resolution = DNFL / $\sqrt{2}$ * 2.35.

Drift correction quality and supported resolution

The quality of a final drift-corrected image may be assessed by characterizing the Point Spread Function (PSF) of a single binding site. A statistical overlay of images of more than
10 thousands of single docking sites may be produced as the reference for the PSF distribution. A 2-D Gaussian fitting may be performed on the statistical overlay to determine the standard deviation (sigma) of the PSF, which in turn determines the best supported resolution of the produced image, given by a similar formula as above: image supported resolution = sigma * 2.35. The isolation and overlay of single isolated docking sites may be performed with the
15 help of an auxiliary DNA nanostructure. This structure has a known pattern of well-separated docking sites (e.g., a lattice grid pattern), and may be the same structure used for the template-based drift correction stage, discussed in more detail below. Because of variation of laser intensity, unevenness of optical surface deposition and other systematic factors, as well as the possible stochastic nature of the imaging process, the above determined
20 image quality of the whole image may not reflect the true imaging quality for each single sample object in the imaging field. Typically, structures closer to the center of the imaging field and better fixed to the optical surface, are better illuminated, and show better resolution than those that are on the periphery and are less well fixed. The image quality and resolution of a single molecule may be determined in a procedure similar to the one above. A projection
25 of a single molecule of an auxiliary DNA nanostructure (same as above) may be taken along a direction that best separates the docking sites (in the case of lattice grid structures, this will be along any of the lattice directions), and a multi-Gaussian fit may be performed on the projected 1-D distribution. The standard deviation of the fitted Gaussian peaks may then be determined and similarly used to infer the resolution of a single-molecule image.

30

Drift assessment and choice of drift correction stages

In some embodiments, the five stages incorporating techniques for performing drift correction, discussed in further detail below, operate in series to reduce the drift of an unprocessed image, where each consecutive stage reduces the drift further, and low enough

for the successful operation of the next stage. Depending on the amount of drift in the captured images, less than all five stages may be used. For example, if it is determined that there is low drift in the original image, the localizations in each frame may be separable, and processing may begin from the second stage without requiring processing by first stage. If it is determined that there is even lower drift in the original image, processing may be begin from the third stage without significant loss of final image quality. Due to the complex origin of drift, which may involve, among other things, thermal fluctuation and expansion, microscope stage movement due to electric motor activation, vibration from the building and optical table complex, controlling drift in the original image tends to be difficult, and including the first two stages is often useful in producing a final image with desired resolution. For example, including all five stages described herein provides a robust strategy for drift correction that is applicable to images taken in most biology labs, without the requirement of specialized hardware or building requirements.

In some embodiments, the amount of drift and overall image quality of the original unprocessed image may be determined using any suitable technique, and the determined image quality may be used to select a choice of drift correction stages to use in performing drift correction. For example, a technique for determining image quality may compare different temporal segments of the same image. In this illustrative technique, the original image may be divided into two halves by separately pooling localizations from the first half and the second half of the movie, respectively. The cross-correlation between the two images may be calculated and a best offset may be estimated to provide an indication of the overall drift. The indication of overall drift may be compared to one or more threshold values to determine whether one or more of the drift correction stages may be skipped in performing drift correction in accordance with the techniques described herein.

FIG. 13 illustrates a process for performing drift correction in accordance with some embodiments. In act 210, drift correction is performed by considering differences in localizations across neighboring frames of a movie. The process then proceeds to act 220, where a single drift marker is selected, a time trace describing the movement of the drift marker over time during the movie is determined, and the time trace for the single drift marker is used to perform drift correction of the image. The process then proceeds to act 230, where time traces are determined for each of a plurality of drift markers identified in the image. As discussed in more detail below, differences between the time traces may be used to provide a further drift correction in the final image. The process then proceeds to act 240, where drift correction using geometrically-constrained templates is performed. The process

then proceeds to act 250, where the image is further drift corrected by smoothing the drift trace using suitable smoothing techniques, as discussed in more detail below. Each of the five stages for performing drift correction in accordance with the techniques described herein are described in further detail below. As should be appreciated from the foregoing

5 discussion, not all embodiments require the use of all five stages for drift correction processing. For example, in some embodiments, only acts 230 and 240 may be performed. In other embodiments, acts 230, 240, and 250 may be performed. In yet other embodiments, acts 220, 230, 240 and 250 may be performed without including act 210.

10 *First stage drift correction*

A first stage of drift correction (*e.g.*, act 210 of FIG. 13) operates by comparing localizations from neighboring frames in a movie. A procedure similar to the DNFL calculation described above (or any other suitable technique) may be used to identify pairs of localizations originating from the same transient event (*e.g.*, a single binding event). All

15 pairs of localizations originating from the same transient event may be pooled to create a bimodal distribution. After creating a bimodal distribution of the localizations from neighboring images, a cutoff value may be automatically determined to separate those pairs of localizations from the same event (close localizations), from those that are different. Next,

20 all pairs of close localizations for the same neighboring frame (NF) pair are pooled, and the offset between each pair is computed. The vector average of all offsets are output as the drift correction. For NF pairs with no qualifying close localizations being identified, a zero drift may be output. The first stage of drift correction typically corrects for global drift with high amplitude (farther than $1\mu\text{m}$ in offset), which effectively removes interference between

different drift markers and allows for incorporation of the next stage. As discussed above, in

25 some embodiments where different drift markers may already be separable from each other, the first stage of processing may be omitted. A determination of whether the first stage of processing may be omitted may be made using an image quality factor analysis, as discussed above, or using any other suitable technique (*e.g.*, manual inspection).

30 *Second stage drift correction*

A second stage of drift correction (*e.g.*, act 220 of FIG. 13) operates on a single drift marker or sample object. The single drift marker for use in this stage of drift correction processing may be selected in any suitable way. For example, in some embodiments, the single drift marker may be randomly selected from the set of all identified drift markers. In

other embodiments, a particular drift marker associated with desirable qualities (*e.g.*, an average amount of drift over the entire movie) may be selected as the single drift marker to use for this stage. After selecting the single drift marker, its time trace is automatically determined, smoothed, and output as the drift correction. Alternatively, a drift trace may be manually drawn in cases where separation between drift markers is hard to identify automatically.

The second stage of drift correction further reduces global drift in the movie (typically <200 nm), and allows automatic batch identification of drift markers in the following stages.

10 *Third stage of drift correction*

A third stage of drift correction (*e.g.*, stage 230 of FIG 2) combines the time traces of a plurality of drift markers to compute drift correction with a finer resolution than the second stage of drift correction. Each drift marker has a large number of docking sites to allow a high temporal coverage; and a large number of these drift markers are deposited onto the imaging surface together with the samples. The number of binding sites on each drift marker and the concentration of drift markers on the surface may be selected appropriately to ensure a high quality drift correction, as the improvement in drift correction in performing this stage is primarily determined by the spread of each drift marker in the image and the effective number of drift markers per frame.

FIG. 14 illustrates a process for performing drift correction corresponding to stage 230 of FIG 2. In act 310, locations of a plurality of drift markers are identified. Identifying locations of the plurality of drift markers may include pooling localizations from all frames of the movie to calculate a two-dimensional (2D) histogram. Then, the locations of drift markers may be identified by appropriately tuning the histogram binning size and a combination of other selection criteria. For example, the binning size of the histogram may be tuned to reflect the feature size of the drift marker, *e.g.*, a fourth or third of their overall size. The range of selection criteria includes, but it is not limited to, a lower-bound threshold of the histogram value and filtering based on geometrical properties (*e.g.*, area, dimensions). Adequate separation between nearby drift markers is often necessary to exclude false localizations, which, for example, arise from spurious localizations of double-binding events. After the identification of a pool (*e.g.*, thousands) of drift markers, the process to act 320, where the time trace for each drift marker is determined and the relative time trace determined as the offset of each time trace from the center of the combined trace is computed. Because the images capture transient events (*e.g.*, nucleic acid-binding events),

not all time traces for a drift marker may cover all time points in the movie. In such cases, the time traces may be linearly interpolated at the “missing points” to achieve a finer and smoother result.

After determining the time trace for each of the plurality of drift markers, the process proceeds to act 330, where the drift correction for the image is determined based on the time traces determined for each drift marker. Any suitable combination of the time traces may be used to determine the drift correction, and embodiments are not limited in this respect. In some embodiments, a weighted average of the time traces is used to determine the drift correction output from this stage. For example, a weighted average of the pool of relative time traces may be computed as the result of drift correction, where the correction is weighted by the quality of drift marker traces. The quality of drift marker traces may be determined in any suitable way including, but not limited to, determining the quality by assessing drift marker quality or individual localization quality. In some embodiments, the quality of each drift marker trace is computed by taking the standard deviation (σ) of the trace over time. The inverse of this measure (*e.g.*, $1/\sigma$) for each trace may be used as the weight factor. Alternatively, the quality of each individual localization within the time traces may be computed as the localization uncertainty given by the formula in *Thomson, 2002*. The inverse of the standard deviation of this calculation may be used as the weight factor for each time trace. After determining the drift correction, the process proceeds to act 340 where the image is corrected using the determined drift correction.

This stage of drift correction may be performed any number of times. In some embodiments, this stage of drift correction is iteratively performed with different parameters used for each iteration. As drift correction proceeds, the remaining drift amplitude is decreased further and further, the spatial spread-out of drift markers becomes smaller and smaller, and selection of drift markers may be performed more and more stringently. Consequently, in initial iterations, the threshold histogram count may be set to a lower value, and this value may be adjusted during later iterations to higher values. Additionally, in some embodiments, the valid area and dimensions of drift markers may be set to larger values in initial iterations, and later adjusted to smaller values during subsequent iterations. Yet further, in some embodiments, separation between drift markers may be shifted from larger values to smaller values in later iterations. In some embodiments, an interactive quality check (either manually or automatically performed) may be determined between iterations to facilitate a determination of further operations.

Depending on the imaging quality, this stage of drift correction typically brings the obtained image resolution to within a factor of two from the best allowed resolution (i.e. if the precision of each individual localization supports resolution of ~5 nm, then this stage usually yields ~10 nm resolution). Typically, with good imaging conditions, a resolution <10 nm may be obtained following processing with this stage.

Fourth stage of drift correction

A fourth stage of drift correction (e.g., act 240 in FIG. 13) uses drift marker "templates," and thus this stage is termed "templated drift correction." One or more drift marker templates (e.g., DNA nanostructures with docking sites in a known and well-separated geometric arrangement) are deposited onto the imaging surface together with "ordinary" drift markers, discussed above in connection with stage three. The separation between these docking sites is preferably chosen not to be not smaller than twice the resulting resolution from the previous stage (e.g., stage three), allowing easy separation between localizations from different docking sites. The number of docking sites on these templates as well as the concentration of the docking sites on the surface, is preferably chosen to achieve effective template correction, similar that described for the third stage.

FIG. 15 illustrates a process for performing drift correction corresponding to stage 240 of FIG 2. In act 410, a plurality of drift correction templates are identified from a 2-D histogram of localizations pooled across all frames of the movie. To distinguish the drift templates from the drift markers used in the third stage, an extra upper-bound threshold in histogram count may be incorporated in addition to the range of selection criteria as mentioned above. After the drift templates have been identified, the process proceeds to act 420, where the time trace of each drift template is determined. Because the docking sites are designed to be well separated in the templates, several non-overlapping time traces from individual docking sites may be isolated from each time trace of a full drift template. This identification and separation step may be carried out in a similar manner as the identification of drift markers from the entire image. For example, a combination of local histogram thresholding and filtering on standard deviation of the individual time traces for each drift template may be used.

After the time trace for each drift template has been determined, the process proceeds to act 430, where the combination of time traces is used to determine a drift correction for this stage. In some embodiments, this is accomplished by computing relative time traces for each time trace, and the relative time traces are used, at least in part, to determine the drift

correction. The relative time traces may be used in any suitable way to determine the drift correction. For example, in some embodiments, a weighted average of all the time traces of individual docking sites may be averaged to produce the final drift correction. The weight factors may be determined in any suitable way. For example, the weight factors may be based on the quality of each individual site, or the quality of each individual localization in the time trace. After determining the drift correction, the process proceeds to act 440 where the image is corrected using the determined drift correction.

Depending on the imaging quality, this stage of drift correction may result in a resolution of the final image being close to the best possible resolution. That is, if the precision of each individual localization supports a resolution of ~5 nm, performing template drift correction in accordance with the techniques described herein may achieve a resolution close to ~6 nm. In some embodiments, for the <10 nm resolution achieved after the third stage, a DNA nanostructure with 12 docking sites arranged in a 4x3 grid of lattice spacing 20 nm may be used as the drift correction template. Template-based drift correction using the techniques described in this section may enable the achievement of 6–7 nm resolution after this stage.

Fifth stage drift correction

A fifth stage of drift correction (*e.g.*, act 250 in FIG. 13) performs smoothing of the drift correction trace, and the smoothed drift correction trace may be used to perform drift correction. For example, after determining a drift correction for one or more of the second, third and fourth stages discussed above, the resultant drift correction may be smoothed using any suitable technique to produce the final drift correction result. Smoothing effectively increases the number of drift markers or drift templates in each frame, by taking the localizations in neighboring frames into account. Smoothing may be performed in any suitable manner using any suitable window period. For example, in some embodiments, smoothing is performed with a robust local regression method that operates over a window period determined by the characteristic drift time scale. A non-limiting example of a smoothing window period may be 10–30 s.

Extension to 3D imaging

All stages described above can be directly applied to correct a 3D image (*e.g.*, super-resolution image) as well. In 3D super-resolution imaging, for example, an astigmatism lens may be introduced in the imaging path and the ellipticity of the resulting Gaussian emission

profile may be used to determine a z-position of a molecule. The above-described origami drift marker structures (*e.g.*, geometric-constrained templates) may be used in a one-to-one fashion to perform the stages of drift correction. For the template-based drift correction stage, a DNA origami structure with a defined 3D shape (*e.g.*, a tetrahedron) may be used.

5 FIG. 16 illustrates 3D tetrahedrons used as templates for 3D drift correction. The four corners are labeled with docking sites. FIG. 16A shows that the four corners are clearly resolved. FIG. 16B illustrates the X-Z projection of the structures with a height of ~85 nm.

Extension to multicolor imaging

10 The above-described techniques for correcting drift in a plurality of time sequence images is described with respect to imaging a transient event identified using a single color in the images. However, it should be appreciated that these techniques may be extended to multicolor imaging in which different transient events (*e.g.*, binding of different nucleic acid drift markers with docking sites) are labeled with different colors that can be identified in the
15 same image. When multicolor imaging is used, the geometric templates discussed above for template-based drift correction may include information describing a particular known geometry for the different transient events that correspond to the different colors. For example, rather than just a single binding event occurring at a single docking site in a 3x4 grid, multiple binding events color-coded using different colors in the same geometric
20 template may be represented, and the drift correction may be performed using information from the multiple binding events. Other processes for extending the techniques described herein to multicolor imaging are also contemplated, and embodiments are not limited in this respect.

Exemplary computer system

25 An illustrative implementation of a computer system 600 that may be used in connection with any of the embodiments of the present disclosure described herein is shown in FIG. 17. The computer system 600 may include one or more processors 610 and one or more computer-readable non-transitory storage media (*e.g.*, memory 620 and one or more
30 non-volatile storage media 630). The processor 610 may control writing data to and reading data from the memory 620 and the non-volatile storage device 630 in any suitable manner, as the aspects of the present disclosure described herein are not limited in this respect. To perform any of the functionality described herein, the processor 610 may execute one or more instructions stored in one or more computer-readable storage media (*e.g.*, the memory 620),

which may serve as non-transitory computer-readable storage media storing instructions for execution by the processor 610.

The above-described embodiments of the present disclosure can be implemented in any of numerous ways. For example, the embodiments may be implemented using hardware, software or a combination thereof. When implemented in software, the software code can be executed on any suitable processor or collection of processors, whether provided in a single computer or distributed among multiple computers. It should be appreciated that any component or collection of components that perform the functions described above can be generically considered as one or more controllers that control the above-discussed functions. The one or more controllers can be implemented in numerous ways, such as with dedicated hardware, or with general purpose hardware (*e.g.*, one or more processors) that is programmed using microcode or software to perform the functions recited above.

In this respect, it should be appreciated that one implementation of the embodiments of the present disclosure comprises at least one non-transitory computer-readable storage medium (*e.g.*, a computer memory, a floppy disk, a compact disk, a tape, etc.) encoded with a computer program (*i.e.*, a plurality of instructions), which, when executed on a processor, performs the above-discussed functions of the embodiments of the present disclosure. The computer-readable storage medium can be transportable such that the program stored thereon can be loaded onto any computer resource to implement the aspects of the present disclosure discussed herein. In addition, it should be appreciated that the reference to a computer program which, when executed, performs the above-discussed functions, is not limited to an application program running on a host computer. Rather, the term computer program is used herein in a generic sense to reference any type of computer code (*e.g.*, software or microcode) that can be employed to program a processor to implement the above-discussed aspects of the present disclosure.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters,

dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

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

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

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

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, *i.e.*, “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, *i.e.*, the inclusion of at least

one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein
5 shall only be interpreted as indicating exclusive alternatives (*i.e.* “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in
10 reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified
15 within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another
20 embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

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

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

EXAMPLES

Example 1: Cellular Imaging

Multiplexed super-resolution imaging of intra-cellular components in fixed cells was achieved by linking docking strands to antibodies (FIG. 2). These antibody-DNA conjugates were formed by first reacting biotinylated docking strands with streptavidin, and then incubating with a biotinylated antibody against the protein of interest. Fixed HeLa cells were then immunostained using a preassembled antibody-DNA conjugate against beta-tubulin. Prior to imaging, ATTO655-labeled imager strands were introduced to the sample in hybridization buffer (1×PBS supplemented with 500 mM NaCl), and single-molecule imaging was carried out using oblique illumination (9). The resulting super-resolution images show a clear increase in spatial resolution in contrast to the diffraction-limited representation (FIGs. 3a–3c). A cross-sectional profile taken at position *i* in FIG. 3B yields a distance of ≈ 79 nm between two adjacent microtubules with an apparent width of ≈ 47 and ≈ 44 nm for each of the microtubules, which is in agreement with earlier reports for immunostained microtubules (13). The antibody-DNA conjugation approach of the present disclosure yields a high labeling density, and little to no non-specific binding of imager strands to non-labeled cellular components occurs.

To demonstrate the multicolor extension of the labeling scheme of the present disclosure, where orthogonal imager strand sequences are coupled to spectrally distinct dyes, the microtubule network in a fixed HeLa cell was labeled with a preassembled antibody-DNA conjugate carrying a docking sequence for Cy3b-labeled strands, and mitochondria were stained using a second antibody linked to an orthogonal sequence for ATTO655 imager sequences. While both Cy3b- and ATTO655-labeled imager strands were present in solution at the same time, imaging was carried out sequentially in the Cy3b and ATTO655 channels and the resulting super-resolution images showed a clear increase in spatial resolution as compared to the diffraction-limited representation (FIGs. 3d–3f). As in the single-color case, little-to-no non-specific binding of the imager strands to non-labeled components in the cellular environment was observed. In addition, similar to the *in vitro* case, no crosstalk between the two colors was observed, indicating a sequence-specific interaction of the imager strands.

Example 2: Multiplexing

Assuming fixed localization accuracy, one can trivially obtain a direct two-fold increase in imaging resolution. This can be realized by spacing imaging spots with the same

docking strand sequence (*e.g.*, docking sites *a* in FIG. 4) farther apart than the actual current resolution limit, thus clearly identifying these sites as single spots in the super-resolved image. As all obtained localizations can now be assigned to a specific site, the obtainable imaging resolution is no longer the full width at half maximum (FWHM) of the reconstructed spots, but rather the standard deviation (≈ 2.35 -times smaller than the FWHM). This is depicted in FIG. 4B1, wherein a set of seven points with 10 nm spacing was imaged with ≈ 14 nm resolution, leaving individual points unresolvable. Cross-sectional histogram data showed a broad peak (bottom). In FIGs. 4B2 and 4B3, imaging every other site at a time permitted the localization of individual spots. These localizations were then combined to form the final composite image with increased resolution.

FIG. 5A shows a single DNA nanostructure, displaying four distinct sets of DNA sequences, designed to resemble the digits from 0 to 3, respectively. Imaging was performed sequentially using a simple flow chamber setup, first flushing in imager strands complementary to the docking strands of the number 0, and then exchanging the solution for imager strands with a sequence complementary to docking strands of the number 1 and so forth. The resulting images were pseudo-colored to represent the respective imaging rounds. The demonstration using DNA origami structures showed the high imaging efficiency as well as no crosstalk between consecutive imaging runs.

To demonstrate ten-“color” super-resolution imaging of DNA structures using Exchange-PAINT, ten unique rectangular DNA origami structures were designed, each displaying a distinct pattern of orthogonal docking strands that resembles a digit between 0 and 9 (see FIG. 5B(ii) for pattern “4”). After surface immobilization of all ten structures, sequential imaging was performed using a custom made fluidic chamber (FIG. 23A) for easy liquid handling. Ten orthogonal imager strands (P1* to P10*), all labeled with Cy3b, were used to perform Exchange-PAINT. The resulting digits from all ten imaging rounds are shown in FIG. 5B(v). Each target is resolved with high spatial resolution. Cross-sectional histograms along the bars of the digits show sub-10 nm FWHM of the distributions (data not shown). Note that high resolution is maintained for all digits, as the same optimized dye (Cy3b) and imaging conditions are used in each cycle.

FIG. 5B(iii) shows a combined image of all ten rounds, demonstrating specific interaction of imager strands with respective targets with no observable crosstalk between cycles. Digits 8 and 9 are not present in the selected area. An apparent “green” digit 5 instead of 2 was observed (*<i>* in FIG. 5B(iii)). This is likely not a falsely imaged digit 5 from crosstalk, but rather a “mirrored” digit 2. A mirrored image likely results from an

origami immobilized upside-down, with docking strands trapped underneath, yet still accessible to imager strands.

The fluidic setup is designed to minimize sample movement by “decoupling” the fluid reservoir and syringe from the actual flow chamber via flexible tubing. To avoid sample distortion, special care was taken to ensure gentle fluid flow during washing steps. To verify that the sample indeed exhibited little movement and little-to-no distortion, a ten-round Exchange-PAINT experiment was performed. The DNA origami was imaged for digit 4 in the first round and reimaged after ten rounds of buffer exchange. The total sample movement (physical movement of the fluidic chamber with respect to the objective) was less than 2 μm , which could easily be corrected using fiducial markers. Normalized cross-correlation analysis for select structures produced a correlation coefficient 0.92, demonstrating almost no sample distortion (also see the discussion in the cellular imaging section).

Finally, using Exchange-PAINT, four different digit patterns were successfully imaged on the same DNA origami structure (FIG. 5B(iv)). Thus Exchange-PAINT is not limited to spatially separate species and can resolve sub-diffraction patterns on the same structure with no observable crosstalk or sample distortion. Aligning images from different Exchange-PAINT rounds is straightforward using DNA origami-based drift markers. Additionally, because imaging is performed using the same dye, no chromatic aberration needs to be corrected between imaging rounds.

The applicability of the methods of the present disclosure in a cellular environment was shown by targeting microtubules and mitochondria in fixed HeLa cells, similar to FIG. 3, but only using a single color fluorophore, or spectrally indistinct imager strands. Imaging was performed sequentially using imager strands labeled with the same dye (two rounds, FIG. 6).

Example 3: Quantitative Imaging

To demonstrate the feasibility of the quantitative methods of the present disclosure, a DNA origami nanostructure with 13 binding sites in a grid-like arrangement was used (FIG. 7C). The incorporation efficiency for docking sites was not 100%, leading to a distribution of actually incorporated sites (FIGs. 7C and 7D1). Nonetheless, the structures were an ideal test system because the number of available sites could be determined visually by counting the number of spots (“direct”) and comparing it with the corresponding number of sites calculated using the proposed binding kinetic analysis (“kinetics”). FIG. 7D1 shows the binding site distribution for 377 origami structures obtained by direct counting. The binding

site distribution for the same structures obtained by binding kinetic analysis is shown in FIG. 7D2. Finally, as a benchmark, the “offset” between direct and kinetic counting was calculated for each structure. The counting “error” or uncertainty for the method was less than 7% (determined by the coefficient of variation of the Gaussian distribution) with an imaging time of ~25 min.

Example 4: Kinetic Barcoding

Highly multiplexed super-resolution barcoding was obtained by binding frequency analysis rather than geometrical or spectral encoding. Binding frequencies of BP-NA conjugates, or docking strands, to a molecule of interest is linearly dependent on the number of binding sites on this molecule. Given a certain concentration of fluorescently-labeled imager strands and association rate, binding frequency scales linearly with the number of binding sites, and can thus be used for identification. For example, 124 distinct dynamic “blinking signatures” were created using 3 colors and 4 levels of binding frequency per color (FIG. 8). Compared to geometrical encoding, this approach features much more compact, unstructured probes. Compared to spectral encoding (14) the method of the present disclosure is more cost effective, scalable, and easier to implement. Only 3 fluorescently-labeled imager strands are required in this frequency encoding method, making it very cost-efficient for high-throughput screening experiments. *In vitro* tests on a DNA origami test structure are shown in FIG. 8C.

In addition to using the inter-event lifetime τ_d or binding frequency to determine the number of available binding sites and to barcode molecules, the fluorescence ON-time or τ_b and thus the dissociation constant k_{off} can also be used to encode information. k_{off} can be precisely tuned by the base-composition and/or length of the duplex of docking and imager strand. The feasibility of this approach is illustrated in FIG. 9: $1/\tau_d$ and $1/\tau_b$ are plotted vs. the length of the docking/imaging duplex. $1/\tau_d$, and thus k_{on} , is independent of the duplex stability. However, extending the imaging/docking duplex from 9 to 10 nt by adding a single CG base pair reduces the kinetic OFF-rate by almost one order of magnitude.

Finally, a “microbarcode,” a minimal barcode that uses our ability to detect differences in thermodynamic stability of the imager/docking duplex was produced to identify a large number of molecules using a short, economical and unstructured probe. The barcode contains only of a single DNA molecule, roughly 50 nt in length, which is used to tag a molecule of interest using a 21 nt target detection domain t^* (FIG. 10A) followed by a

~30 nt long “barcode” region with a combination of 8, 9, or 10 nt long binding domain for red, green, or blue imager strands. Despite being only 30 nt in length, it can be used to present $3^3 = 27$ different barcodes with only three spectrally distinct colors and three thermodynamically distinct sequence lengths. FIG. 10 illustrates a 8, 9, or 10 nt long docking strands for three colors with a k_{off} of 10, 1 and 0.1 per second, respectively. FIG. 10A shows an example barcode consisting of an 8 nt long binding domain for a red imager strand, two 9 nt long binding domains for the green, and blue imager strand, respectively. This produces characteristic intensity vs. time traces with increased fluorescence ON-times τ_b for the 9 nt interaction domain compared to the 8 nt interaction domain (FIG. 10B). Stochastic simulations show that it is clearly possible to distinguish between k_{off} values of 10, 1 and 0.1 per second, respectively (FIG. 10C).

Example 5: Genetically Encoded Live-Cell Super-Resolution Imaging

A significant advantage of fluorescence imaging lies in its potential to visualize biomolecular processes in living cells. There are however challenges in demonstrating live cell super-resolution imaging (ref. 22-22). One such challenge is in the delivery of a sufficient concentration of synthetic imaging probes to living cells in a biocompatible manner while also allowing for proper imaging conditions. Another challenge is the extent of background fluorescence for unbound probes in the context of a live cell environment where macromolecular crowding may be more of a significant factor compared to fixed cell environments, and “live” helicases may influence binding kinetics.

This disclosure addresses these and other challenges by utilizing a genetically encoded RNA probe that can specifically bind to a target molecule in a living cell, and only then start to fluoresce and blink to enable super-resolution imaging of the specific target.

This conditional “blinking” probe is a small single-stranded RNA (<100 nt) with a target binding domain (TBD) and a conditional blinking domain (CBD). The CBD is under mechanical control of the TBD and is dark when the TBD is not bound to a target T. The binding of TBD with target “T” results in a reconfiguration of the CBD and enables it to blink with an intensity and frequency suitable for the super-resolution imaging of T.

The blinking RNA probe is based on the Spinach aptamer system (ref. 19), a fluorescent RNA mimic of GFP (FIG. 19). Spinach can turn on the fluorescence of an initially dark small molecule DFHBI (similar to DMHBI), which is non-toxic and cell-

permeable. By tagging Spinach to a target RNA, the expression of the target RNA in bacteria and mammalian cells can be imaged (ref. 19).

As the small molecule DFHBI binds and unbinds Spinach, it will alternate between a fluorescence ON- and OFF-state. The resulting blinking behavior is used to perform super-resolution fluorescence microscopy, which is referred to herein as “Spinach-PAINT” (FIG. 20). Unlike most live cell super-resolution imaging techniques, Spinach-PAINT needs no special imaging conditions such as special buffer systems or external photoswitching or activation. More importantly, DFHBI is a small, cell-permeable molecule and has been shown to provide non-toxic imaging for living cells. The necessary “blinking” behavior for super-resolution imaging can be obtained by altering the fluorescence ON- and OFF-times by adjusting the DFHBI concentration in solution and modifying/mutating Spinach to enhance/weaken the binding of DFHBI to it in the same spirit as one would tune the DNA-DNA binding interaction of an imaging strand with its partner. It is possible to characterize and optimize the binding kinetics of DFHBI to surface-immobilized Spinach based on single-molecule measurements (FIG. 20). The binding kinetics are checked for compatibility with super-resolution microscopy based on transient binding.

Generate spinach variants with optimized blinking properties for super-resolution imaging: Based on DNA-PAINT, a starting point is to obtain Spinach variants that show ON-times of at least 50 ms, occurring at a rate of ≈ 2 Hz. It has been found that Spinach exhibits a k_{off} of 0.02 s^{-1} , resulting in a residence time of ≈ 50 s. This is markedly longer than needed for super-resolution imaging. To identify Spinach variants with a k_{off} between $1 - 20\text{ s}^{-1}$, a “doped” library of Spinach variants will be prepared, using a dNTP mixture containing the dNTP that is found in Spinach for each position and the other three dNTPs in a 2:1:1:1 ratio. This approach is typically used to optimize aptamer sequences (SELEX) (ref. 23). The Spinach variants will then be screened for variants with shorter DFHBI residence times. After 5-10 rounds of SELEX, clones will be individually characterized. A first step is to confirm that all the Spinach variants still exhibit Spinach-like fluorescence with comparable quantum yield and extinction coefficient upon binding DFHBI. It is generally easy to weaken rather than to strengthen the binding interaction between an aptamer and a small molecule.

A second step involves measuring the binding and unbinding rate constants of these Spinach variants by single-molecule imaging. Spinach variants that exhibit binding durations

that provide sufficient photon counts for obtaining precise super-resolution imaging will be chosen.

To optimize the blinking frequency, we will titrate with DFHBI, as the rate of RNA-fluorophore complex formation is determined by both k_{on} and the DFHBI concentration, and will identify the concentration that produces a blinking rate of 5–10 Hz. Finally, we will have identified a set of Spinach variants that exhibit optimized blinking needed for super-resolution imaging.

As a testing platform, we will optimize the super-resolution ability of Spinach-PAINT by *in vitro* “imaging” of a DNA-based nanostructure (*e.g.*, a nanostructure made by DNA origami). The DNA origami substrate has the advantage of providing a programmable environment for placing multiple Spinach molecules in a defined distance and geometry. This nanoscopic ruler system will enable us to precisely quantify the obtainable resolution of this super-resolution imaging technique (FIG. 21).

RNAs that Exhibit Blinking upon Binding Tubulin for Super-Resolution Imaging of Cytoskeleton Proteins: Spinach-based sensors that are activated by metabolites (ref. 24) and by proteins (ref. 25) have been generated. The approach of this disclosure will be used to generate Spinach-based sensors that are activated by binding tubulin, using tubulin-binding aptamers and the blinking Spinach variants described herein.

The allosteric form of Spinach is comprised of the modified Spinach aptamer domain fused to a “control” or “sensing” module consisting of an aptamer that binds a target of interest (ref. 24). Binding of the target results in the allosteric folding of the modified Spinach aptamer into an “active” conformation, enabling DFHBI binding and fluorescence (FIG. 22). Several tubulin-binding DNA aptamers have been described (ref. 26). This disclosure provides for the evolution of control modules for Spinach capable of sensing tubulin using previously established protocols. Briefly, candidate aptamers will be fused to Spinach to obtain tubulin-dependent Spinach fluorescence, and Spinach activation only in the presence of tubulin (which will be Cy5 labeled and polymerized *in vitro*) will be verified.

Using the conditional Spinach probe generated above and based on the super-resolution conditions tested for *in vitro* imaging, Spinach-PAINT can be used for, for example, super-resolution imaging of microtubules in live mammalian cells.

Example 6: Flow Chamber

FIG. 23A shows an example of an experimental setup used for *in vitro* DNA origami experiments, as provided herein. The sample is immobilized on a glass coverslip in a PDMS

channel. Imaging and washing buffers are added to a reservoir and pulled through the channel by a syringe. Reservoirs and syringes are connected to the PDMS channel via flexible tubing and are, thus, mechanically decoupled. FIG. 23B shows an experimental setup used for *in situ* cell imaging. Cells are imaged in a Lab-Tek II chamber. One syringe supplies new buffer solution, the second one removes the previous buffer.

An exemplary protocol for Exchange-PAINT imaging using a flow chamber, for example, as depicted in FIGs. 23A and 23B, is as follow:

PDMS flow chamber volume: 40 μ l

- Rinse flow chamber with 100 μ l 1 M KOH
- 10 • Rinse flow chamber with 100 μ l buffer A twice
- Incubate for 5 min
- Rinse flow chamber with 100 μ l buffer A
- Rinse flow chamber with 50 μ l 1mg/ml BSA-Biotin in buffer A
- Incubate for 2 min
- 15 • Rinse flow chamber with 50 μ l 1mg/ml BSA-Biotin in buffer A
- Incubate for 2 min
- Rinse flow chamber with 100 μ l buffer A twice
- Rinse flow chamber with 50 μ l 0.5 mg/ml Streptavidin in buffer A
- Incubate for 2 min
- 20 • Rinse flow chamber with 50 μ l 0.5 mg/ml Streptavidin in buffer A
- Incubate for 2 min
- Rinse flow chamber with 100 μ l buffer A twice
- Rinse flow chamber with 100 μ l buffer B twice
- Incubate for 30 min
- 25 • Rinse flow chamber with 100 μ l buffer B twice
- Rinse flow chamber with 50 μ l 1nM origami in buffer B
- Incubate for 10 min
- Rinse flow chamber with 100 μ l buffer B twice
- Attach tubing
- 30 • Operate in buffer B.

Additional Materials and Methods

Materials. Unmodified DNA oligonucleotides were purchased from Integrated DNA Technologies. Fluorescently modified DNA oligonucleotides were purchased from

Biosynthesis. Biotinylated monoclonal antibodies against β -tubulin (9F3; Catalog number: 6181) and COX IV (3E11; Catalog number: 6014) were purchased from Cell Signaling. Anti-PMP70 (Catalog number: ab28499) was purchased from Abcam. Anti-TGN46 (Catalog number: NBP1-49643B) was purchased from VWR. Streptavidin was purchased from

5 Invitrogen (Catalog number: S-888). Bovine serum albumin (BSA), and BSA-biotin was obtained from Sigma Aldrich (Catalog Number: A8549). Glass slides and coverslips were purchased from VWR. Lab-Tek II chambered cover glass were purchased from Thermo Fisher Scientific. M13mp18 scaffold was obtained from New England Biolabs. p8064 scaffold for microtubule-like DNA origami structures was prepared as described 19. 'Freeze

10 N Squeeze' columns were ordered from Bio- Rad. TetraSpeck Beads were purchased from Life Technologies. Paraformaldehyde, glutaraldehyde and TEM grids (FORMVAR 400 Mesh Copper Grids) were obtained from Electron Microscopy Sciences. Three buffers were used for sample preparation and imaging: Buffer A (10 mM Tris-HCl, 100 mM NaCl, 0.05 % Tween-20, pH 7.5), buffer B (5 mM Tris-HCl, 10 mM MgCl₂, 1 mM EDTA, 0.05 % Tween-

15 20, pH 8), and buffer C (1×PBS, 500 mM NaCl, pH 8).

Optical setup. Fluorescence imaging was carried out on an inverted Nikon Eclipse Ti microscope (Nikon Instruments) with the Perfect Focus System, applying an objective-type TIRF configuration using a Nikon TIRF illuminator with an oil-immersion objective (CFI

20 Apo TIRF 100×, NA 1.49, Oil). For 2D imaging an additional 1.5 magnification was used to obtain a final magnification of \approx 150-fold, corresponding to a pixel size of 107 nm. Three lasers were used for excitation: 488 nm (200 mW nominal, Coherent Sapphire), 561 nm (200 mW nominal, Coherent Sapphire) and 647 nm (300 mW nominal, MBP Communications). The laser beam was passed through cleanup filters (ZT488/10, ZET561/10, and ZET640/20,

25 Chroma Technology) and coupled into the microscope objective using a multi-band beam splitter (ZT488rdc/ZT561rdc/ZT640rdc, Chroma Technology). Fluorescence light was spectrally filtered with emission filters (ET525/50m, ET600/50m, and ET700/75m, Chroma Technology) and imaged on an EMCCD camera (iXon X3 DU-897, Andor Technologies).

30 **DNA origami self-assembly.** The microtubule-like DNA origami structures were formed in a one-pot reaction with 40 μ l total volume containing 10 nM scaffold strand (p8064), 500 nM folding staples and biotin handles, 750 nM biotin anti-handles and 1.1 μ M DNA-PAINT docking strands in folding buffer (1×TAE Buffer with 20 mM MgCl₂). The solution was annealed using a thermal ramp¹³ cooling from 80 °C to 14 °C over the course of 15 h. After

self-assembly, monomeric structures were purified by agarose gel electrophoresis (1.5 % agarose, 0.5×TBE, 10 mM MgCl₂, 1×SybrSafe) at 4.5 V/cm for 1.5 h. Gel bands were cut, crushed and filled into a 'Freeze 'N Squeeze' column and spun for 5 min at 1000×g at 4 °C.

Polymerization was carried out at 30 °C for 48 h with a 5-fold excess of polymerization

5 staples in folding buffer. Polymerized structures were used for imaging without further purification. DNA origami drift markers were self-assembled in a one-pot reaction (40 µl total volume, 20 nM M13mp18 scaffold, 100 nM biotinylated staples, 530 nM staples with DNA-PAINT docking sites, 1×TAE with 12.5 mM MgCl₂). Self-assembled structures were purified as described before. DNA origami structures for the 4-“color” *in vitro* Exchange-

10 PAINT demonstration were self-assembled in a one-pot reaction (40 µl total volume, 30 nM M13mp18 scaffold, 470 nM biotinylated staples, 400 nM staples with docking sites for number imaging, 370 nM core structure staples, 1×TAE with 12.5 mM MgCl₂). Self-

assembled structures were purified as described before. DNA origami structures for the 10-“color” *in vitro* Exchange-PAINT demonstration were self-assembled in a one-pot reaction

15 (40 µl total volume, 30 nM M13mp18 scaffold, 36 nM biotinylated staples, 750 nM staples with docking sites for number imaging, 300 nM core structure staples, 1×TAE with 12.5 mM MgCl₂). Structures were not purified. Excessive staples are washed out of the sample after immobilization of the structure on the surface. DNA strand sequences for the microtubule-like DNA origami structures can be found in Table 1. DNA strand sequences for DNA

20 origami drift markers can be found in Table 2. DNA strand sequences for DNA origami structures for 10-“color” *in vitro* Exchange-PAINT demonstration can be found in Tables 3 and 4 for odd and even digits, respectively. DNA strand sequences for DNA origami

structures for *in vitro* Exchange-PAINT demonstration (digits 0 to 3) can be found in Table 5.

The scaffold sequence for p8064 and M13mp18 correspond to SEQ ID NO: 882 and 883,

25 respectively. DNA-PAINT imager and docking sequences as well as sequences for surface attachment via Biotin are listed in Table 6.

Antibody-DNA conjugates. Antibody-DNA conjugates used to specifically label proteins of interest with DNA-PAINT docking sites were preassembled in two steps: First, 3.2 µl of 1
30 mg/ml streptavidin (dissolved in buffer A) was reacted with 0.5 µl biotinylated DNA-PAINT docking strands at 100 µM and an additional 5.3 µl of buffer A for 30 min at room temperature (RT) while gently shaking. The solution was then incubated in a second step with 1 µl of monoclonal biotinylated antibodies at 1 mg/ml against the protein of interest for

30 min at RT. Filter columns (Amicon 100 kDa, Millipore) were used to purify the preassembled conjugates from unreacted streptavidin-oligo conjugates.

Cell immunostaining. HeLa and DLD1 cells were cultured with Eagle's minimum essential

5 medium fortified with 10 % FBS with penicillin and streptomycin and were incubated at 37 °C with 5 % CO₂. Approximately 30 % confluence cells per well were seeded into Lab-Tek II chambered cover glass 24 h before fixation. Microtubules, mitochondria, Golgi complex, and peroxisomes were immunostained using the following procedure: washing in PBS; fixation in a mixture of 3 % paraformaldehyde and 0.1 % glutaraldehyde in PBS for 10 min; 10 3-times washing with PBS; reduction with ≈ 1 mg/ml NaBH₄ for 7 min; 3-times washing with PBS; permeabilization with 0.25 % (v/v) Triton X-100 in PBS for 10 min; 3-times washing with PBS; blocking with 3 % (w/v) bovine serum albumin for 30 min and staining over night with the preassembled antibody-DNA conjugates against β -tubulin, COX IV, PMP70, or TGN46 (conjugates were diluted to 10 μ g/ml in 5 % BSA); 3-times washing with PBS; post- 15 fixation in a mixture of 3 % paraformaldehyde and 0.1 % glutaraldehyde in PBS for 10 min; and 3-times washing with PBS.

Super-resolution DNA-PAINT imaging of microtubule-like DNA origami structures.

For sample preparation, a piece of coverslip (No. 1.5, 18×18 mm², ≈ 0.17 mm thick) and a 20 glass slide (3×1 inch², 1 mm thick) were sandwiched together by two strips of double-sided tape to form a flow chamber with inner volume of ≈ 20 μ l. First, 20 μ l of biotin-labeled bovine albumin (1 mg/ml, dissolved in buffer A) was flown into the chamber and incubated for 2 min. The chamber was then washed using 40 μ l of buffer A. 20 μ l of streptavidin (0.5 mg/ml, dissolved in buffer A) was then flown through the chamber and allowed to bind for 2 25 min. After washing with 40 μ l of buffer A and subsequently with 40 μ l of buffer B, 20 μ l of biotin-labeled microtubule-like DNA structures (≈ 300 pM monomer concentration) and DNA origami drift markers (≈ 100 pM) in buffer B were finally flown into the chamber and incubated for 5 min. The chamber was washed using 40 μ l of buffer B. The final imaging buffer solution contained 1.5 nM Cy3b-labeled imager strands in buffer B. The chamber was 30 sealed with epoxy before subsequent imaging. The CCD readout bandwidth was set to 1 MHz at 16 bit and 5.1 pre-amp gain. No EM gain was used. Imaging was performed using TIR illumination with an excitation intensity of 294 W/cm² at 561 nm.

Super-resolution Exchange-PAINT imaging of DNA nanostructures. For fluid exchange, a custom flow chamber was constructed. A detailed preparation protocol can be found in Example 6. Prior to functionalizing the imaging channel with BSA-biotin, it was rinsed with 1 M KOH for cleaning. Binding of the origami structures to the surface of the flow chamber was performed as described before. Each image acquisition step was followed with a brief ~1–2 min washing step consisting of at least three washes using 200 µl of buffer B each. Then the next imager strand solution was introduced. The surface was monitored throughout the washing procedure to ensure complete exchange of imager solutions. Acquisition and washing steps were repeated until all ten targets were imaged. The CCD readout bandwidth was set to 3 MHz at 14 bit and 5.1 pre-amp gain. No EM gain was used. Imaging was performed using TIR illumination with an excitation intensity of 166 W/cm² at 561 nm (Ten-“color” Exchange-PAINT with 3 nM Cy3b-labeled imager strands in buffer B, FIG. 5B(iii) and 5B(v)) and 600 W/cm² at 647 nm (Four-“color” Exchange-PAINT with 3 nM ATTO655-labeled imager strands in buffer B, FIG. 5B(iv)).

Super-resolution DNA-PAINT imaging of cells. All data was acquired with an EMCCD readout bandwidth of 5 MHz at 14 bit, 5.1 pre-amp gain and 255 electron-multiplying gain. Imaging was performed using HILO illumination¹¹. The laser power densities were 283 W/cm² at 647 nm in FIG. 3A, 142 W/cm² at 647 nm and 19 W/cm² at 561 nm in FIG. 3D. Imaging conditions: FIG. 3A: 700 pM ATTO655-labeled imager strands in buffer C. FIG. 3D: 600 pM Cy3b-labeled imager strands and 1.5 nM ATTO655-labeled imager strands in buffer C.

Super-resolution Exchange-PAINT imaging of cells. A Lab-Tek II chamber was adapted for fluid exchange. 2D images (FIGs. 6B and 6C) were acquired with an EMCCD readout bandwidth of 5 MHz at 14 bit, 5.1 preamp gain and 255 EM gain. 3D images (FIG. 6) were acquired with a CCD readout bandwidth of 3 MHz at 154 bit, 5.1 pre-amp gain and no EM gain. Imaging was performed using HILO illumination in both cases. Sequential imaging was done as described for the 2D origami nanostructures, but the washing steps were performed using buffer C.

3D DNA-PAINT imaging. 3D images were acquired with a cylindrical lens in the detection path (Nikon). The N-STORM analysis package for NIS Elements (Nikon) was used for data processing. Imaging was performed without additional magnification in the detection path,

yielding 160 nm pixel size. 3D calibration was carried out according to the manufacturer's instructions.

Imager strand concentration determination. Optimal imager concentrations are determined empirically according to the labeling density. Generally, a high enough fluorescence OFF/ON-ratio has to be ensured in order to guarantee binding of only a single imager strand per diffraction-limited area. Additionally, a sufficient imager strand concentration (and thus sufficiently low fluorescence OFF-time) is necessary to ensure sufficient binding events and thereby robust detection of every docking strand during image acquisition.

Super-resolution data processing. Super-resolution DNA-PAINT images were reconstructed using spot-finding and 2D-Gaussian fitting algorithms programmed in LabVIEW10. A simplified version of this software is available for download at the DNA-Paint website (org suffix).

Normalized cross-correlation analysis. Normalized cross-correlation coefficients were obtained by first normalizing the respective reconstructed gray-scale super-resolution images and subsequently performing a cross-correlation analysis in MATLAB R2013b (MathWorks, Natick, MA, USA).

Drift correction and channel alignment. DNA origami structures are used for drift correction and as alignment markers in *in vitro* DNA-PAINT and Exchange-PAINT imaging. Drift correction was performed by tracking the position of each origami drift marker throughout the duration of each movie. The trajectories of all detected drift markers were then averaged and used to globally correct the drift in the final super-resolution reconstruction. For channel alignment between different imaging cycles in Exchange-PAINT, these structures are used as alignment points by matching their positions in each Exchange-PAINT image. For cellular imaging, 100 nm gold nanoparticles (Sigma Aldrich; 10 nM in buffer C, added before imaging) were used as drift and alignment markers. The gold nanoparticles adsorb non-specifically to the glass bottom of the imaging chambers. Drift correction and alignment is performed in a similar fashion as for the origami drift markers. Again, the apparent movement of all gold nanoparticles in a field of view is tracked throughout the movie. The obtained trajectories are then averaged and used for global drift

correction of the final super-resolution image. For the dual-color image of mitochondria and microtubules in FIG. 3D–3F, the gold particles are visible in both color channels. The same gold nanoparticles are also used for drift-correction and re-alignment of the different imaging rounds in the *in situ* Exchange-PAINT experiments (FIG. 6).

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Transmission electron microscopy imaging. For imaging, 3.5 μ l of undiluted microtubules-like DNA structures were adsorbed for 2 minutes onto glow-discharged, carbon-coated TEM grids. The grids were then stained for 10 seconds using a 2 % aqueous ultra-filtrated (0.2 μ m filter) uranyl formate solution containing 25 mM NaOH. Imaging was performed using a

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Atomic force microscopy imaging. Imaging was performed using tapping mode on a Multimode VIII atomic force microscope (AFM) with an E-scanner (Bruker). Imaging was performed in TAE/Mg²⁺ buffer solution with DNP-S oxide-sharpened silicon nitride cantilevers and SNL sharp nitride levers (Bruker Probes) using resonance frequencies between 7–9 kHz of the narrow 100 μ m, 0.38 N/m force constant cantilever. After self-assembly of the origami structure \approx 20 μ l of TAE/Mg²⁺ buffer solution was deposited onto a freshly cleaved mica surface (Ted Pella) glued to a metal puck (Ted Pella). After 30 s the mica surface was dried using a gentle stream of N₂ and 5 μ l of the origami solution was

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deposited onto the mica surface. After another 30 s, 30 μ l of additional buffer solution was added to the sample. Imaging parameters were optimized for best image quality while maintaining the highest possible setpoint to minimize damage to the samples. Images were post-processed by subtracting a 1st order polynomial from each scan line. Drive amplitudes were approximately 0.11 V, integral gains \approx 2, and proportional gains \approx 4.

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In Tables 1-5 below, each oligonucleotide position in a described structure is set forth. Each oligonucleotide position (*e.g.*, n[n]n[n]) in the first column is separated by a comma and corresponds respectively to a sequence identification number in the second column within the same row. Each sequence identification number identifies a corresponding oligonucleotide sequence in the attached sequence listing, incorporated by reference herein. For example, in

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Table 1, position 0[39]21[39] corresponds to the oligonucleotide represented by SEQ ID NO: 1; position 0[79]1[79] corresponds to the oligonucleotide represented by SEQ ID NO: 2, and so forth.

Table 1. Staple sequences for microtubule analog DNA structure.

Position*	Sequence Identifiers	Description
0[39]21[39], 0[79]1[79], 0[167]22[168], 0[199]2[200], 0[239]21[231], 1[24]18[24], 1[96]17[95], 1[120]1[151], 1[152]19[167], 2[39]23[55], 2[79]23[71], 2[103]3[119], 2[127]31[143], 2[199]23[207], 2[231]5[231], 3[16]31[31], 3[56]19[55], 3[80]2[80], 3[120]24[128], 3[168]5[175], 4[71]5[87], 4[135]22[120], 4[207]6[184], 5[16]22[16], 5[32]25[31], 5[52]3[55], 5[88]23[103], 5[152]4[136], 5[176]22[184], 6[95]4[72], 6[127]8[120], 6[151]22[144], 6[159]26[160], 6[183]2[184], 6[207]4[208], 6[231]24[208], 7[16]4[16], 7[48]5[51], 7[80]25[95], 7[112]6[96], 7[176]25[191], 8[39]11[31], 8[95]12[80], 8[159]10[160], 8[191]8[160], 9[48]25[63], 9[104]24[112], 9[120]10[136], 9[136]24[144], 9[176]11[191], 9[208]25[223], 10[31]27[23], 10[55]8[40], 10[95]27[95], 10[135]26[112], 10[159]27[159], 10[183]27[191], 10[215]10[184], 11[192]26[208], 12[31]29[23], 12[55]28[32], 12[79]11[63], 12[119]16[120], 12[183]29[191], 12[215]27[215], 13[40]27[55], 13[72]30[80], 13[104]9[103], 13[144]17[159], 13[168]25[183], 13[216]14[224], 14[55]16[40], 14[87]28[72], 14[119]15[135], 14[223]30[208], 15[32]29[55], 15[80]18[80], 15[96]28[104], 15[160]28[168], 16[119]31[111], 16[143]30[120], 16[191]14[160], 16[207]19[207], 16[239]29[231], 17[24]14[24], 17[40]18[56], 17[64]14[56], 17[160]31[175], 17[224]31[239], 18[55]2[40], 18[79]21[79], 18[111]0[96], 18[183]30[160], 18[239]2[232], 19[56]30[64], 19[96]30[96], 19[168]3[167], 19[192]30[184], 19[208]30[224], 20[159]21[135], 20[223]21[199], 21[16]1[23], 21[40]4[32], 21[80]22[96], 21[136]2[128], 21[200]1[215], 21[232]6[232], 22[95]3[79], 22[119]2[104], 22[143]21[159], 22[167]7[175], 22[183]18[184], 22[207]21[223], 23[56]1[55], 23[72]24[56], 23[104]5[103], 23[208]22[208], 24[79]7[79], 24[127]9[135], 24[143]5[151], 24[231]26[216], 25[64]24[80], 25[224]10[216], 26[159]12[144], 26[207]6[208], 26[239]7[247], 27[96]10[96], 27[112]14[120], 27[136]7[151], 28[191]15[183], 29[56]15[79], 29[136]27[135], 29[192]13[215], 29[232]28[216], 30[95]14[88], 30[119]29[135], 30[183]16[192], 30[207]17[223], 30[223]15[239], 31[112]1[119], 31[144]19[143], 1[56]0[40], 1[80]19[95], 1[216]0[200], 2[183]19[191], 5[104]6[128], 8[63]10[56], 9[80]8[64], 11[64]9[79], 11[216]9[239], 14[159]15[159], 15[184]16[208], 17[96]15[95], 19[128]18[112], 19[144]19[127], 24[55]7[47], 24[111]7[111], 24[183]6[160], 24[207]8[192], 25[32]9[47], 25[96]8[96], 25[192]9[207], 27[32]10[32], 27[160]9[175], 30[63]17[63], 30[79]0[80], 30[159]16[144], 31[32]15[31], 31[176]0[168], 13[136]12[120], 15[136]13[135], 27[24]13[39], 27[56]12[56], 27[216]12[216], 28[71]13[71], 28[103]13[103], 28[167]13[167], 28[215]12[184]	SEQ ID NO: 1- SEQ ID NO: 178	Structure Strand
1[56]0[40], 1[80]19[95], 1[216]0[200], 2[183]19[191], 5[104]6[128], 8[63]10[56], 9[80]8[64], 11[64]9[79], 11[216]9[239], 14[159]15[159], 15[184]16[208], 17[96]15[95], 19[128]18[112], 19[144]19[127], 24[55]7[47], 24[111]7[111], 24[183]6[160], 24[207]8[192], 25[32]9[47], 25[96]8[96], 25[192]9[207], 27[32]10[32], 27[160]9[175], 30[63]17[63], 30[79]0[80], 30[159]16[144], 31[32]15[31], 31[176]0[168]	SEQ ID NO: 179- SEQ ID NO: 206	DNA-PAINT, docking site
2[9]2[10], 4[15]6[248], 5[232]5[15], 6[247]3[15], 7[248]24[232], 9[240]11[7], 11[8]25[23], 13[8]26[240], 14[23]16[240], 15[240]28[8], 17[8]18[240], 19[3]0[248], 22[15]21[15], 24[23]7[15], 27[237]13[7], 28[7]27[236], 30[23]17[7], 31[16]31[15], 31[240]0[240]	SEQ ID NO: 207- SEQ ID NO: 225	Connector strand
13[136]12[120], 15[136]13[135], 27[24]13[39], 27[56]12[56], 27[216]12[216], 28[71]13[71], 28[103]13[103], 28[167]13[167], 28[215]12[184]	SEQ ID NO: 226- SEQ ID NO: 234	3'-Biotin, docking site

Table 2. Staple sequences for drift markers.

Position*	Sequence Identifiers	Description
0[111]1[95], 0[143]1[127], 0[175]0[144], 0[207]1[191], 0[239]1[223], 1[32]3[31], 1[96]3[95], 1[224]3[223], 2[79]0[80], 2[111]0[112], 2[143]1[159], 2[175]0[176], 2[207]0[208], 3[32]5[31], 3[96]5[95], 3[160]4[144], 3[224]5[223], 4[143]3[159], 5[32]7[31], 5[96]7[95], 5[224]7[223], 6[47]4[48], 6[79]4[80], 6[111]4[112], 6[143]5[159], 6[175]4[176], 6[207]4[208], 6[239]4[240], 6[271]4[272], 7[32]9[31], 7[96]9[95], 7[160]8[144], 7[224]9[223], 8[143]7[159], 9[32]11[31], 9[64]11[63], 9[96]11[95], 9[128]11[127], 9[192]11[191], 9[224]11[223], 9[256]11[255], 10[47]8[48], 10[79]8[80], 10[111]8[112], 10[143]9[159], 10[175]8[176], 10[207]8[208], 10[239]8[240], 10[271]8[272], 12[143]11[159], 13[32]15[31], 13[64]15[63], 13[96]15[95], 13[128]15[127], 13[192]15[191], 13[224]15[223], 13[256]15[255], 14[271]12[272], 15[32]17[31], 15[96]17[95], 15[160]16[144], 15[224]17[223], 16[143]15[159], 17[32]19[31], 17[96]19[95], 17[224]19[223], 18[47]16[48], 18[79]16[80], 18[111]16[112], 18[143]17[159], 18[175]16[176], 18[207]16[208], 18[239]16[240], 18[271]16[272], 19[32]21[31], 19[96]21[95], 19[160]20[144], 19[224]21[223], 20[143]19[159], 21[96]23[95], 21[160]22[144], 21[224]23[223], 22[47]20[48], 22[79]20[80], 22[111]20[112], 22[143]21[159], 22[175]20[176], 22[207]20[208], 22[239]20[240], 22[271]20[272], 23[64]22[80], 23[96]22[112], 23[128]23[159], 23[160]22[176], 23[192]22[208], 7[56]9[63], 7[120]9[127], 7[184]9[191], 7[248]9[255], 11[32]13[31], 11[64]13[63], 11[96]13[95], 11[128]13[127], 11[160]12[144], 11[192]13[191], 11[224]13[223], 11[256]13[255], 14[47]12[48], 14[79]12[80], 14[111]12[112], 14[143]13[159], 14[175]12[176], 14[207]12[208], 14[239]12[240], 21[120]23[127], 21[184]23[191], 1[160]2[144], 4[47]2[48], 4[79]2[80], 4[111]2[112], 4[175]2[176], 4[207]2[208], 4[239]2[240], 4[271]2[272], 5[160]6[144], 8[47]6[48], 8[79]6[80], 8[111]6[112], 8[175]6[176], 8[207]6[208], 8[239]6[240], 8[271]6[272], 9[160]10[144], 12[47]10[48], 12[79]10[80], 12[111]10[112], 12[175]10[176], 12[207]10[208], 12[239]10[240], 12[271]10[272], 13[160]14[144], 16[47]14[48], 16[79]14[80], 16[111]14[112], 16[175]14[176], 16[207]14[208], 16[239]14[240], 16[271]14[272], 17[160]18[144], 20[47]18[48], 20[79]18[80], 20[111]18[112], 20[175]18[176], 20[207]18[208], 20[239]18[240], 20[271]18[272], 0[47]1[31], 0[79]1[63], 0[271]1[255], 2[47]0[48], 2[239]0[240], 2[271]0[272], 21[32]23[31], 21[56]23[63], 21[248]23[255], 23[32]22[48], 23[224]22[240], 23[256]22[272]	SEQ ID NO: 235- SEQ ID NO: 402	DNA-PAINT, docking site
4[63]6[56], 4[127]6[120], 4[191]6[184], 4[255]6[248], 18[63]20[56], 18[127]20[120], 18[191]20[184], 18[255]20[248]	SEQ ID NO: 403- SEQ ID NO: 410	5'-Biotin modification
1[64]4[64], 1[128]4[128], 1[192]4[192], 1[256]4[256], 15[64]18[64], 15[128]18[128], 15[192]18[192], 15[256]18[256],	SEQ ID NO: 411- SEQ ID NO: 418	Structure strand

5 Table 3. Staple sequences for DNA origami structures for 10-“color” *in vitro* Exchange-PAINT demonstration (odd digits).

Position*	Sequence Identifiers	Description (number)
0[111]1[95], 0[143]1[127], 0[175]0[144], 0[79]1[63], 1[160]2[144], 2[47]0[48], 3[160]4[144], 5[160]6[144], 7[160]8[144]	SEQ ID NO: 419- SEQ ID NO: 427	5,9
10[271]8[272], 11[160]12[144], 12[271]10[272], 13[160]14[144], 14[271]12[272], 15[160]16[144], 16[271]14[272], 17[160]18[144], 18[271]16[272], 19[160]20[144], 2[271]0[272], 20[271]18[272],	SEQ ID NO: 428- SEQ ID NO: 445	3,5,9

21[160]22[144], 22[271]20[272], 4[271]2[272], 6[271]4[272], 8[271]6[272], 9[160]10[144]		
0[47]1[31], 1[32]3[31], 11[32]13[31], 13[32]15[31], 15[32]17[31], 17[32]19[31], 19[32]21[31], 3[32]5[31], 5[32]7[31], 7[32]9[31], 9[32]11[31]	SEQ ID NO: 446- SEQ ID NO: 456	3,5,7,9
21[120]23[127], 21[56]23[63], 21[96]23[95], 23[32]22[48], 23[64]22[80], 23[96]22[112]	SEQ ID NO: 457- SEQ ID NO: 462	1,3,7,9
21[184]23[191], 21[224]23[223], 21[248]23[255], 21[32]23[31], 23[128]23[159], 23[160]22[176], 23[192]22[208], 23[224]22[240], 23[256]22[272]	SEQ ID NO: 463- SEQ ID NO: 471	1,3,5,7,9
2[111]0[112], 2[79]0[80]	SEQ ID NO: 472- SEQ ID NO: 473	9
0[207]1[191], 0[239]1[223], 0[271]1[255], 1[128]4[128], 1[192]4[192], 1[224]3[223], 1[256]4[256], 1[64]4[64], 1[96]3[95], 10[111]8[112], 10[143]9[159], 10[175]8[176], 10[207]8[208], 10[239]8[240], 10[47]8[48], 10[79]8[80], 11[128]13[127], 11[192]13[191], 11[224]13[223], 11[256]13[255], 11[64]13[63], 11[96]13[95], 12[111]10[112], 12[143]11[159], 12[175]10[176], 12[207]10[208], 12[239]10[240], 12[47]10[48], 12[79]10[80], 13[128]15[127], 13[192]15[191], 13[224]15[223], 13[256]15[255], 13[64]15[63], 13[96]15[95], 14[111]12[112], 14[143]13[159], 14[175]12[176], 14[207]12[208], 14[239]12[240], 14[47]12[48], 14[79]12[80], 15[128]18[128], 15[192]18[192], 15[224]17[223], 15[256]18[256], 15[64]18[64], 15[96]17[95], 16[111]14[112], 16[143]15[159], 16[175]14[176], 16[207]14[208], 16[239]14[240], 16[47]14[48], 16[79]14[80], 17[224]19[223], 17[96]19[95], 18[111]16[112], 18[143]17[159], 18[175]16[176], 18[207]16[208], 18[239]16[240], 18[47]16[48], 18[79]16[80], 19[224]21[223], 19[96]21[95], 2[143]1[159], 2[175]0[176], 2[207]0[208], 2[239]0[240], 20[111]18[112], 20[143]19[159], 20[175]18[176], 20[207]18[208], 20[239]18[240], 20[47]18[48], 20[79]18[80], 22[111]20[112], 22[143]21[159], 22[175]20[176], 22[207]20[208], 22[239]20[240], 22[47]20[48], 22[79]20[80], 3[224]5[223], 3[96]5[95], 4[111]2[112], 4[143]3[159], 4[175]2[176], 4[207]2[208], 4[239]2[240], 4[47]2[48], 4[79]2[80], 5[224]7[223], 5[96]7[95], 6[111]4[112], 6[143]5[159], 6[175]4[176], 6[207]4[208], 6[239]4[240], 6[47]4[48], 6[79]4[80], 7[120]9[127], 7[184]9[191], 7[224]9[223], 7[248]9[255], 7[56]9[63], 7[96]9[95], 8[111]6[112], 8[143]7[159], 8[175]6[176], 8[207]6[208], 8[239]6[240], 8[47]6[48], 8[79]6[80], 9[128]11[127], 9[192]11[191], 9[224]11[223], 9[256]11[255], 9[64]11[63], 9[96]11[95]	SEQ ID NO: 474- SEQ ID NO: 594	Structure staple
4[63]6[56], 4[127]6[120], 4[191]6[184], 4[255]6[248], 18[63]20[56], 18[127]20[120], 18[191]20[184], 18[255]20[248]	SEQ ID NO: 595- SEQ ID NO: 602	5'-Biotin

Table 4. Staple sequences for DNA origami structures for 10-“color” *in vitro* Exchange-PAINT demonstration (even digits).

Position*	Sequence Identifiers	Description (number)
1[160]2[144], 11[160]12[144], 13[160]14[144], 15[160]16[144], 17[160]18[144], 19[160]20[144], 21[160]22[144], 3[160]4[144], 5[160]6[144], 7[160]8[144], 9[160]10[144]	SEQ ID NO: 603- SEQ ID NO: 613	2,4,6,8
21[224]23[223], 21[248]23[255]	SEQ ID NO: 614- SEQ ID NO: 615	0,4,8
0[111]1[95], 0[143]1[127], 0[79]1[63], 2[111]0[112], 2[47]0[48], 2[79]0[80], 21[184]23[191], 23[160]22[176], 23[192]22[208], 23[224]22[240]	SEQ ID NO: 606- SEQ ID NO: 625	0,4,6,8
1[32]3[31], 11[32]13[31], 13[32]15[31], 15[32]17[31], 17[32]19[31], 19[32]21[31], 3[32]5[31], 5[32]7[31], 7[32]9[31], 9[32]11[31]	SEQ ID NO: 626- SEQ ID NO: 635	0,2,8
0[207]1[191], 0[239]1[223], 0[271]1[255], 10[271]8[272], 12[271]10[272], 14[271]12[272], 16[271]14[272], 18[271]16[272], 2[175]0[176], 2[207]0[208], 2[239]0[240], 2[271]0[272], 20[271]18[272], 22[271]20[272], 4[271]2[272], 6[271]4[272], 8[271]6[272]	SEQ ID NO: 636- SEQ ID NO: 652	0,2,6,8

21[32]23[31], 21[56]23[63], 21[96]23[95], 23[32]22[48], 23[64]22[80], 23[96]22[112]	SEQ ID NO: 653- SEQ ID NO: 658	0,2,4,8
0[175]0[144], 0[47]1[31], 23[128]23[159], 23[256]22[272]	SEQ ID NO: 659- SEQ ID NO: 662	0,2,4,6,8
21[120]23[127]	SEQ ID NO: 663	0,2,4
1[128]4[128], 1[192]4[192], 1[224]3[223], 1[256]4[256], 1[64]4[64], 1[96]3[95], 10[111]8[112], 10[143]9[159], 10[175]8[176], 10[207]8[208], 10[239]8[240], 10[47]8[48], 10[79]8[80], 11[128]13[127], 11[192]13[191], 11[224]13[223], 11[256]13[255], 11[64]13[63], 11[96]13[95], 12[111]10[112], 12[143]11[159], 12[175]10[176], 12[207]10[208], 12[239]10[240], 12[47]10[48], 12[79]10[80], 13[128]15[127], 13[192]15[191], 13[224]15[223], 13[256]15[255], 13[64]15[63], 13[96]15[95], 14[111]12[112], 14[143]13[159], 14[175]12[176], 14[207]12[208], 14[239]12[240], 14[47]12[48], 14[79]12[80], 15[128]18[128], 15[192]18[192], 15[224]17[223], 15[256]18[256], 15[64]18[64], 15[96]17[95], 16[111]14[112], 16[143]15[159], 16[175]14[176], 16[207]14[208], 16[239]14[240], 16[47]14[48], 16[79]14[80], 17[224]19[223], 17[96]19[95], 18[111]16[112], 18[143]17[159], 18[175]16[176], 18[207]16[208], 18[239]16[240], 18[47]16[48], 18[79]16[80], 19[224]21[223], 19[96]21[95], 2[143]1[159], 20[111]18[112], 20[143]19[159], 20[175]18[176], 20[207]18[208], 20[239]18[240], 20[47]18[48], 20[79]18[80], 22[111]20[112], 22[143]21[159], 22[175]20[176], 22[207]20[208], 22[239]20[240], 22[47]20[48], 22[79]20[80], 3[224]5[223], 3[96]5[95], 4[111]2[112], 4[143]3[159], 4[175]2[176], 4[207]2[208], 4[239]2[240], 4[47]2[48], 4[79]2[80], 5[224]7[223], 5[96]7[95], 6[111]4[112], 6[143]5[159], 6[175]4[176], 6[207]4[208], 6[239]4[240], 6[47]4[48], 6[79]4[80], 7[120]9[127], 7[184]9[191], 7[224]9[223], 7[248]9[255], 7[56]9[63], 7[96]9[95], 8[111]6[112], 8[143]7[159], 8[175]6[176], 8[207]6[208], 8[239]6[240], 8[47]6[48], 8[79]6[80], 9[128]11[127], 9[192]11[191], 9[224]11[223], 9[256]11[255], 9[64]11[63], 9[96]11[95]	SEQ ID NO: 664- SEQ ID NO: 778	Structure Staple
4[63]6[56], 4[255]6[248], 4[191]6[184], 4[127]6[120], 18[63]20[56], 18[255]20[248], 18[191]20[184], 18[127]20[120],	SEQ ID NO: 779- SEQ ID NO: 786	5'-Biotin

Table 5. Staple sequences for DNA origami structures for in vitro Exchange-PAINT demonstration (digits 0 to 3)

Position*	Sequence Identifiers	Description (number)
2[47]0[48], 2[79]0[80], 2[111]0[112], 2[143]1[159], 2[175]0[176], 2[207]0[208], 2[239]0[240], 6[47]4[48], 6[239]4[240], 10[47]8[48], 10[239]8[240], 14[47]12[48], 14[239]12[240], 18[47]16[48], 18[239]16[240], 22[47]20[48], 22[79]20[80], 22[111]20[112], 22[143]21[159], 22[175]20[176], 22[207]20[208], 22[239]20[240]	SEQ ID NO: 787- SEQ ID NO: 808	0
9[64]11[63], 9[96]11[95], 9[128]11[127], 9[192]11[191], 9[224]11[223], 9[256]11[255], 11[64]13[63], 11[96]13[95], 11[128]13[127], 11[160]12[144], 11[192]13[191], 11[224]13[223], 11[256]13[255], 12[47]10[48], 12[79]10[80], 12[111]10[112], 12[175]10[176], 12[207]10[208], 12[239]10[240], 13[160]14[144], 14[79]12[80], 14[111]12[112], 14[175]12[176], 14[207]12[208]	SEQ ID NO: 809- SEQ ID NO: 832	1
0[175]0[144], 0[207]1[191], 0[239]1[223], 0[271]1[255], 1[32]3[31], 4[143]3[159], 4[271]2[272], 5[32]7[31], 8[143]7[159], 8[271]6[272], 9[32]11[31], 12[143]11[159], 12[271]10[272], 13[32]15[31], 16[143]15[159], 16[271]14[272], 17[32]19[31], 20[143]19[159], 20[271]18[272], 21[32]23[31], 21[56]23[63], 21[96]23[95], 21[120]23[127], 21[160]22[144], 23[256]22[272]	SEQ ID NO: 833- SEQ ID NO: 857	2
0[47]1[31], 2[271]0[272], 3[32]5[31], 6[143]5[159], 6[271]4[272], 7[32]9[31], 10[143]9[159], 10[271]8[272], 11[32]13[31], 14[143]13[159], 14[271]12[272], 15[32]17[31], 18[143]17[159], 18[271]16[272], 19[32]21[31], 19[160]20[144],	SEQ ID NO: 858- SEQ ID NO: 881	3

22[271]20[272], 23[32]22[48], 23[64]22[80], 23[96]22[112], 23[128]23[159],
23[160]22[176], 23[192]22[208], 23[224]22[240]

Table 6. DNA-PAINT docking and imager sequences and biotin docking sequence

Description	SEQ ID NO:	Sequence
Imager P1*	884	5'-CTAGATGTAT-dye
Imager P2*	885	5'-TATGTAGATC-dye
Imager P3*	886	5'-GTAATGAAGA-dye
Imager P4*	887	5'-GTAGATTCAT-dye
Imager P5*	888	5'-CTTTACCTAA-dye
Imager P6*	889	5'-GTACTCAATT-dye
Imager P7*	890	5'-CATCCTAATT-dye
Imager P8*	891	5'-GATCCATTAT-dye
Imager P9*	892	5'-CACCTTATTA-dye
Imager P10*	893	5'-CCTTCTCTAT-dye
Imager P11*	894	5'-GTATCATCAA-dye
Imager P12*	895	5'-GAATCACTAT-dye
9nt P1 docking site	896	Strand-TTATACATCTA-3'
9nt P2 docking site	897	Strand-TTATCTACATA-3'
10nt P2 docking site	898	Strand-TTGATCTACATA-3'
9nt P3 docking site	899	Strand-TTCTTCATTA-3'
9nt P4 docking site	900	Strand-TTATGAATCTA-3'
9nt P5 docking site	901	Strand-TTTTAGGTAAA-3'
9nt P6 docking site	902	Strand-TTAATTGAGTA-3'
9nt P7 docking site	903	Strand-TTAATTAGGAT-3'
9nt P8 docking site	904	Strand-TTATAATGGAT-3'
9nt P9 docking site	905	Strand-TTTAATAAGGT-3'
9nt P10 docking site	906	Strand-TTATAGAGAAG-3'
9nt P11 docking site	907	Strand-TTTTGATGATA-3'
9nt P12 docking site	908	Strand-TTATAGTGATT-3'
Biotinylated P1 docking site for antibody coupling	909	Biotin-TTATACATCTA-3'
Biotinylated P2 docking site for antibody coupling	910	Biotin-TTATCTACATA-3'
Biotinylated P3 docking site for antibody coupling	911	Biotin-TTCTTCATTA-3'
Biotinylated P4 docking site for antibody coupling	912	Biotin-TTATGAATCTA-3'
Biotinylated docking site for microtubule-like structure	913	Biotin-GAATCGGTCACAGTACAACCG-3'

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CLAIMS

What is claimed is:

1. A protein-nucleic acid conjugate, comprising a protein linked to a docking strand that
5 is capable of transiently binding to a complementary labeled imager strand.
2. The protein-nucleic acid conjugate of claim 1, wherein the docking strand is transiently bound to the complementary labeled imager strand.
- 10 3. The protein-nucleic acid conjugate of claim 1 or 2, wherein the protein is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.
4. The protein-nucleic acid conjugate of any one of claims 1-3, wherein the protein is linked to the docking strand through an intermediate linker.
- 15 5. The protein-nucleic acid conjugate of claim 4, wherein the intermediate linker comprises biotin and streptavidin.
6. The protein-nucleic acid conjugate of any one of claims 3-5, wherein the antibody is a
20 monoclonal antibody.
7. The protein-nucleic acid conjugate of any one of claims 1-6, wherein the complementary labeled imager strand is a complementary fluorescently-labeled imager strand.
- 25 8. The protein-nucleic acid conjugate of claim 7, wherein the complementary fluorescently-labeled imager strand comprises at least one fluorophore.
9. The protein-nucleic acid conjugate of any one of claims 1-8, wherein the
30 complementary labeled imager strand is about 4 to about 30 nucleotides in length.
10. The protein-nucleic acid conjugate of claim 9, wherein the complementary labeled imager strand is about 8 to about 10 nucleotides in length.

11. A target bound to at least one protein-nucleic acid conjugate of any one of claims 1-10.

12. The target of claim 11, wherein the target is a protein.

13. A plurality of the protein-nucleic acid conjugates of any one of claims 1-10.

14. The plurality of claim 13, wherein the plurality comprises at least two subsets of the protein-nucleic acid conjugates, and the protein-nucleic acid conjugates of each subset bind to different targets.

15. A composition comprising the plurality of protein-nucleic acid conjugates of claim 13 or 14, wherein at least one of the protein-nucleic acid conjugates is bound to at least one target.

16. A composition comprising:
at least one protein-nucleic acid conjugate that comprises a protein linked to a docking strand, wherein the at least one protein-nucleic acid conjugate is bound to a target;
and

at least one complementary labeled imager strand that is transiently bound to the at least one protein-nucleic acid conjugate.

17. The composition of claim 16, comprising at least two complementary labeled imager strands, wherein the at least two complementary labeled imager strands are identical.

18. The composition of claim 16, comprising at least two complementary labeled imager strands, wherein the at least two complementary labeled imager strands are different.

19. The composition of any one of claims 16-18, wherein the number of complementary labeled imager strands is less than, greater than or equal to the number of protein-nucleic acid conjugates.

20. The composition of any one of claims 13-19, wherein the composition comprises at least 2 different complementary labeled imager strands.

21. The composition of any one of claims 13-20, wherein the composition comprises at least 5 different complementary labeled imager strands.

5 22. The composition of any one of claims 13-21, wherein the composition comprises at least 10 different complementary labeled imager strands.

23. The composition of claim 22, wherein the composition comprises at least 100 different complementary labeled imager strands.

10

24. The composition of any one of claims 16-23, wherein the complementary labeled imager strands are complementary fluorescently-labeled imager strands.

15 25. An antibody-DNA conjugate, comprising a monoclonal antibody linked to a docking strand that is bound to a complementary labeled imager strand, wherein the antibody and the docking strand are each biotinylated and linked to each other through a biotin-streptavidin linker.

20 26. The antibody-DNA conjugate of claim 25, wherein the complementary labeled imager strand is a complementary fluorescently-labeled imager strand.

25 27. The antibody-DNA conjugate of any one of claims 1-26, wherein the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary labeled imager strand.

28. The antibody-DNA conjugate of claim 27, wherein the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

30 29. The antibody-DNA conjugate of any one of claims 21-28, wherein the thermal stability of a docking strand transiently bound to a complementary labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

30. An aptamer-nucleic acid conjugate, comprising a nucleic acid aptamer linked to a docking strand that is transiently bound to a complementary labeled imager strand.

31. The aptamer-nucleic acid conjugate of claim 30, wherein the complementary labeled
5 imager strand is a complementary fluorescently-labeled imager strand.

32. The aptamer-nucleic acid conjugate of claim 30 or 31, wherein the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary labeled imager strand.

10 33. The aptamer-nucleic acid conjugate of claim 32, wherein the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

15 34. The aptamer-nucleic acid conjugate of any one of claims 30-33, wherein the thermal stability of a docking strand transiently bound to a complementary labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

20 35. A method of detecting a target in a sample, the method comprising:
contacting a sample with (a) at least one protein-nucleic acid conjugate that comprises a protein linked to a docking strand and (b) at least one fluorescently-labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one protein-nucleic acid conjugate; and

25 determining whether the at least one protein-nucleic acid conjugate binds to the target in the sample.

30 36. The method of claim 35, wherein the determining step comprises imaging transient binding of the at least one fluorescently-labeled imager strand to the docking strand of the at least one protein-nucleic acid conjugate.

37. The method of claim 35 or 36, wherein the protein of the protein-nucleic acid conjugate is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

38. The method of claim 36, wherein the antibody is a monoclonal antibody.

39. The method of any one of claims 35-38, wherein the protein of the protein-nucleic acid conjugate is linked to the docking strand through an intermediate linker.

5

40. The method of claim 39, wherein the intermediate linker comprises biotin and/or streptavidin.

10 41. The method of any one of claims 35-40, wherein the complementary fluorescently-labeled imager strand comprises at least one fluorophore.

42. The method of any one of claims 35-41, wherein the complementary fluorescently-labeled imager strand is about 4 to about 30 nucleotides in length.

15 43. The method of claim 42, wherein the complementary fluorescently-labeled imager strand is about 8 to about 10 nucleotides in length.

44. The method of any one of claims 35-43, wherein the sample is a cell or cell lysate.

20 45. The method of any one of claims 35-44, wherein the target is a protein

46. The method of any one of claims 35-45, wherein the target is obtained from a cell or cell lysate.

25 47. The method of any one of claims 35-46, wherein the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary labeled imager strand.

30 48. The method of claim 47, wherein the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

49. The method of any one of claims 35-48, wherein the thermal stability of a docking strand transiently bound to a labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

50. A method of detecting at least one target in a sample, the method comprising:
contacting a sample with (a) at least two protein-nucleic acid conjugates, each
comprising a protein linked to a docking strand, and (b) at least two labeled imager strands
5 that are complementary to and transiently bind to respective docking strands of the at least
two different protein-nucleic acid conjugates; and
determining whether the at least two protein-nucleic acid conjugates bind to at least
one target in the sample.

10 51. The method of claim 50, wherein the determining step comprises, in the following
order,

imaging transient binding of one of the at least two labeled imager strands to a
docking strand of one of the at least two protein-nucleic acid conjugates to produce a first
image of signal, and

15 imaging transient binding of another of the at least two labeled imager strands to a
docking strand of another of the at least two protein-nucleic acid conjugates to produce at
least one other image of signal.

20 52. The method of claim 51, further comprising combining the first image and the at least
one other image to produce a composite image of signal, wherein the signal of the composite
image is representative of the at least one target.

25 53. The method of any one of claims 50-52, wherein the protein of the protein-nucleic
acid conjugate is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

54. The method of claims 53, wherein the antibody is a monoclonal antibody.

55. The method of any one of claims 50-54 wherein the protein of the protein-nucleic acid
conjugate is linked to the docking strand through an intermediate linker.

30 56. The method of claim 55, wherein the intermediate linker comprises biotin and
streptavidin.

57. The method of any one of claims 50-56, wherein each of the at least two labeled imager strands are fluorescently-labeled imager strands.

58. The method of claim 57, wherein the at least two fluorescently-labeled imager strands are spectrally distinct, fluorescently-labeled imager strands.

59. The method of any claim 57 or 58, wherein each of the at least two labeled imager strands comprises at least one fluorophore.

60. The method of any one of claims 50-59, wherein each of the at least two labeled imager strands is about 4 to about 30 nucleotides in length.

61. The method of claim 60, wherein each of the at least two labeled imager strands is about 8 to about 10 nucleotides in length.

62. The method of any one of claims 50-61, wherein the sample is a cell or cell lysate.

63. The method of any one of claims 50-62, wherein the at least one target is a protein.

64. The method of any one of claims 50-63, wherein the at least one target is obtained from a cell or cell lysate.

65. The method of any one of claims 50-64, wherein step of determining whether the at least two protein-nucleic acid conjugates bind to at least one target in the sample comprises determining whether the at least two protein-nucleic acid conjugates bind to at least two targets in the sample.

66. The method of any one of claims 50-65, wherein the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary labeled imager strand.

67. The method of claim 66, wherein the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

68. The method of any one of claims 50-67, wherein the thermal stability of a docking strand transiently bound to a labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

5 69. A method of detecting at least one protein target in a sample, comprising:
(a) contacting a sample with at least two protein-nucleic acid conjugates, each comprising a protein linked to a docking strand and (b) sequentially contacting the sample with at least two labeled imager strands that are complementary to and transiently bind to respective docking strands of the at least two protein-nucleic acid conjugates; and
10 determining whether the at least two protein-nucleic acid conjugates bind to at least one protein target in the sample.

70. The method of claim 69, comprising, in the following ordered steps:
contacting the sample with a first protein-nucleic acid conjugate and at least one other
15 protein-nucleic acid conjugate;
contacting the sample with a first labeled imager strand that is complementary to and transiently binds to the docking strand of the first protein-nucleic acid conjugate;
imaging the sample to obtain a first image, optionally using time-lapsed imaging;
removing the first labeled imager strand;
20 contacting the sample with at least one other labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other protein-nucleic acid conjugate; and
imaging the sample to obtain at least one other image, optionally using time-lapsed imaging.
25

71. The method of claim 69, comprising, in the following ordered steps:
contacting the sample with a first protein-nucleic acid conjugate;
contacting the sample with a first labeled imager strand that is complementary to and transiently binds to the docking strand of the first protein-nucleic acid conjugate;
30 imaging the sample to obtain a first image, optionally using time-lapsed imaging;
removing the first labeled imager strand;
contacting the sample with at least one other protein-nucleic acid conjugate;

contacting the sample with at least one other labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other protein-nucleic acid conjugate; and

5 imaging the sample to obtain at least one other image, optionally using time-lapsed imaging.

72. The method of claim 70 or 71, further comprising determining whether the first protein-DNA conjugate binds to a first target and/or whether the at least one other protein-DNA conjugate binds to at least one other target.

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73. The method of claim 72, further comprising assigning a pseudo-color to the signal in the first image, and assigning at least one other pseudo-color to the signal in the at least one other image.

15 74. The method of claim 73, further comprising combining the first image and the at least one other image to produce a composite image of the pseudo-colored signals, wherein the pseudo-colored signals of the composite image are representative of the at least two targets.

20 75. The method of any one of claims 69-74, wherein the protein of the protein-nucleic acid conjugate(s) is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

76. The method of claim 75, wherein the antibody is a monoclonal antibody.

25 77. The method of any one of claims 69-76, wherein the protein of the protein-nucleic acid conjugate(s) is linked to the docking strand through an intermediate linker.

78. The method of claim 77, wherein the intermediate linker comprises biotin and/or streptavidin.

30 79. The method of any one of claims 69-78, wherein each of the labeled imager strands is a fluorescently-labeled imager strand.

80. The method of claim 69, wherein each of the labeled imager strands is a spectrally distinct, fluorescently-labeled imager strand.

81. The method of claims 69 or 70, wherein each of the labeled imager strands comprises at least one fluorophore.

5 82. The method of any one of claims 69-81, wherein each of the labeled imager strands is about 4 to about 30 nucleotides in length.

83. The method of claim 82, wherein each of the labeled imager strands is about 8 to about 10 nucleotides in length.

10

84. The method of any one of claims 69-83, wherein the sample is a cell or cell lysate.

85. The method of any one of claims 69-84, wherein the target(s) is a protein.

15 86. The method of any one of claims 69-85, wherein the target(s) is obtained from a cell or cell lysate.

87. The method of any one of claims 69-86, wherein the step of determining whether the at least two protein-nucleic acid conjugates bind to at least one protein target in the sample
20 comprises determining whether the at least two protein-nucleic acid conjugates bind to at least two protein targets in the sample.

88. The method of any one of claims 69-87, wherein each of the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary
25 labeled imager strand.

89. The method of any one of claims 69-88, wherein each of the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

30

90. The method of any one of claims 69-89, wherein the thermal stability of a docking strand transiently bound to a labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands.

91. A method of detecting a molecule, comprising
contacting a sample containing at least one target with (a) at least one BP-NA
conjugate, each BP-NA conjugate comprising a binding partner linked to a docking strand
and (b) at least one labeled imager strand that is complementary to and transiently binds the
5 docking strand of the at least one BP-NA conjugate; and
determining whether the at least one BP-NA conjugate binds to at least one target in
the sample.
92. The method of claim 91, wherein the determining step comprises imaging transient
10 binding the at least one labeled imager strand to the docking strand of the at least one BP-NA
conjugate.
93. The method of claim 91 or 92, wherein the sample is a cell or cell lysate.
- 15 94. The method of any one of claims 91-93, wherein the at least one target is obtained
from a cell or cell lysate.
95. The method of any one of claims 91-94, wherein the target is a naturally-occurring
biomolecule.
20
96. The method of claim 95, wherein the naturally-occurring biomolecule is a protein.
97. The method of claim 96, wherein the protein is an antibody, an antigen-binding
antibody fragment, or a peptide aptamer.
25
98. The method of claim 97, wherein the antibody is a monoclonal antibody.
99. The method of any one of claims 91-98, wherein the target is linked to the docking
strand through an intermediate linker.
30
100. The method of claim 99, wherein the intermediate linker comprises biotin and/or
streptavidin.

101. The method of claim 95, wherein the naturally-occurring biomolecule is a nucleic acid.

102. The method of claim 101, wherein the nucleic acid is a nucleic acid aptamer.

5

103. The method of any one of claims 91-103, wherein the labeled imager strand is a fluorescently-labeled imager strand.

104. The method of claim 103, wherein the fluorescently-labeled imager strand comprises at least one fluorophore.

10

105. The method of any one of claims 91-104, wherein the fluorescently-labeled imager strand is about 4 to about 30 nucleotides in length.

106. The method of claim 105, wherein the fluorescently-labeled imager strand is about 8 to about 10 nucleotides in length.

15

107. The method of any one of claims 91-106, wherein the binding partner is a protein or nucleic acid.

20

108. The method of any one of claims 91-107, wherein the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary labeled imager strand.

109. The method of claim 108, wherein the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

25

110. The method of any one of claims 91-109, wherein the thermal stability of a docking strand transiently bound to a labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands..

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111. A method of detecting a naturally-occurring biomolecule, comprising contacting a sample containing at least one target with (a) at least two different BP-NA conjugates, each BP-NA conjugate comprising a binding partner linked to a docking

strand and (b) at least two labeled imager strands that are complementary to and transiently bind to respective docking strands of the at least two BP-NA conjugates; and

determining whether the at least two BP-NA conjugates bind to at least one target in the sample.

5

112. The method of claim 111, comprising, in the following ordered steps:

contacting the sample with a first BP-NA conjugate and at least one other BP-NA conjugate; and

10 contacting the sample with a first labeled imager strand that is complementary to and transiently binds to the docking strand of the first BP-NA conjugate;

imaging the sample to obtain a first image, optionally using time-lapsed imaging;

removing the first fluorescently-labeled imager strand;

15 contacting the sample with at least one other labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other BP-NA conjugate; and

imaging the sample to obtain at least one other image, optionally using time-lapsed imaging.

113. The method of claim 111, comprising, in the following ordered steps:

20 contacting the sample with a first BP-NA conjugate;

contacting the sample with a first labeled imager strand that is complementary to and transiently binds to the docking strand of the first BP-NA conjugate;

imaging the sample to obtain a first image, optionally using time-lapsed imaging;

removing the first labeled imager strand;

25 contacting the sample with at least one other BP-NA conjugate;

contacting the sample with at least one other labeled imager strand that is complementary to and transiently binds to the docking strand of the at least one other BP-NA conjugate; and

30 imaging the sample to obtain a at least one other image, optionally using time-lapsed imaging.

114. The method of claim 112 or 113, further comprising determining whether the first protein-DNA conjugate binds to a first target and/or whether the at least one other protein-DNA conjugate binds to at least one other target.

115. The method of claim 114, further comprising assigning a pseudo-color to the signal in the first image, and assigning at least one other pseudo-color to the signal in the at least one other image.

5

116. The method of claim 115, further comprising combining the first image and the at least one other image to produce a composite image of the pseudo-colored signals, wherein the pseudo-colored signals of the composite image are representative of the at least one target.

10

117. The method of any one of claims 111-116, wherein the sample is a cell or cell lysate.

118. The method of any one of claims 111-117, wherein the at least one target is obtained from a cell or cell lysate.

15

119. The method of any one of claims 111-118, wherein the target is a naturally-occurring biomolecule.

120. The method of claim 119, wherein the naturally-occurring biomolecule is a protein.

20

121. The method of claim 120, wherein the protein is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

122. The method of claim 121, wherein the antibody is a monoclonal antibody.

25

123. The method of any one of claims 111-122, wherein the target is linked to the docking strand through an intermediate linker.

124. The method of claim 123, wherein the intermediate linker comprises biotin and/or streptavidin.

30

125. The method of claim 119, wherein the naturally-occurring biomolecule is a nucleic acid.

126. The method of claim 125, wherein the nucleic acid is a nucleic acid aptamer.

127. The method of any one of claims 111-126, wherein the first labeled imager strand is a fluorescently-labeled imager strand.

5

128. The method of any one of claims 111-126, wherein the at least one other labeled imager strand is a fluorescently-labeled imager strand.

129. The method of claim 127 or 128, wherein the first labeled imager strand and/or the at least one other labeled imager strand comprises at least one fluorophore.

10

130. The method of any one of claims 111-129, wherein at least one other labeled imager strand about 4 to about 30 nucleotides in length.

131. The method of claim 130, at least one other labeled imager strand is about 8 to about 10 nucleotides in length.

15

132. The method of any one of claims 111-131, wherein the binding partner is a protein or nucleic acid.

20

133. The method of any one of claims 111-132, wherein the docking strand is a DNA docking strand.

134. The method of any one of claims 111-133, wherein the docking strand comprises at least two domains, wherein each domain binds to a respectively complementary labeled imager strand.

25

135. The method of claim 134, wherein the docking strand comprises at least three domains, wherein each domain binds to a respectively complementary labeled imager strand.

30

136. The method of any one of claims 111-135, wherein the thermal stability of a docking strand transiently bound to a labeled imager strand is within 0.5 kcal/mol of the thermal stability of other docking strands transiently bound to their respective labeled imager strands..

137. A method of determining the number of targets in a test sample, comprising:
 obtaining a sample that comprises targets transiently bound directly or indirectly to
 labeled imager strands;
 obtaining a time-lapsed image of the sample;
 5 performing spot detection and localization on the image to obtain a high-resolution
 image of the sample;
 calibrating $k_{on} \cdot c_{imager}$, wherein k_{on} is a second order association constant, and c_{imager} is
 concentration of labeled imager strands in the test sample;
 determining variable τ_d ; and
 10 determining the number of test targets in the sample based on the equation,
 $number\ of\ test\ targets = (k_{on} \cdot c_{imager} \cdot \tau_d)^{-1}$.

138. The method of claim 137, wherein the test targets are protein targets.

15 139. The method of claim 138, wherein the protein targets are bound to protein-nucleic
 acid conjugates that comprise a protein linked to a docking strand, and the labeled imager
 strands are complementary to and transiently bind to respective docking strands of the
 protein-nucleic acid conjugates.

20 140. The method of claim 139, wherein the protein of the protein-nucleic acid conjugate is
 an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

141. The method of claim 137, wherein the test targets are single-stranded nucleic acids.

25 142. The method of claim 141, wherein the single-stranded nucleic acids are DNA or
 RNA.

143. The method of any one of claims 137-142, wherein each of the labeled imager strands
 is a fluorescently-labeled imager strand.

30 144. The method of any one of claims 137-143, wherein each of the fluorescently-labeled
 imager strands comprises at least one fluorophore.

145. The method of any one of claims 137-144, wherein each of the fluorescently-labeled imager strands is about 3 to about 30 nucleotides in length.

146. The method of claim 144, wherein each of the fluorescently-labeled imager strands is about 8 to about 10 nucleotides in length.

147. The method of any one of claims 137-146, wherein the time-lapsed image is obtained over a period of about 24 minutes.

148. The method of any one of claims 137-147, wherein the time-lapsed image is a time-lapsed diffraction-limited fluorescence image.

149. The method of any one of claims 137-148, wherein the step of performing localization on the image comprises performing Gaussian fitting on the image.

150. The method of any one of claims 137-149, wherein the step of calibrating $k_{on} \cdot C_{imager}$ comprising calibrating $k_{on} \cdot C_{imager}$ with a control sample with a known number of targets.

151. The method of any one of claims 137-150, wherein the step of determining variable τ_d comprises determining variable τ_d by fitting the signal OFF-time distribution to a cumulative distribution function.

152. The method of any one of claims 137-151, wherein the number of test targets is determined with an accuracy of greater than 90%.

153. A method of determining a relative amount of targets in a test sample, comprising:
obtaining a sample that comprises targets transiently bound directly or indirectly to labeled imager strands;

obtaining a time-lapsed image of the sample;

performing spot detection and localization on the image to obtain a high-resolution image of the sample;

determining variable τ_d ; and

determining the relative amount of two or more test targets in the sample based on τ_d .

154. The method of claim 153, wherein the test targets are protein targets.

155. The method of claim 154, wherein the protein targets are bound to protein-nucleic acid conjugates that comprise a protein linked to a docking strand, and the labeled imager strands are complementary to and transiently bind to respective docking strands of the protein-nucleic acid conjugates.

156. The method of claim 155, wherein the protein of the protein-nucleic acid conjugate is an antibody, an antigen-binding antibody fragment, or a peptide aptamer.

157. The method of claim 153, wherein the test targets are single-stranded nucleic acids.

158. The method of claim 157, wherein the single-stranded nucleic acids are DNA or RNA.

159. The method of any one of claims 153-158, wherein each of the labeled imager strands is a fluorescently-labeled imager strand.

160. The method of claim 159, wherein wherein each of the labeled imager strands comprises at least one fluorophore.

161. The method of any one of claims 153-160, wherein each of the labeled imager strands is about 4 to about 30 nucleotides in length.

162. The method of claim 161, wherein each of the labeled imager strands is about 8 to about 10 nucleotides in length.

163. The method of any one of claims 153-162, wherein the time-lapsed image is obtained over a period of about 25 minutes.

164. The method of any one of claims 153-163, wherein the time-lapsed image is a time-lapsed diffraction-limited fluorescence image.

165. The method of any one of claims 153-164, wherein the step of performing localization on the image comprises performing Gaussian fitting on the image.

166. The method of any one of claims 153-165, wherein the step of determining variable τ_d comprises determining variable τ_d by fitting the signal OFF-time distribution to a cumulative distribution function.

167. The method of any one of claims 153-166, wherein the number of test targets is determined with an accuracy of greater than 90%.

168. A single-stranded DNA probe comprising a target binding domain of about 20 nucleotides in length linked to a docking domain comprising at least one subdomain complementary to at least one labeled imager strand of about 4 to 30 nucleotides in length, and wherein the target binding domain is bound to a complementary domain of a single-stranded mRNA target strand.

169. The single-stranded DNA probe of claim 168, wherein the at least one subdomain is transiently bound to the at least one labeled imager strand.

170. The single-stranded DNA probe of claim 169, wherein the at least one labeled imager strand is fluorescently labeled.

171. The single-stranded DNA probe of claim 170, wherein the at least one labeled imager strand comprises a fluorophore.

172. The single-stranded DNA probe of claim 168, wherein the docking domain comprises at least two subdomains, wherein the at least two subdomains are respectively complementary to at least two labeled imager strands of about 4 to 30 nucleotides in length.

173. The single-stranded DNA probe of claim 172, wherein the at least two subdomains are respectively complementary to at least two labeled imager strands of about 8 to 10 nucleotides in length.

174. The single-stranded DNA probe of claim 172 or 173, wherein the at least two subdomains are transiently bound to respectively complementary labeled imager strands.

175. The single-stranded DNA probe of any one of claims 172-174, wherein the
5 respectively complementary labeled imager strands are distinctly labeled imager strands.

176. The single-stranded DNA probe of any one of claims 172-175, wherein the respectively complementary labeled imager strands are respectively complementary fluorescently-labeled imager strands.

10 177. The single-stranded DNA probe of claim 176, the respectively complementary labeled imager strands each comprise a fluorophore.

178. The single-stranded DNA probe of claim 168, wherein the docking domain comprises
15 at least three subdomains, wherein the at least three subdomains are respectively complementary to at least three labeled imager strands of about 4 to 30 nucleotides in length.

179. The single-stranded DNA probe of claim 178, wherein the at least three subdomains are transiently bound to respectively complementary labeled imager strands.

20 180. The single-stranded DNA probe of claim 179, wherein the respectively complementary labeled imager strands are distinctly labeled imager strands.

181. The single-stranded DNA probe of claim 179 or 180, wherein the respectively
25 complementary labeled imager strands are respectively complementary fluorescently-labeled imager strands.

182. The single-stranded DNA probe of claim 180 or 181, wherein the respectively complementary labeled imager strands each comprise a fluorophore.

30 183. The single-stranded DNA probe of any one of claims 168-182, wherein the target binding domain of about 20 nucleotides in length is linked at its 3' end to a docking domain.

184. The The single-stranded DNA probe of any one of claims 168-182, wherein the target binding domain of about 20 nucleotides in length is linked at its 5' end to a docking domain.

185. A method of performing drift correction for a plurality of images, wherein each of the plurality of images comprises a frame of a time sequence of images, wherein the time sequence of images captures a plurality of transient events, the method comprising:

determining a time trace for each of a plurality of drift markers identified in the plurality of images, wherein a time trace for each drift marker corresponds to movement of an object in the image over the time sequence of images;

determining, with at least one computer processor, a first drift correction from at least one of the plurality of drift markers based, at least in part, on the time traces for the at least one of the plurality of drift markers;

determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images, wherein each drift template in the plurality of drift templates describes a geometrical relationship between the plurality of geometrically-addressable marker sites of transient events in the drift template;

determining a second drift correction based, at least in part, on the time traces for the plurality of geometrically-addressable marker sites from the plurality of drift templates;

correcting the plurality of images based, at least in part, on the first drift correction and the second drift correction; and

outputting a final image based on the corrected plurality of images.

186. The method of claim 185, further comprising:

identifying a plurality of localizations in each of the plurality of images;

creating a two-dimensional histogram of the plurality of localizations; and

identifying locations of the plurality of drift markers based, at least in part, on the two-dimensional histogram;

wherein determining the time traces for each of the plurality of drift markers comprises determining the time traces based, at least in part, on the locations of the plurality of drift markers.

187. The method of claim 186, wherein identifying a plurality of localizations comprises: identifying a plurality of spots on each of the plurality of images; and

determining a fitted center position of each of the plurality of spots using a local Gaussian fitting algorithm;

wherein each of the plurality of localizations comprises the spot identified on an image and its associated fitted center position.

5

188. The method of claim 187, wherein each of the plurality of localizations further comprises a detected photon count corresponding to the localization.

189. The method of claim 186, wherein creating the two-dimensional histogram of the plurality of localizations comprises binning all localizations into a two-dimensional grid and using a total number of localizations in each bin as a histogram count.

10

190. The method of claim 186, wherein creating the two-dimensional histogram of the plurality of localizations comprises binning all localizations into a two-dimensional grid and using a total number of photon count of the plurality of localizations in each bin as a histogram count.

15

191. The method of claim 186, wherein identifying locations of the plurality of drift markers based, at least in part, on the two-dimensional histogram comprises at least one of the following:

20

binarizing the two-dimensional histogram using one or more selection criteria, wherein the one or more selection criteria include a lower-bound threshold of a histogram value or an upper-bound threshold of a histogram value;

25

partitioning the binarized image into partitions and filtering the partitions based on one or more selection criteria, wherein the one or more selection criteria include one or more of a lower-bound threshold of an area of a partition area, an upper-bound threshold of the area, a lower-bound or an upper-bound of a longest or shortest linear dimension of a partition longest, and a lower-bound or an upper-bound of an eccentricity of a partition; and

30

expanding and shrinking the binarized image using one or more binary image operations, wherein the one or more binary image operations include one or more of the following: dilate, erode, bridge, close, open, fill, clean, top-hat, bottom-hat, thicken, thin, and more.

192. The method of claim 185, wherein determining a first drift correction based, at least in part, on the time traces for the plurality of drift markers comprises:

determining a relative time trace for each of the plurality of drift markers, wherein the relative time trace is determined by comparing the time trace for the drift marker with the
5 average position of the same trace; and

determining a combined time trace based on the relative time traces for each of the plurality of drift markers;

wherein determining the first drift correction based, at least in part, on the time traces for the plurality of drift markers comprises determining the first drift correction based, at
10 least in part, on the relative time traces for each of the plurality of drift markers.

193. The method of claim 192, wherein determining the first drift correction based, at least in part, on the relative time traces for each of the plurality of drift markers comprises performing a weighted average of the relative time traces for each of the plurality of drift
15 markers.

194. The method of claim 193, wherein performing the weighted average comprises:

determining a quality score for each of the relative time traces, wherein the quality score is determined based, at least in part, on a measure of variability over time associated
20 with the time trace and/or a measure of localization uncertainty of individual localizations within the time trace.

195. The method of claim 194, wherein the measure of variability over time comprises a standard deviation of the time trace over time.
25

196. The method of claim 194, wherein the measure of localization uncertainty of individual localizations comprises, at least in part, an estimate of uncertainty from a Gaussian fitting or a comparison with other simultaneous localizations, wherein the other simultaneous localizations are from within a same image and from other time traces from the plurality of
30 drift markers, wherein the comparison comprises a mean and standard deviation of all simultaneous localizations.

197. The method of claim 185, further comprising:

determining that a first drift marker of the plurality of drift markers is not present in at least one frame of the time sequence of images; and

linearly interpolating the time trace for the first drift marker for the at least one frame to produce a smoothed time trace for the first drift marker.

198. The method of claim 185, wherein determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images comprises:

identifying a plurality of localizations in each of the plurality of images;

creating a two-dimensional histogram of the plurality of localizations; and

identifying the plurality of drift templates based, at least in part, on the two-dimensional histogram;

wherein identifying the plurality of drift templates comprises evaluating the two-dimensional histogram using an lower-bound and/or an upper-bound threshold in a histogram count.

199. The method of claim 185, wherein determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images comprises determining a time trace for each of a plurality of geometrically-addressable marker sites within each of the plurality of drift templates, and wherein determining the second drift correction comprises determining the second drift correction based, at least in part, on the time traces for each of the plurality of marker sites within each of the plurality of drift templates.

200. The method of claim 185, wherein determining the second drift correction based, at least in part, on the time traces for each of the plurality of geometrically-addressable marker sites from each of the plurality of drift templates comprises:

identifying a plurality of geometrically-addressable marker sites within each of the plurality of drift templates; and

determining a relative time trace for each of a plurality of geometrically-addressable drift markers for each of the plurality of drift templates;

wherein determining the second drift correction based, at least in part, on the time traces for the plurality of drift templates comprises determining the second drift correction

based, at least in part, on the relative time traces for each of the plurality of drift markers within each of the plurality of drift templates.

201. The method of claim 200, wherein identifying a plurality of geometrically addressable marker sites from each of the plurality of drift templates comprises determining a plurality of marker sites based on, at least in part, a two-dimensional histogram of the plurality of localizations in the corresponding drift template, and/or one or more selection criteria, wherein the one or more selection criteria include one or more of a total number of localizations, a surface density of localizations, and standard deviation of localizations.

202. The method of claim 200, wherein determining the second drift correction based, at least in part, on the relative time traces for each of the plurality of drift markers within each of the plurality of drift templates comprises performing a weighted average of the relative time traces for each of the plurality of drift markers within each of the drift templates.

203. The method of claim 202, wherein performing the weighted average comprises:
determining a quality score for each of the relative time traces, wherein the quality score is determined based, at least in part, on a measure of variability over time associated with the time trace and/or a localization uncertainty within the time trace.

204. The method of claim 203, wherein the measure of variability over time comprises a standard deviation of the time trace over time.

205. The method of claim 203, wherein the measure of localization uncertainty of individual localizations comprises an estimate of uncertainty from a Gaussian fitting or a comparison with other simultaneous localizations, wherein the other simultaneous localizations are from within a same image and from the other time traces from the plurality of marker sites from the plurality of drift templates, wherein the comparison comprises a mean and standard deviation of all simultaneous localizations.

206. The method of claim 185, wherein correcting the plurality of images based, at least in part, on the first drift correction and the second drift correction comprises correcting the plurality images using the first drift correction to produce a first corrected plurality of images, and wherein determining a time trace for each of a plurality of drift templates identified from

the plurality of images comprises determining a time trace for each of the plurality of drift templates identified from the first corrected plurality of images.

207. The method of claim 185, further comprising:

5 smoothing the first drift correction prior to correcting the plurality of images using the first drift correction.

208. The method of claim 207, wherein smoothing the first drift correction comprises processing the first drift correction using local regression with a window determined by a
10 characteristic drift time scale of the first drift correction.

209. The method of claim 185, further comprising smoothing the second drift correction prior to correcting the plurality of images using the second drift correction.

15 210. The method of claim 209, wherein smoothing the second drift correction comprises processing the second drift correction using local regression with a window determined by a characteristic drift time scale of the second drift correction.

211. The method of claim 185, further comprising:

20 selecting a single drift marker of the plurality of drift markers; and
 determining a third drift correction based, at least in part, on the selected single drift marker;

 wherein correcting the plurality of images comprises correcting the plurality of images based, at least in part, on the third drift correction.

25

212. The method of claim 211, wherein correcting the plurality of images based, at least in part, on the third drift correction is performed prior to correcting the plurality of images based, at least in part on the first drift correction and the second drift correction.

30 213. The method of any of claims 185 and 211, further comprising:

 identifying locations of a first plurality of points in a first image of the plurality of frames;

identifying locations of a second plurality of points in a second image of the plurality of images, wherein the second image corresponds to a neighboring frame of the first image in the time sequence of images; and

5 determining a fourth drift correction based, at least in part, on differences between the locations of the first plurality of points and the second plurality of points;
wherein correcting the plurality of images comprises correcting the plurality of images based, at least in part, on the fourth drift correction.

214. The method of claim 213, wherein the second image corresponds to a frame
10 immediately following the frame corresponding to the first image in the time sequence of images.

215. The method of claim 213, wherein determining the fourth drift correction based, at least in part, on differences between the locations of the first plurality of points and the
15 second plurality of points comprises:
creating a histogram of distances between the locations of the first plurality of points and the second plurality of points;
determining based, at least in part, on the histogram, pairs of points between the first image and the second image that correspond to the same transient event; and
20 determining a location offset between each of the determined pairs of points;
wherein determining the fourth drift correction is based on a vector average of the location offsets for each of the determined pairs of points.

216. The method of claim 185, wherein the plurality of images correspond to DNA-based
25 images and wherein the plurality of transient events are binding events between an imaging strand and a DNA docking strand.

217. The method of claim 216, wherein the imaging strand is a fluorescent imaging probe configured to fluoresce when associated with the DNA docking strand.

30 218. The method of claim 185, wherein at least one of the drift markers is a DNA based nanostructure.

219. The method of claim 218, wherein the DNA based nanostructure is a DNA origami nanostructure with docking strands.

220. The method of claim 185, wherein at least one of the drift templates is a DNA based nanostructure.

221. The method of claim 220, wherein the DNA based nanostructure is a DNA origami nanostructure with docking strands.

222. The method of claim 185, wherein at least one of the drift templates is a three-dimensional drift template.

223. The method of claim 222, wherein the three-dimensional drift template is a tetrahedron.

224. The method of claim 185, wherein at least one of the drift templates includes multiple colors corresponding to different types of transient events.

225. The method of claim 224, wherein the different types of transient events include a first binding event of a first imaging strand with a first type of DNA docking strand and a second binding event of a second imaging strand with a second type of DNA docking strand.

226. The method of claim 185, wherein outputting the final image comprises displaying the final image on a display.

227. The method of claim 185, wherein outputting the final image comprises sending the final image to a computer via at least one network.

228. The method of claim 185, wherein outputting the final image comprises storing the final image on at least one storage device.

229. A non-transitory computer readable medium encoded with a plurality of instructions that, when executed by at least one computer processor, performs a method of performing drift correction for a plurality of images, wherein each of the plurality of images comprises a

frame of a time sequence of images, wherein the time sequence of images captures a plurality of transient events, the method comprising:

determining a time trace for each of a plurality of drift markers identified in the plurality of images, wherein a time trace for each drift marker corresponds to movement of an object in the image over the time sequence of images;

determining a first drift correction from at least one of the plurality of drift markers based, at least in part, on the time traces for the at least one of the plurality of drift markers;

determining a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images, wherein each drift template in the plurality of drift templates describes a geometrical relationship between the plurality of geometrically-addressable marker sites of transient events in the drift template;

determining a second drift correction based, at least in part, on the time traces for the plurality of geometrically-addressable marker sites from the plurality of drift templates;

correcting the plurality of images based, at least in part, on the first drift correction and the second drift correction; and

outputting a final image based on the corrected plurality of images.

230. A computer, comprising:

an input interface configured to receive a plurality of images, wherein each of the plurality of images comprises a frame of a time sequence of images, wherein the time sequence of images captures a plurality of transient events;

at least one processor programmed to:

determine a time trace for each of a plurality of drift markers identified in the plurality of images, wherein a time trace for each drift marker corresponds to movement of an object in the image over the time sequence of images;

determine a first drift correction from at least one of the plurality of drift markers based, at least in part, on the time traces for the at least one of the plurality of drift markers;

determine a time trace for each of a plurality of geometrically-addressable marker sites from a plurality of drift templates identified from the plurality of images, wherein each drift template in the plurality of drift templates describes a geometrical relationship between the plurality of geometrically-addressable marker sites of transient events in the drift template;

determine a second drift correction based, at least in part, on the time traces for the plurality of geometrically-addressable marker sites from the plurality of drift templates;

5 correct the plurality of images based, at least in part, on the first drift correction and the second drift correction; and

 determine a final image based on the corrected plurality of images; and
an output interface configured to output the final image.

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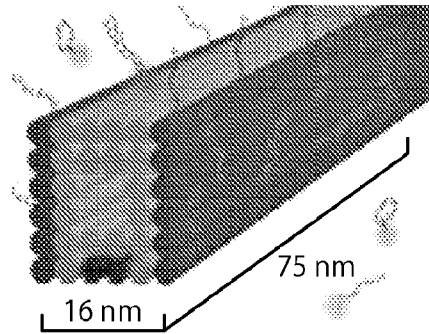


Fig. 1A

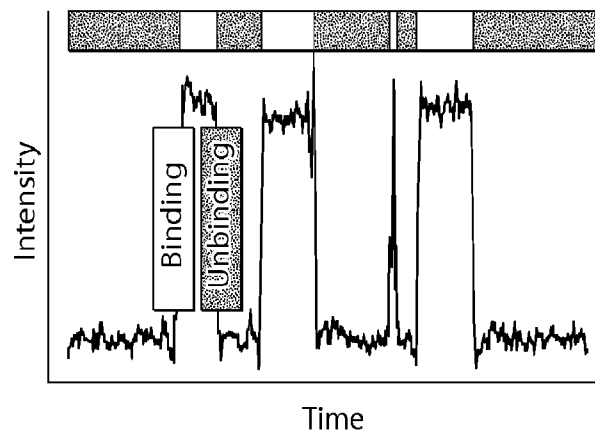


Fig. 1B

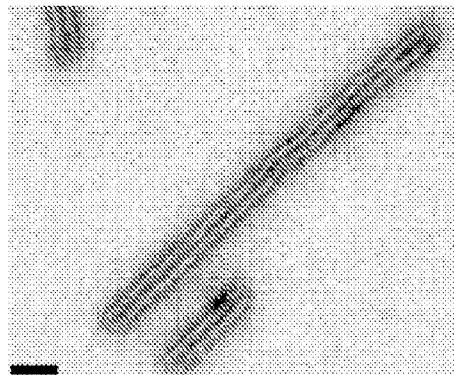


Fig. 1C

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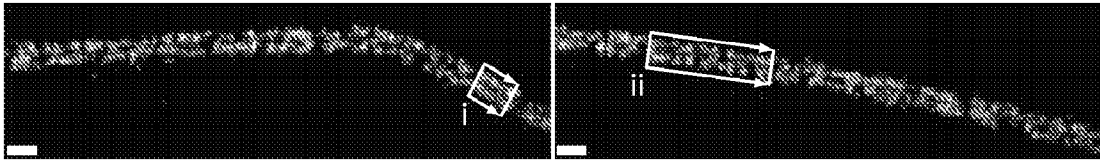


Fig. 1D

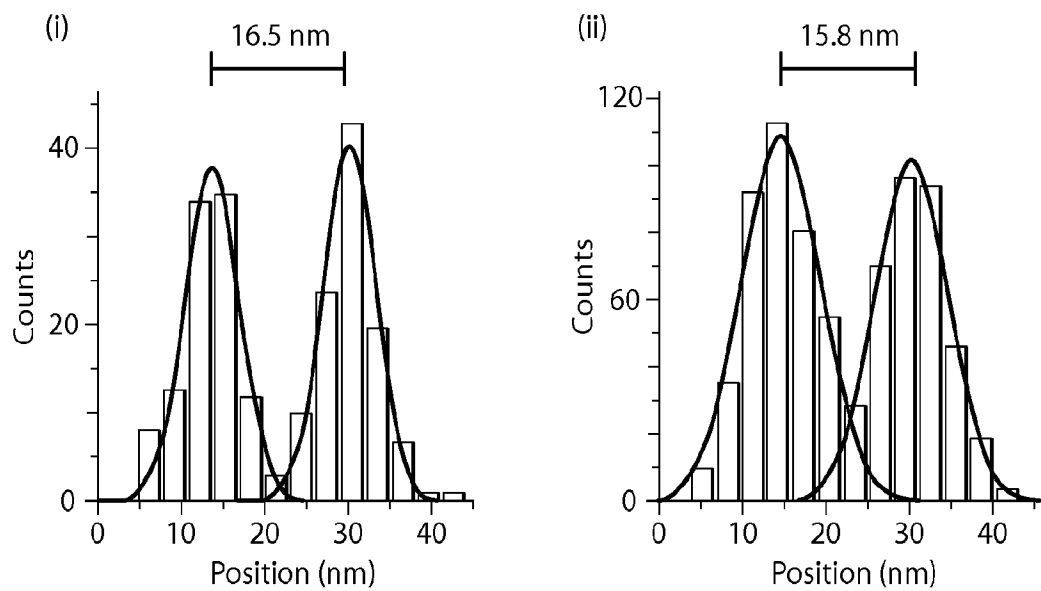


Fig. 1E

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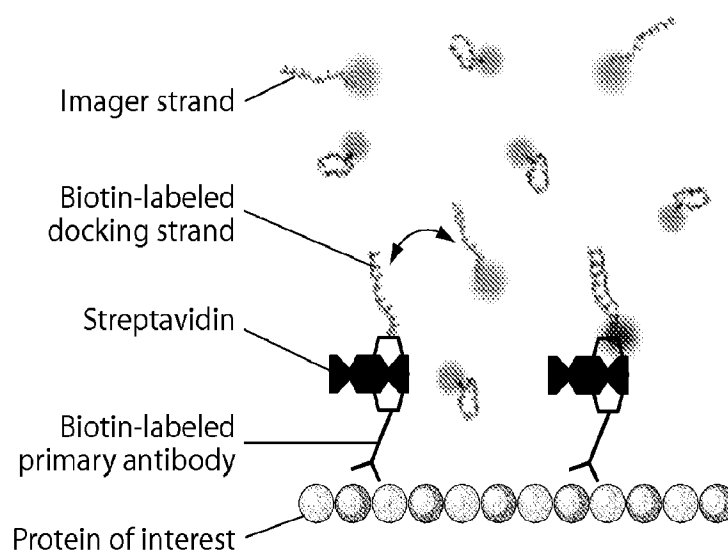


Fig. 2

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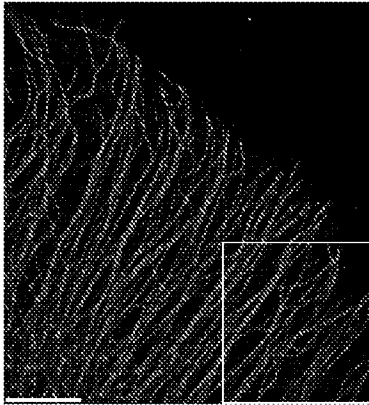


Fig. 3A

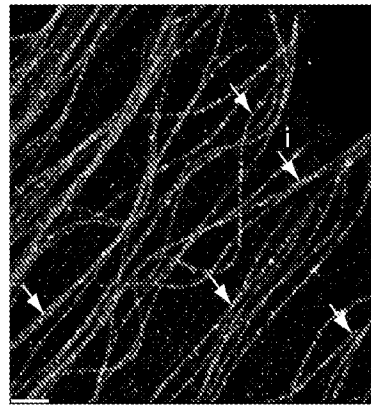


Fig. 3B

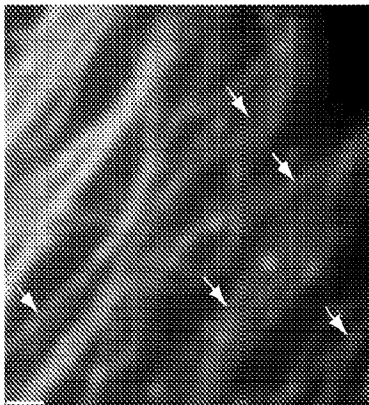


Fig. 3C

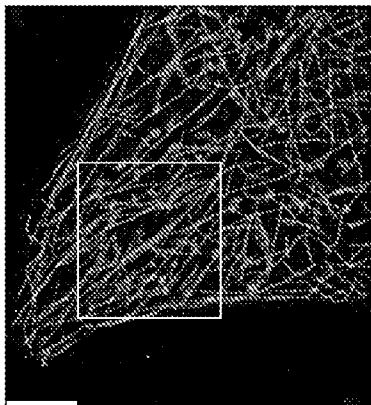


Fig. 3D

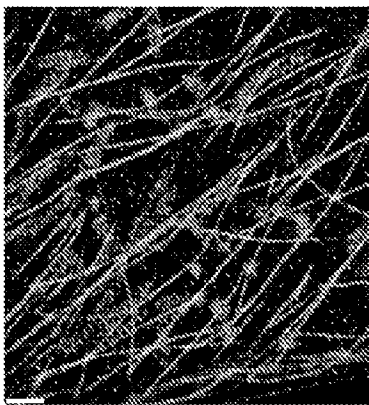


Fig. 3E

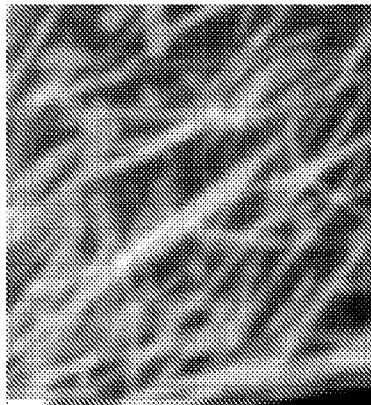


Fig. 3F

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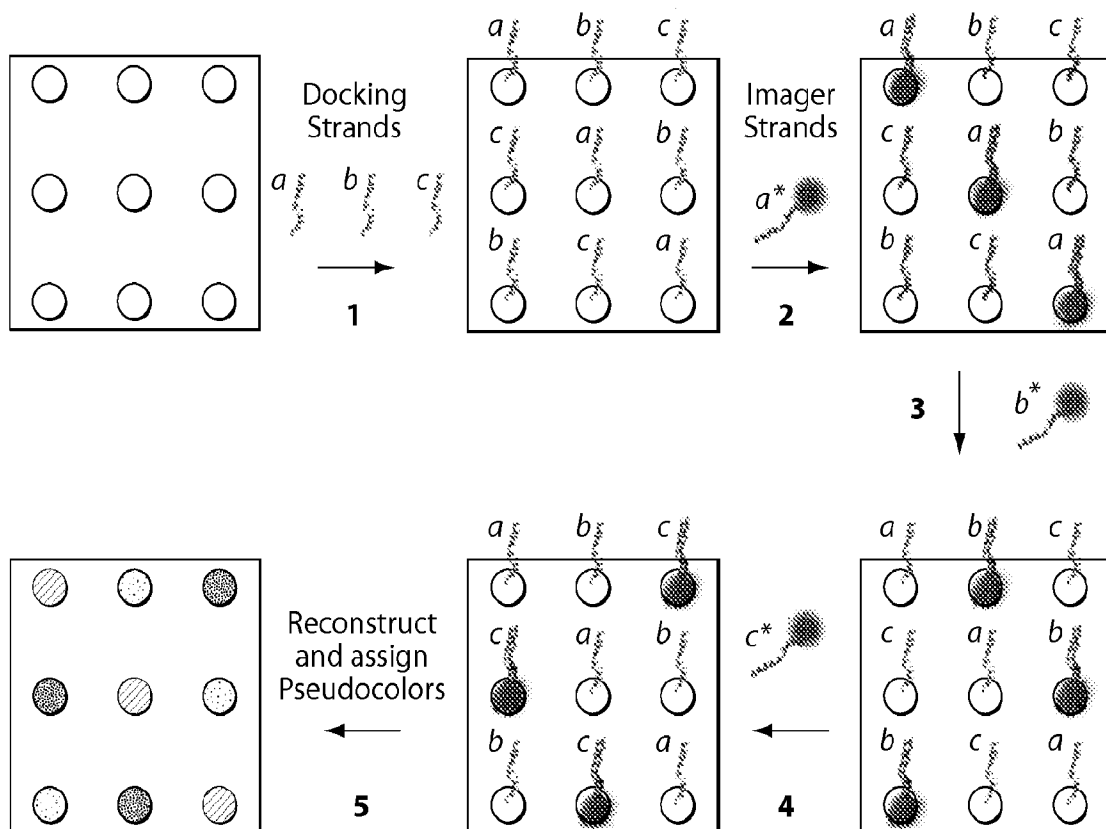


Fig. 4A

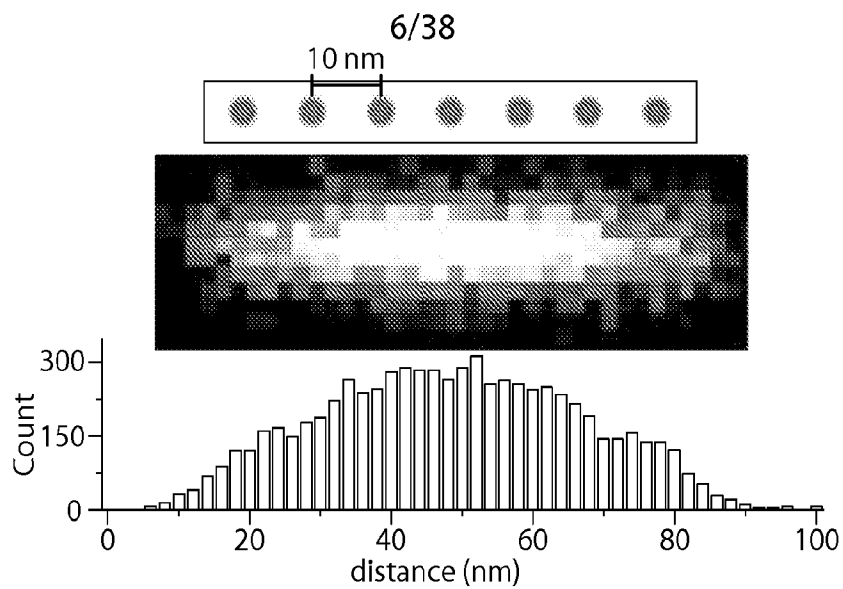


Fig. 4B1

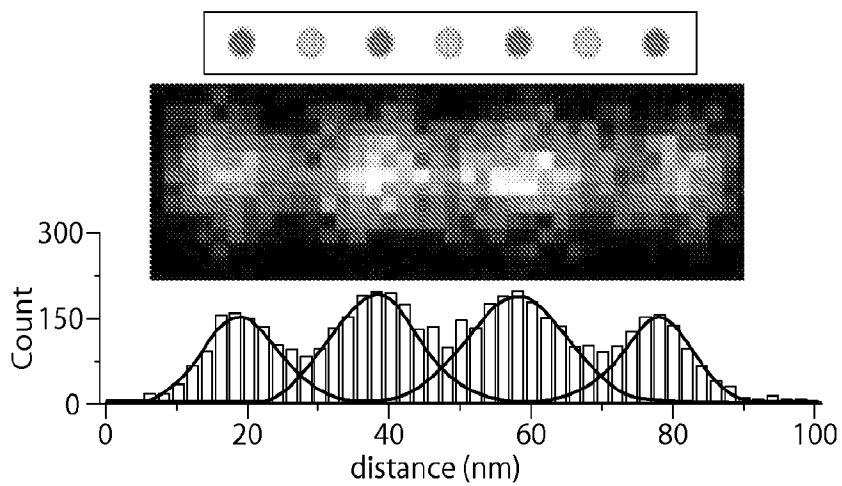


Fig. 4B2

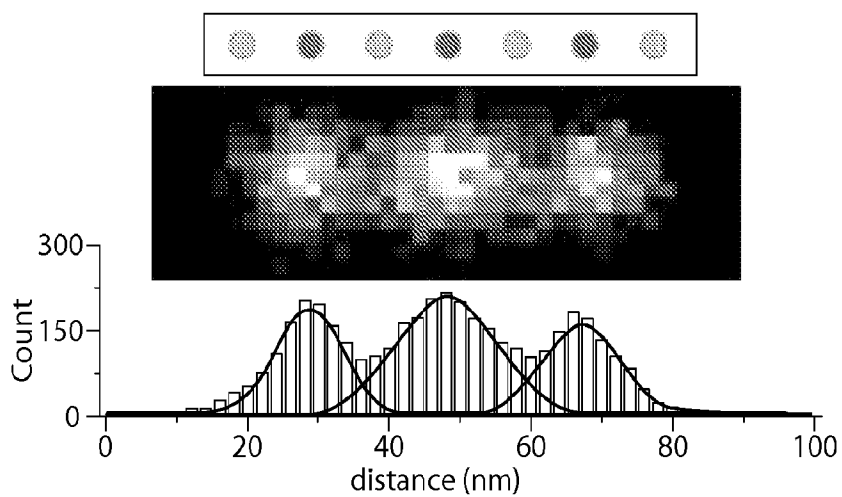
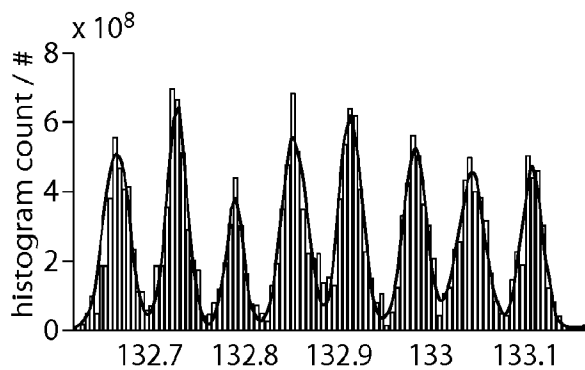
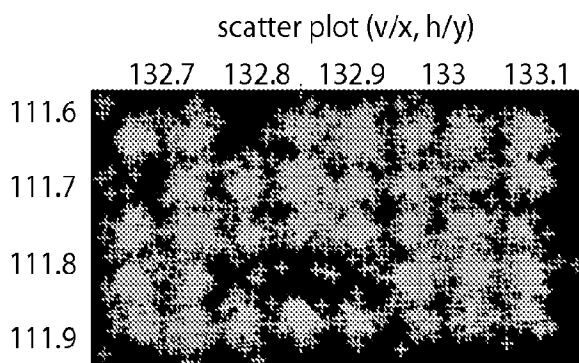
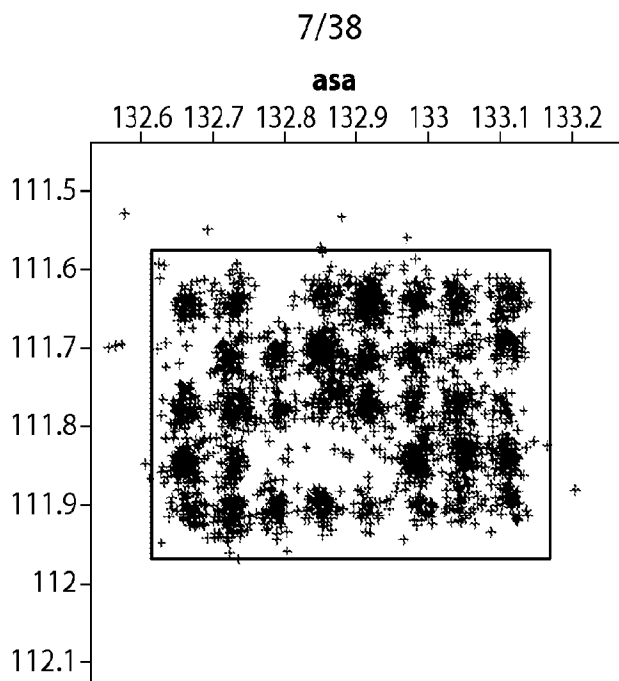


Fig. 4B3



Fitting results:

Intervals:	10.4661	9.94118	10.1858	9.83788	10.978	9.80175	10.4411	nm
Sigmas:	2.1757	1.7805	1.5213	2.163	1.9209	1.9914	2.3486	1.8586 nm
Normalized residue = 3.3e+006, modified Chi squared = 0.024								

Fig. 4C

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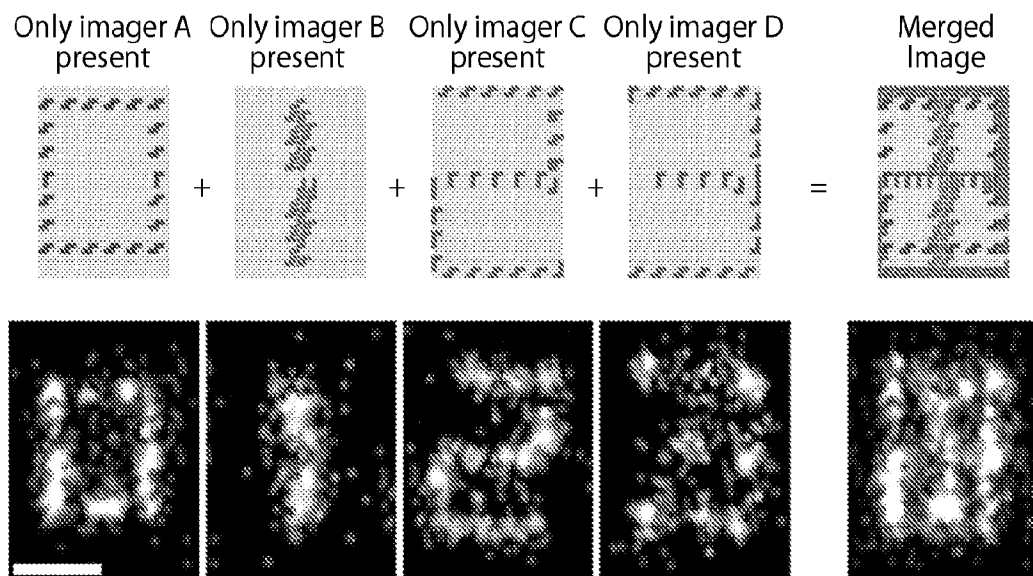


Fig. 5A

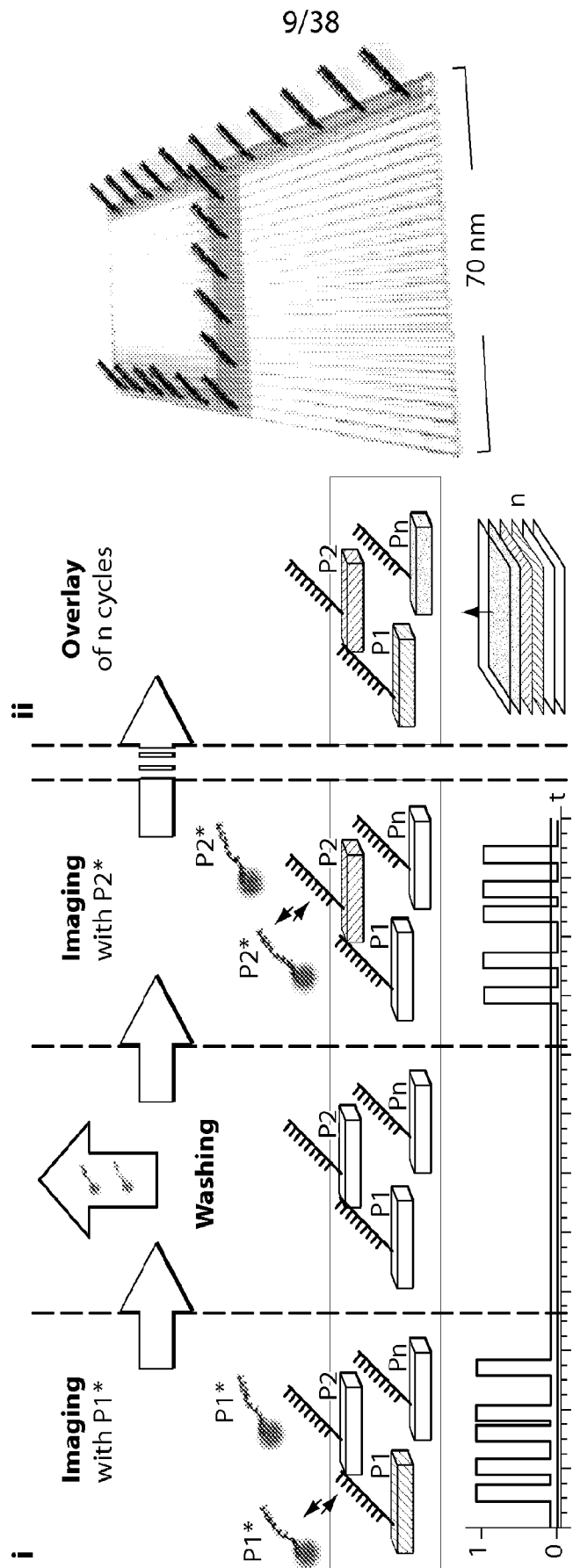


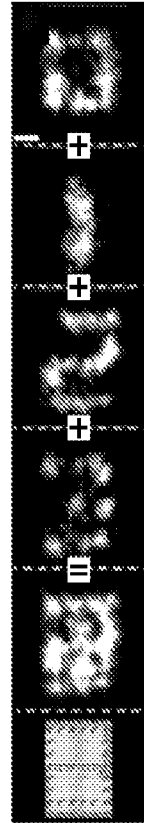
Fig. 5B

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iii



iv



v

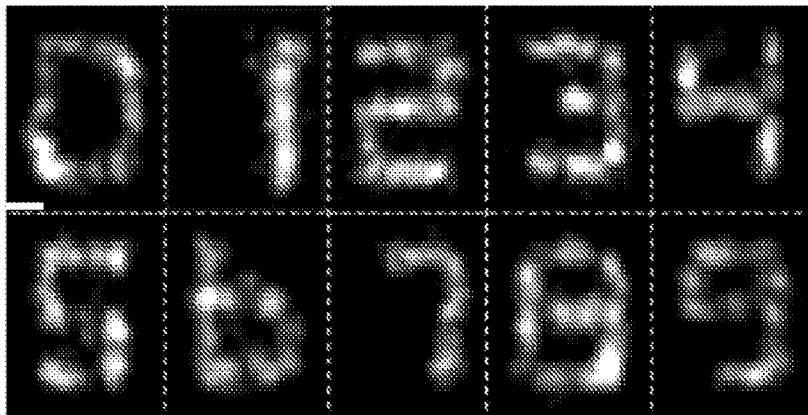


Fig. 5B (Continued)

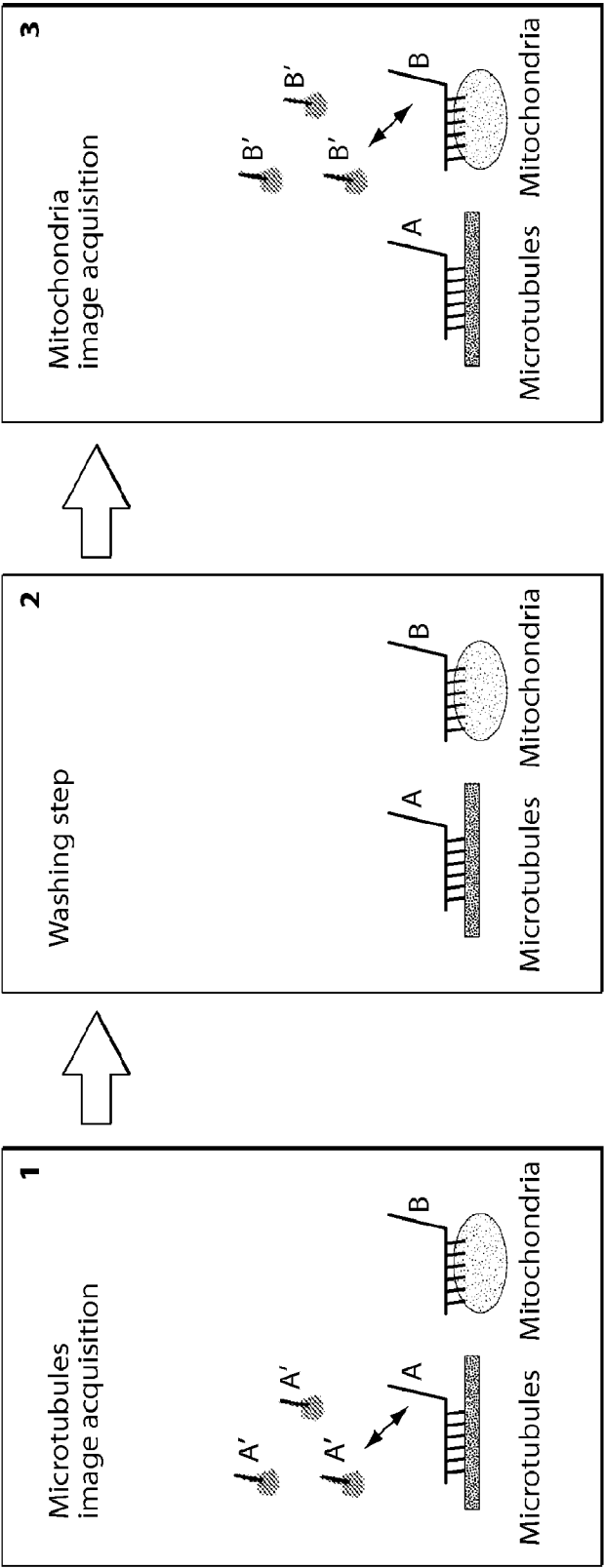


Fig. 6A

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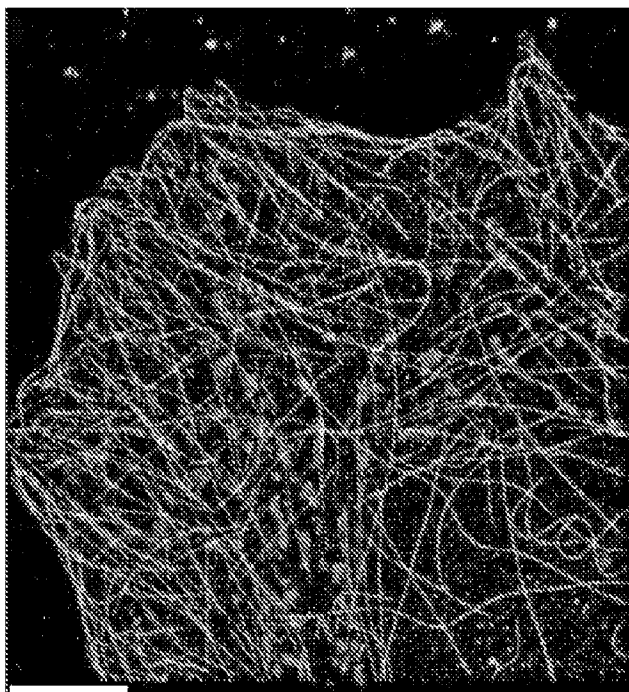


Fig. 6B

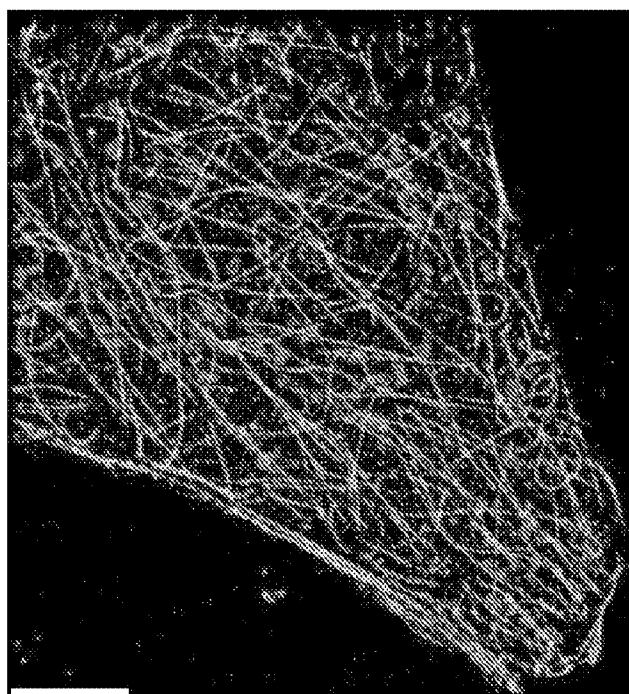


Fig. 6C

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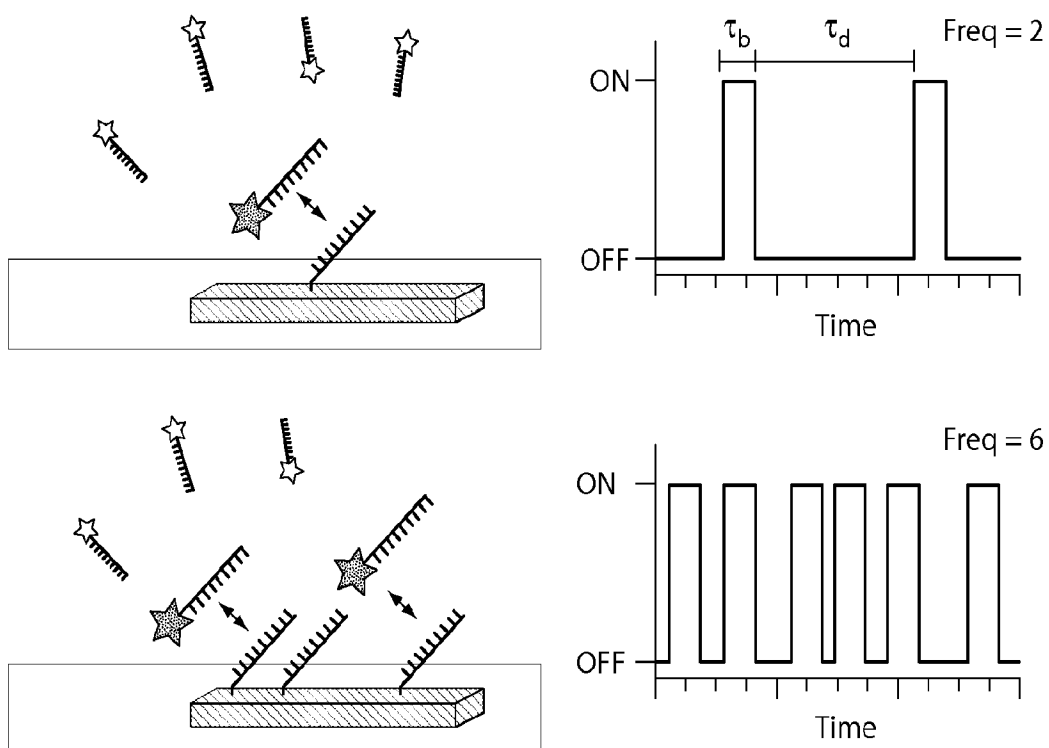


Fig. 7A

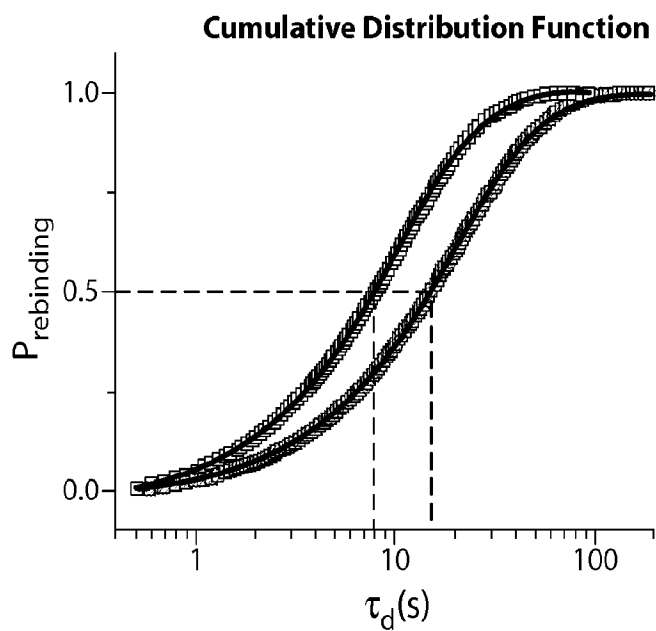


Fig. 7B

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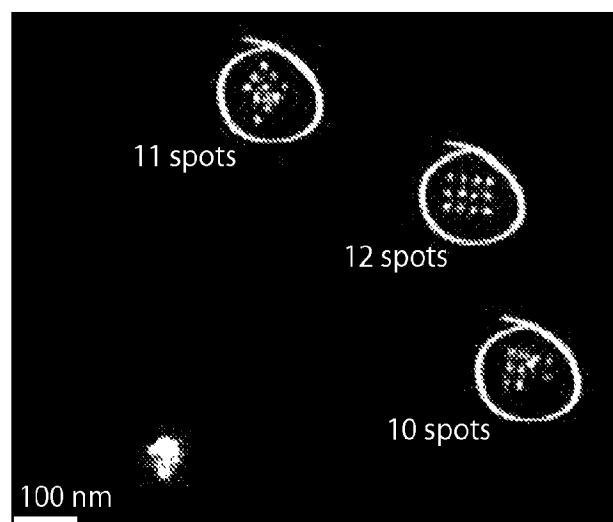


Fig. 7C

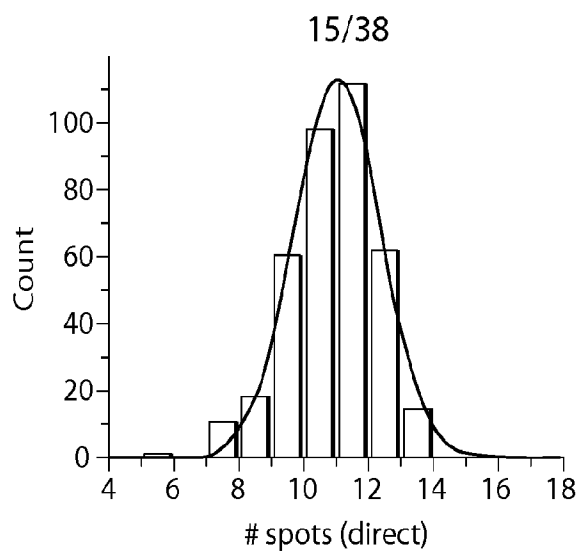


Fig. 7D1

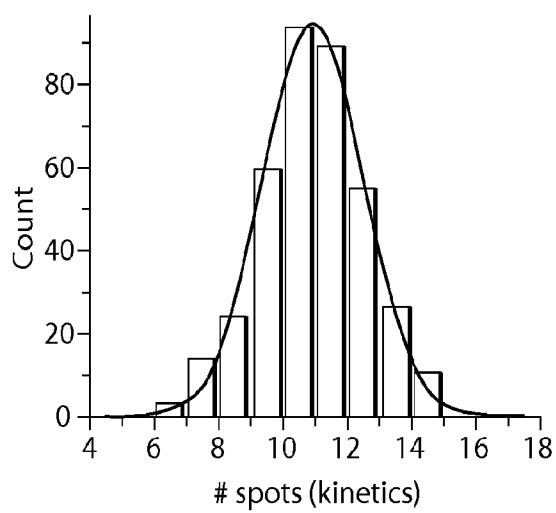


Fig. 7D2

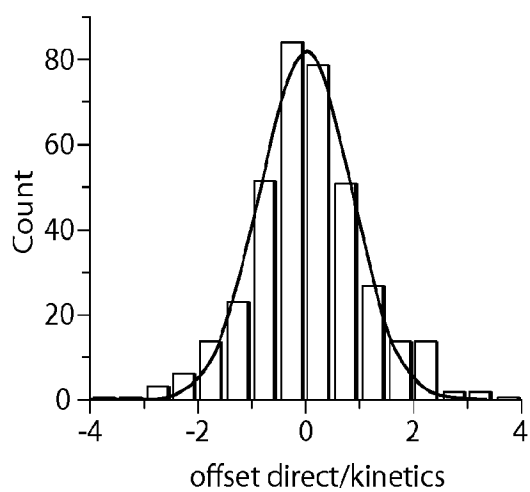


Fig. 7D3

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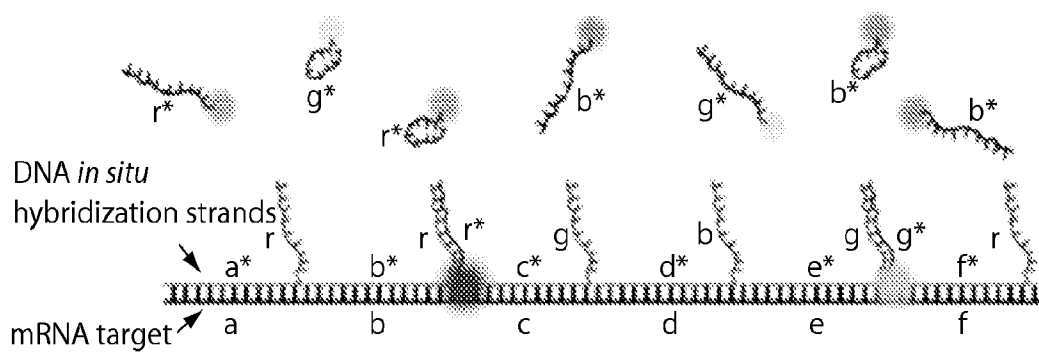


Fig. 8A

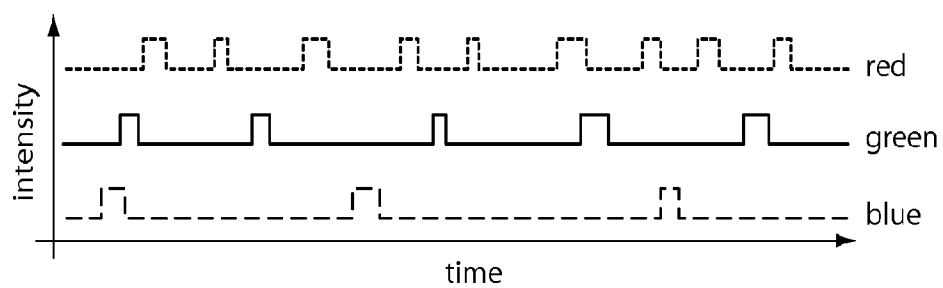


Fig. 8B

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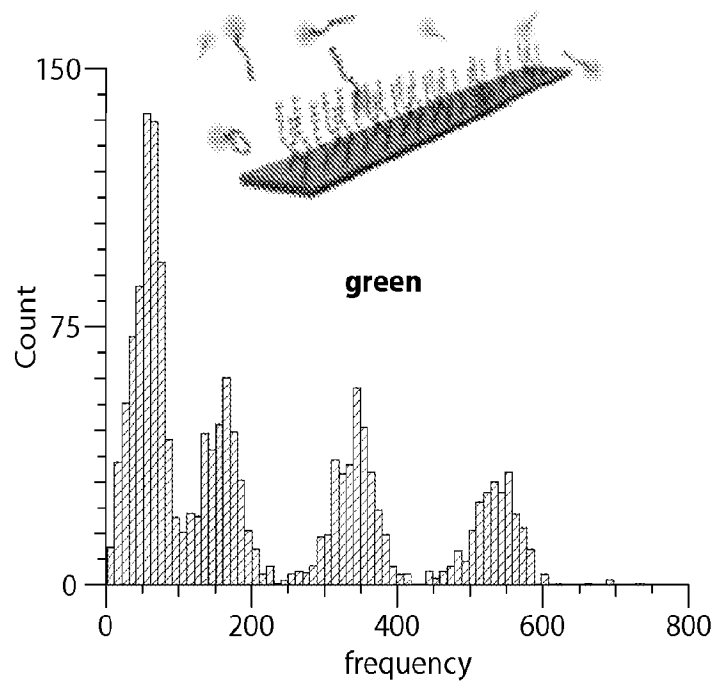
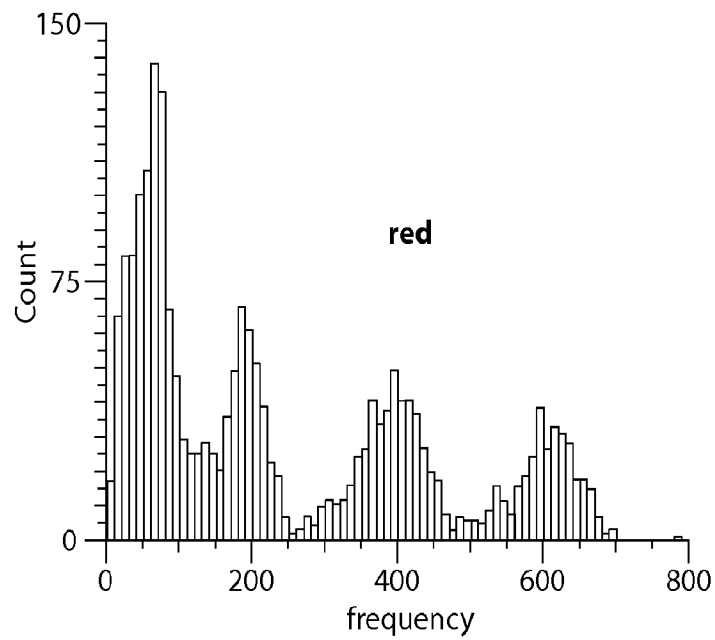


Fig. 8C

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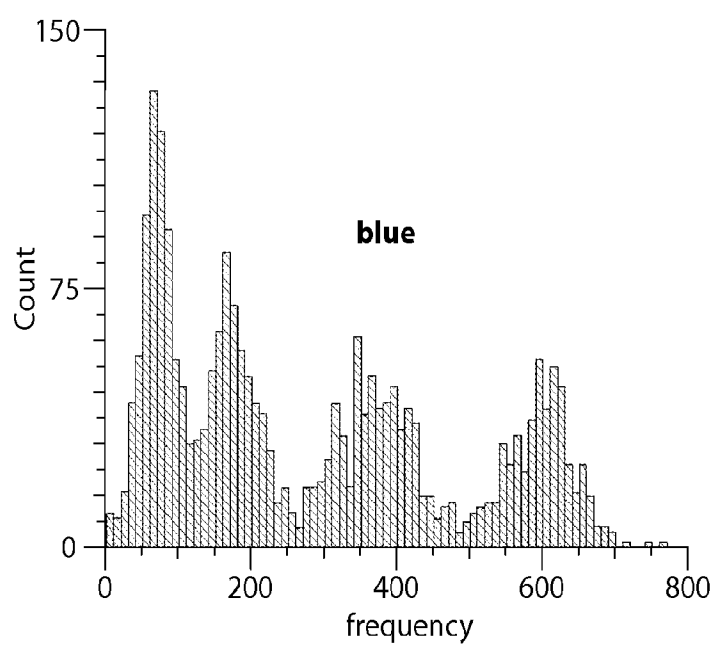


Fig. 8C (Continued)

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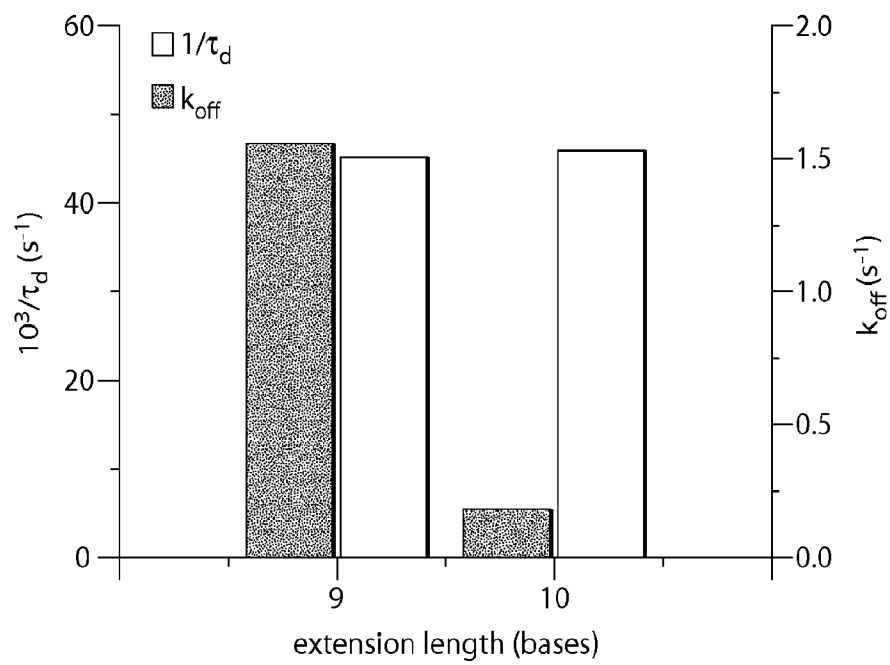


Fig. 9

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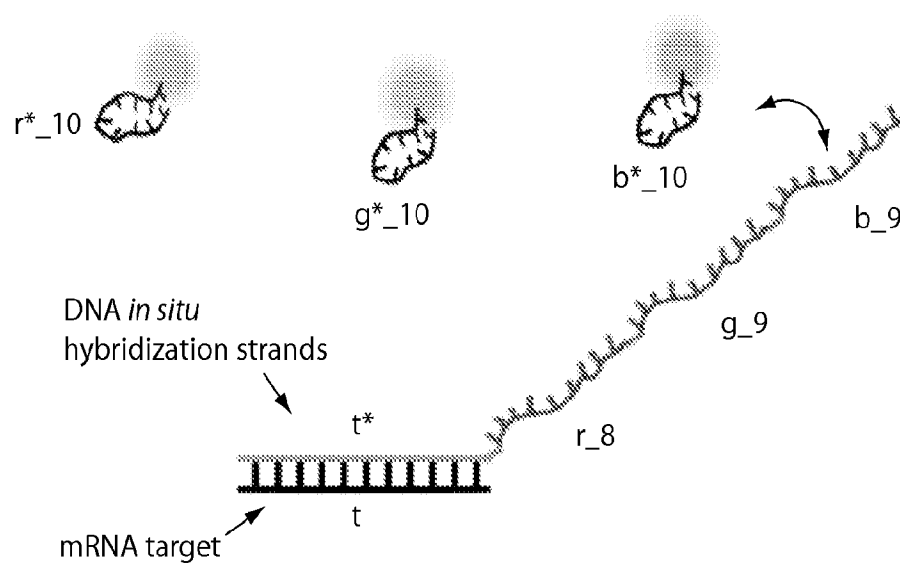


Fig. 10A

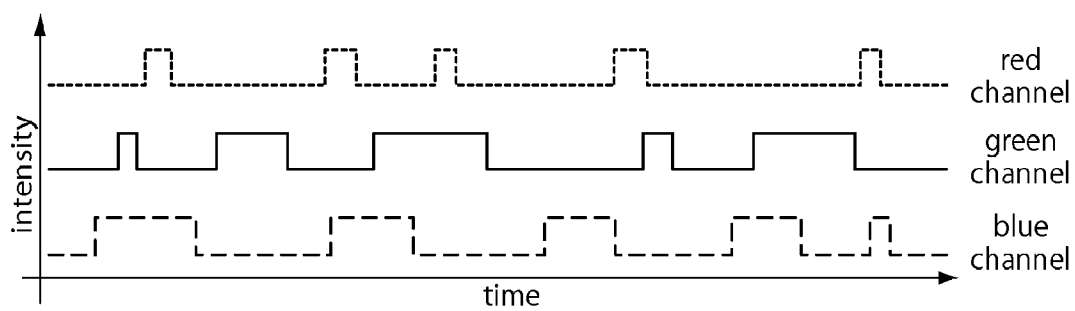


Fig. 10B

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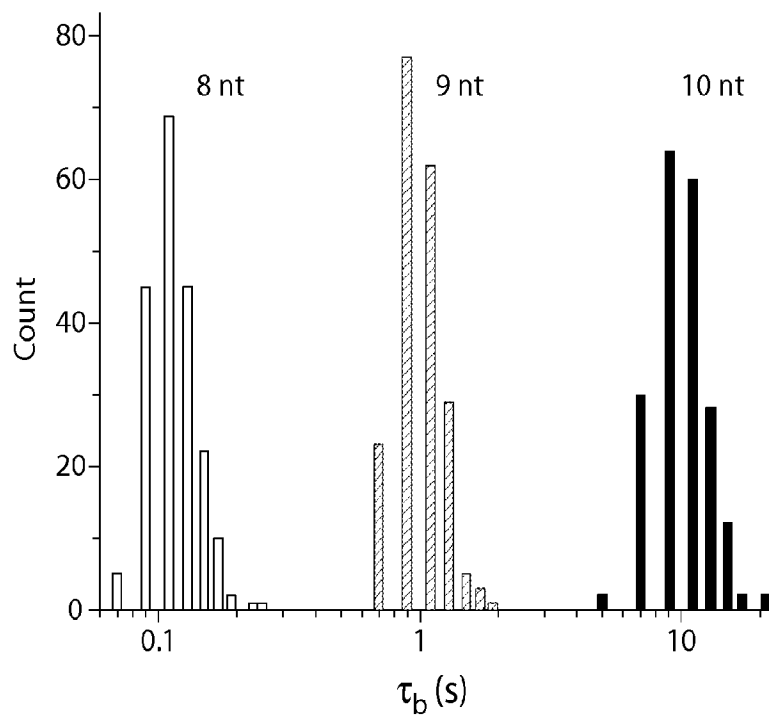


Fig. 10C

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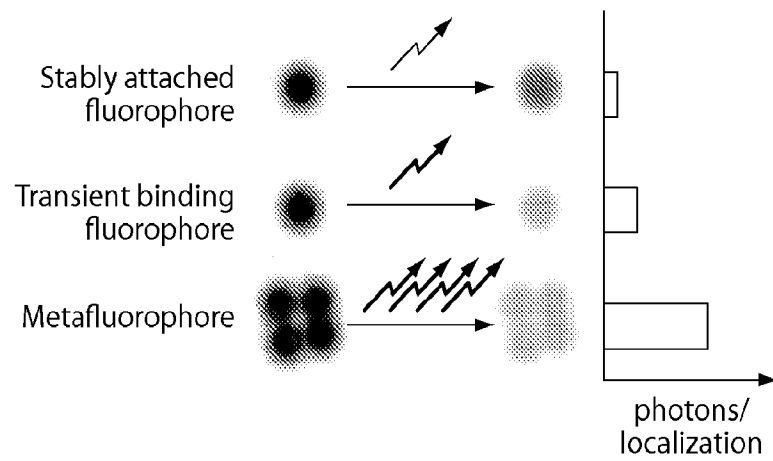


Fig. 11A



Fig. 11B1

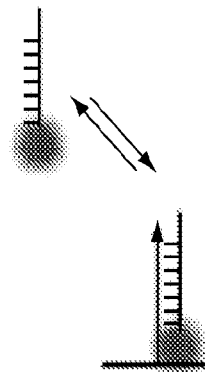


Fig. 11B2

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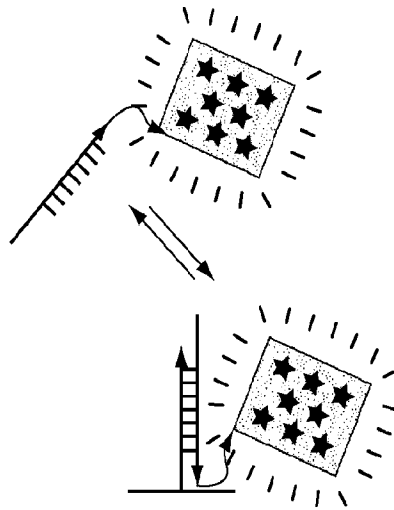


Fig. 11B3

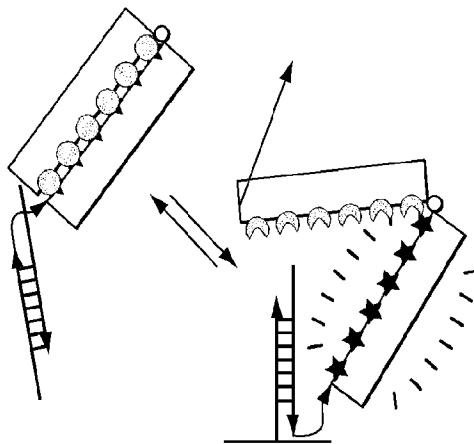


Fig. 11B4

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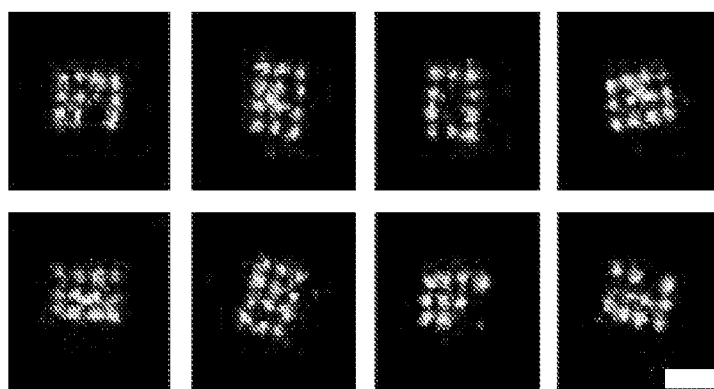
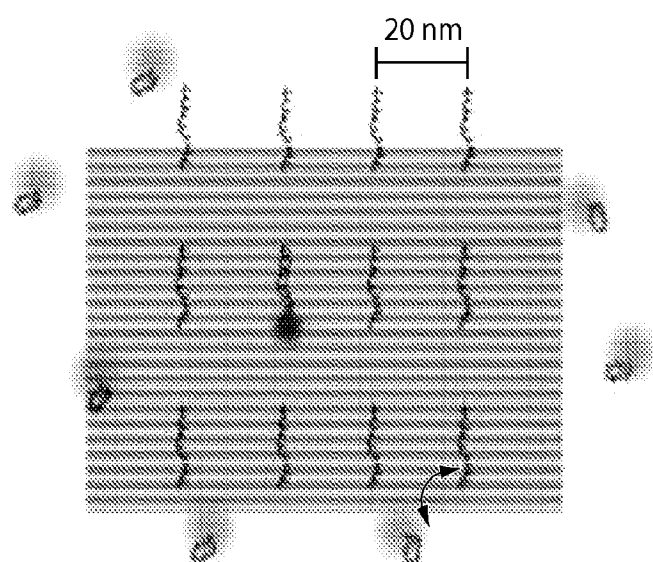


Fig. 11C

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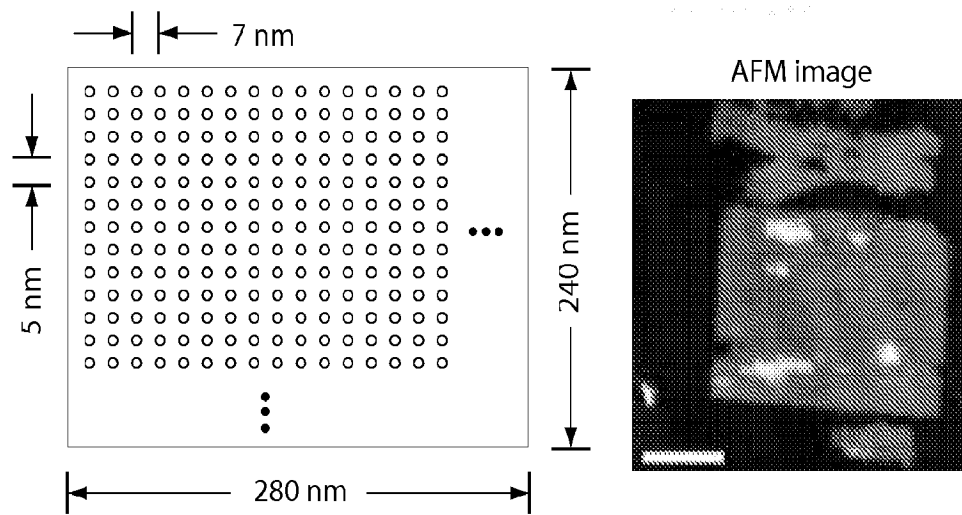
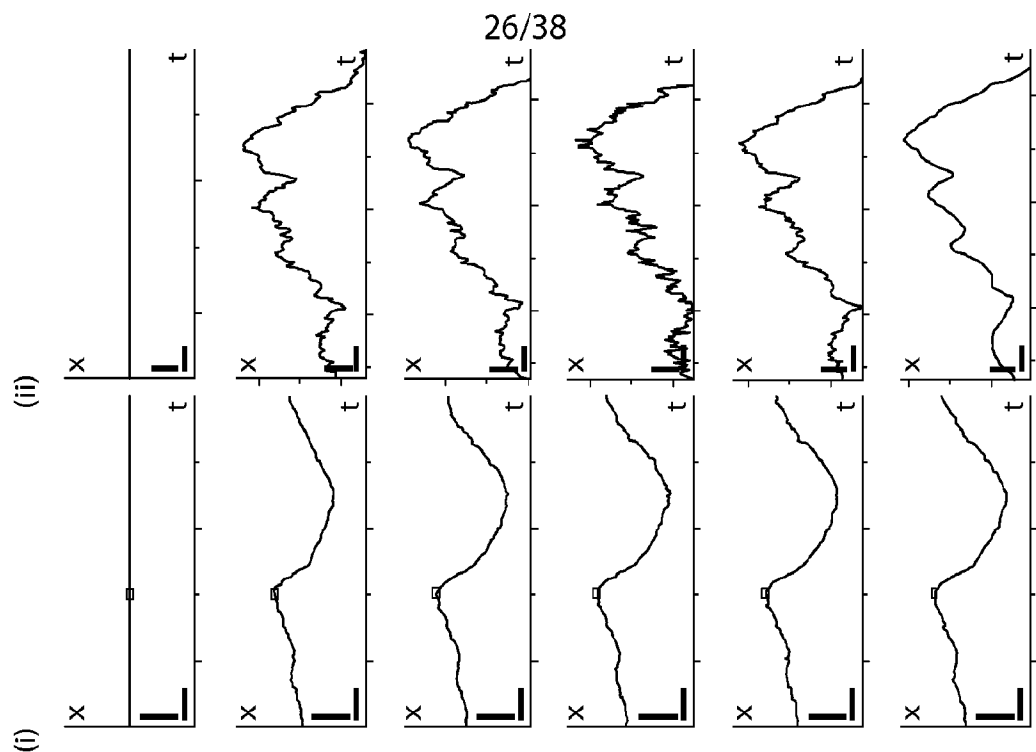
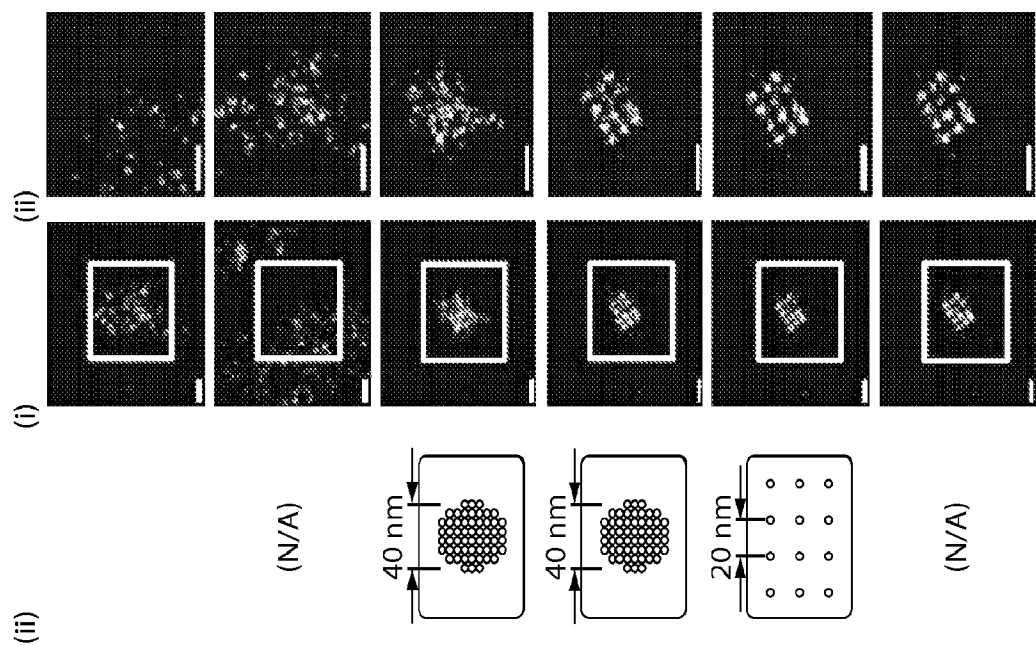
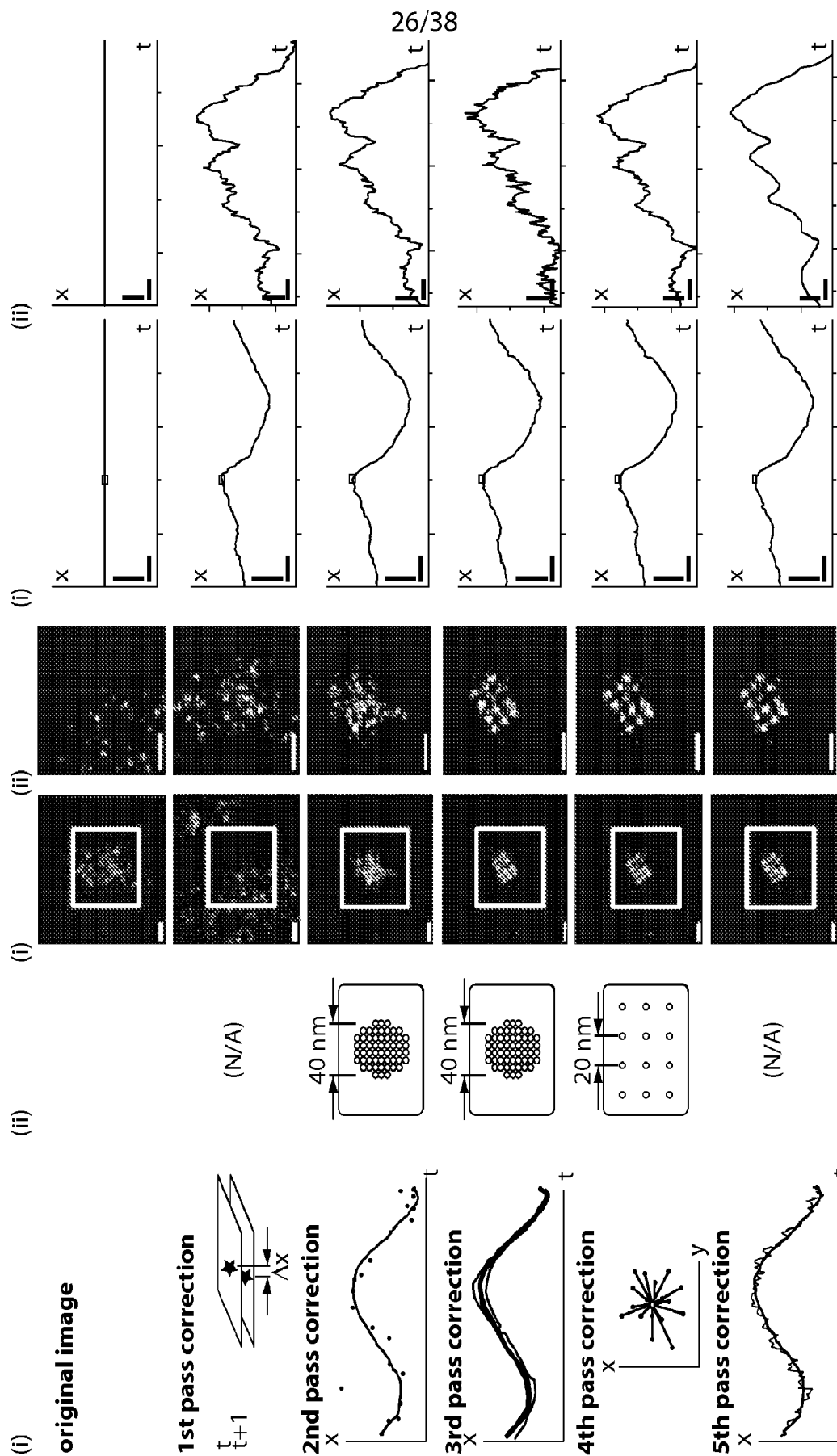


Fig. 11D



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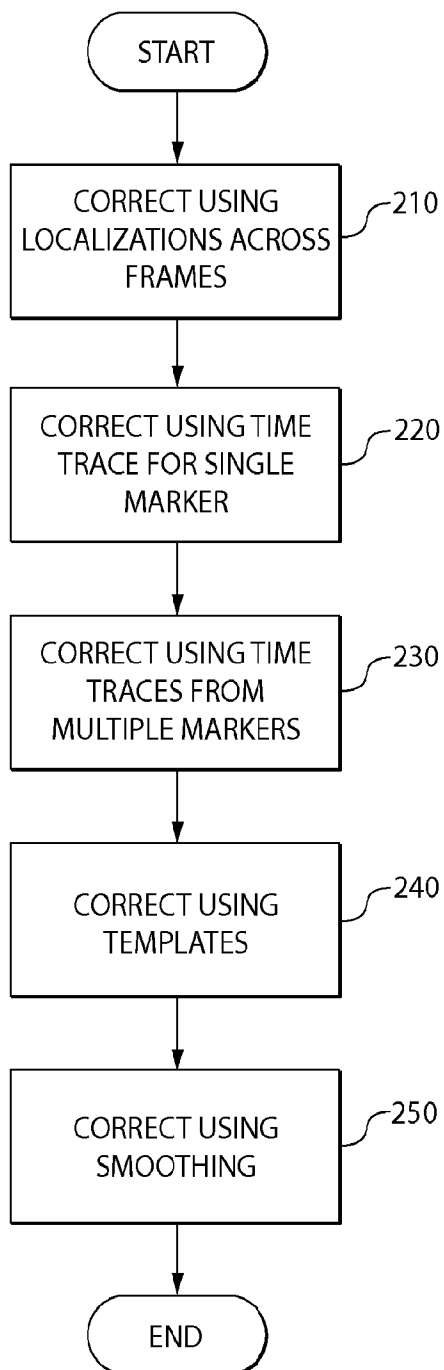


Fig. 13

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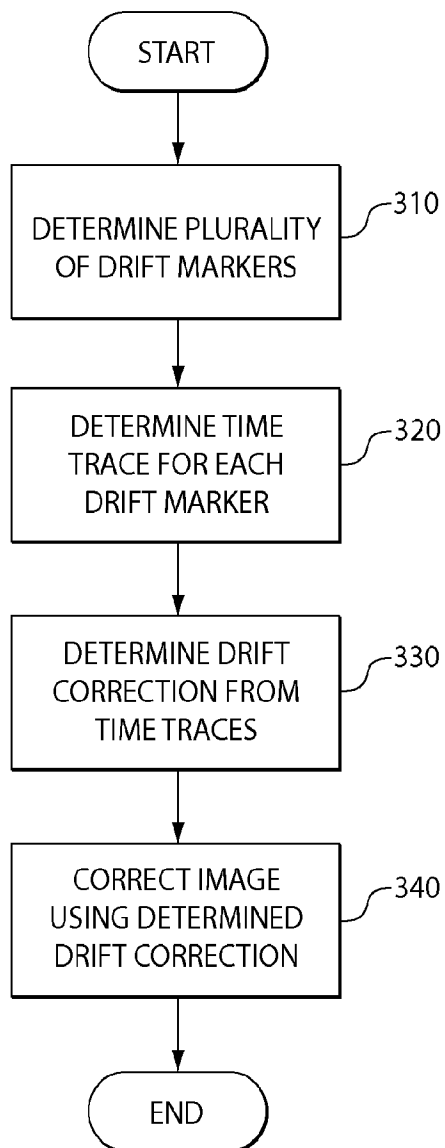


Fig. 14

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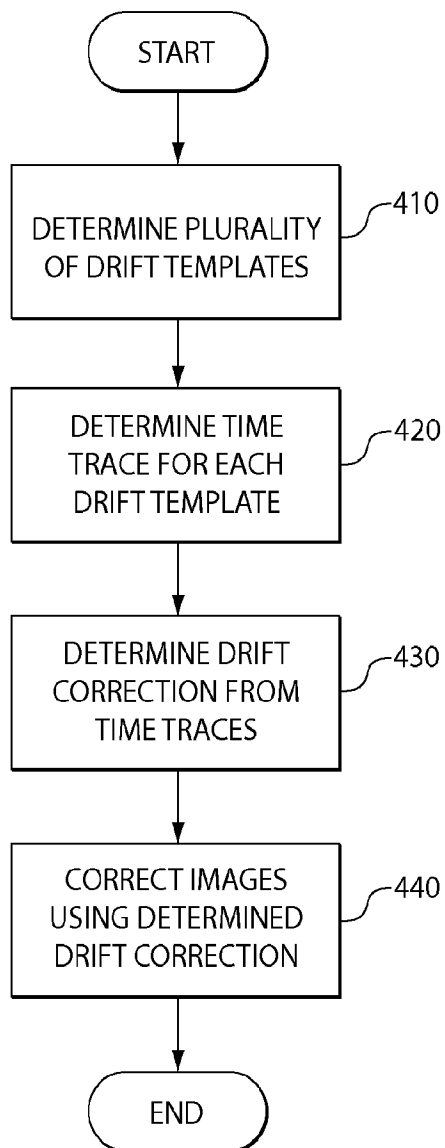


Fig. 15

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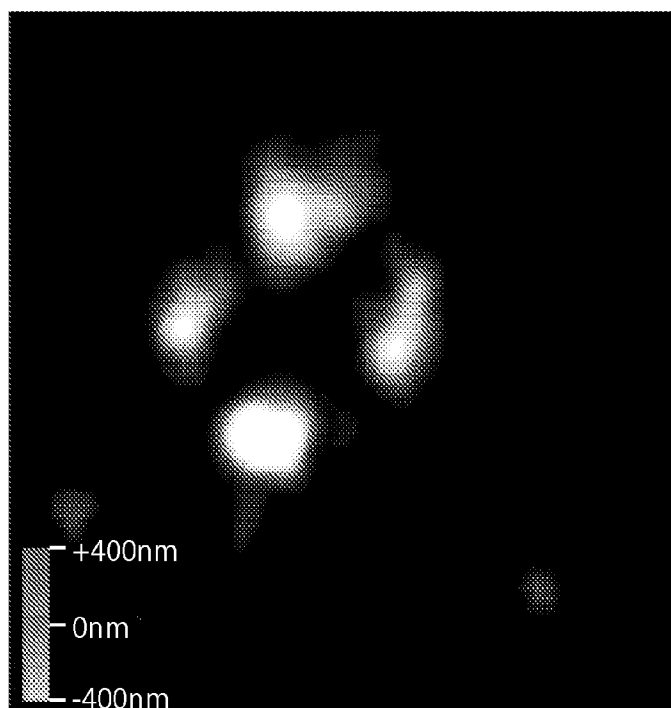


Fig. 16A

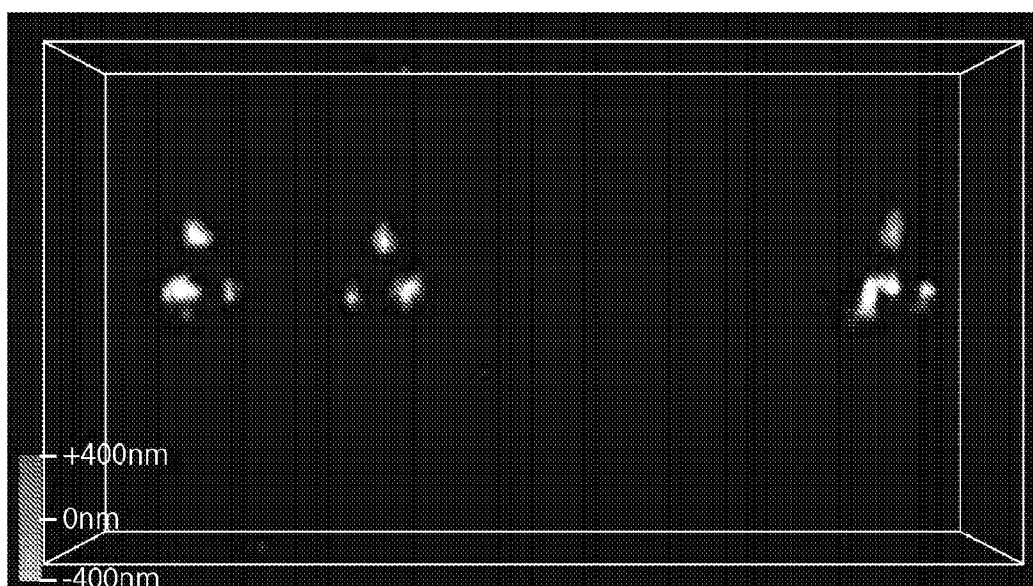


Fig. 16B

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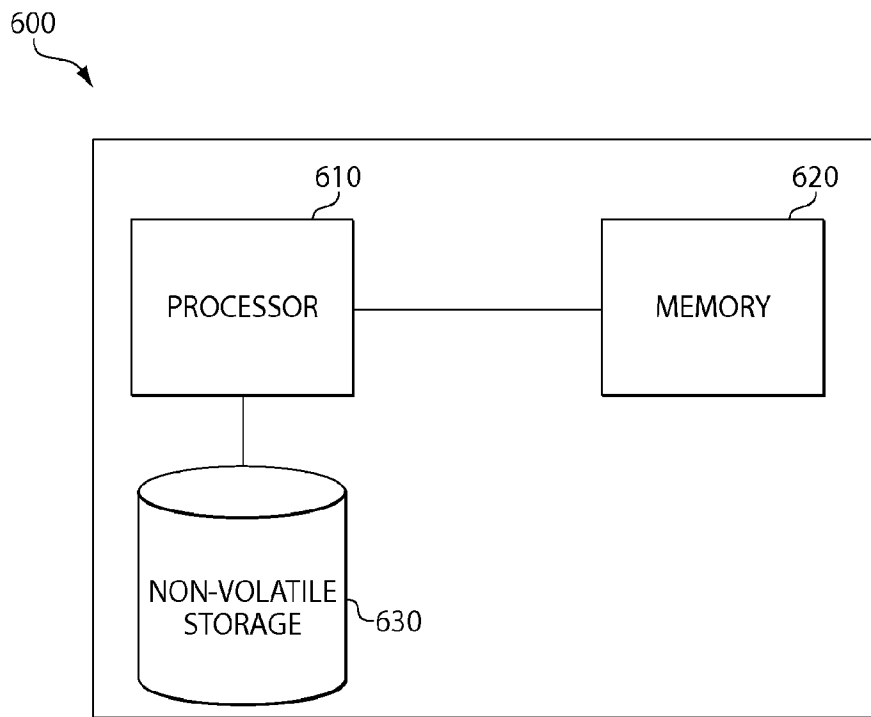


Fig. 17

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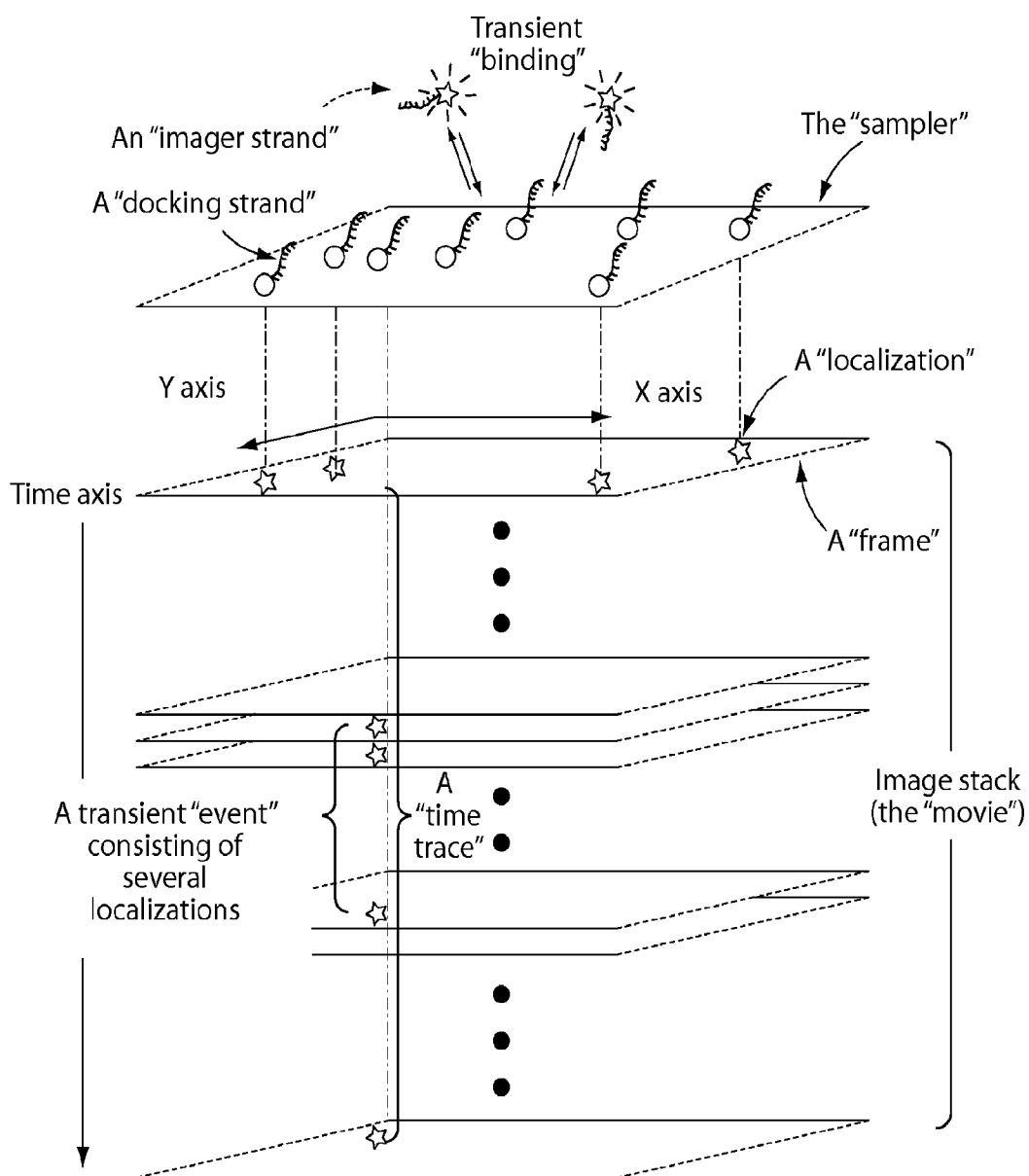
The imaging process

Fig. 18A

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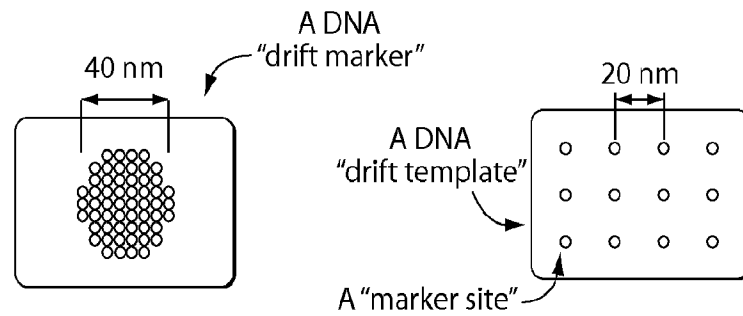
The drift markers

Fig. 18B

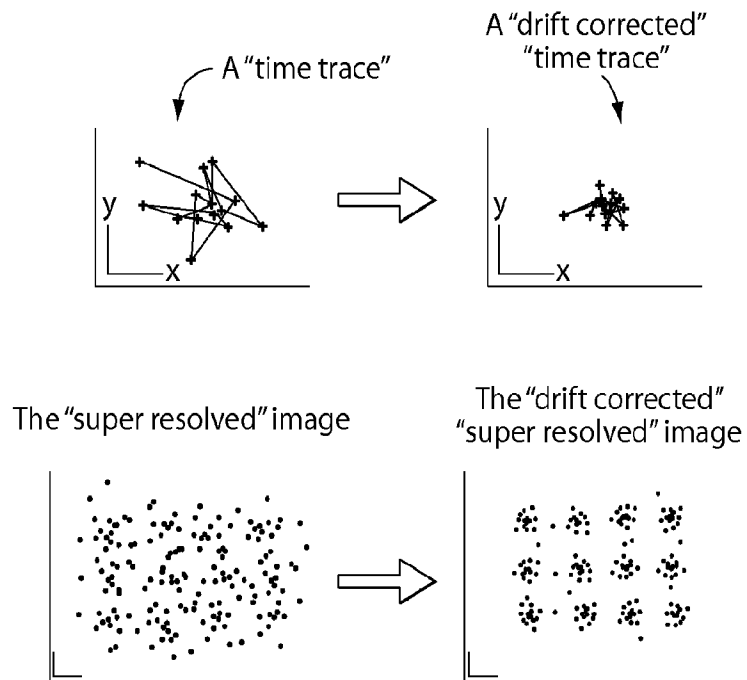
The drift correction principle

Fig. 18C

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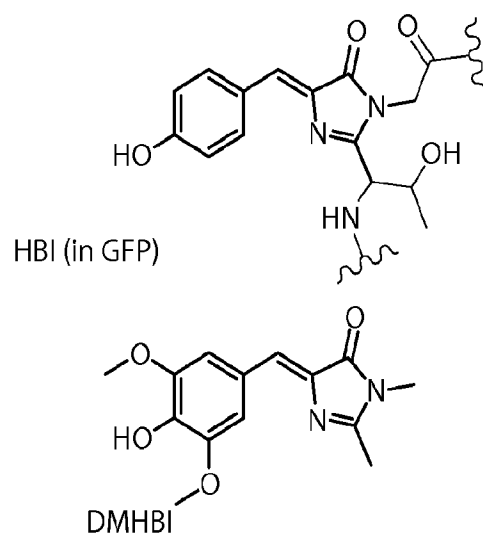


Fig. 19A

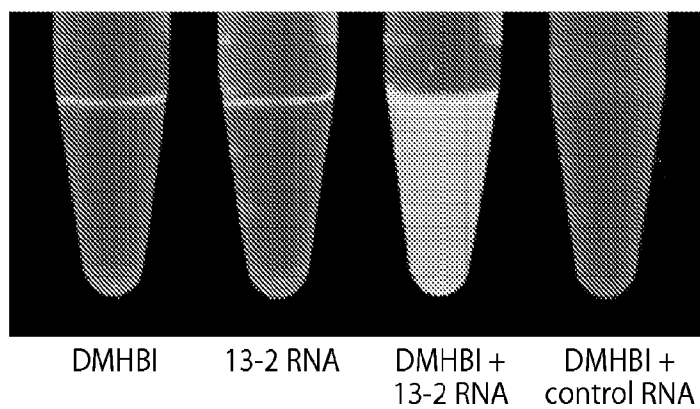


Fig. 19B

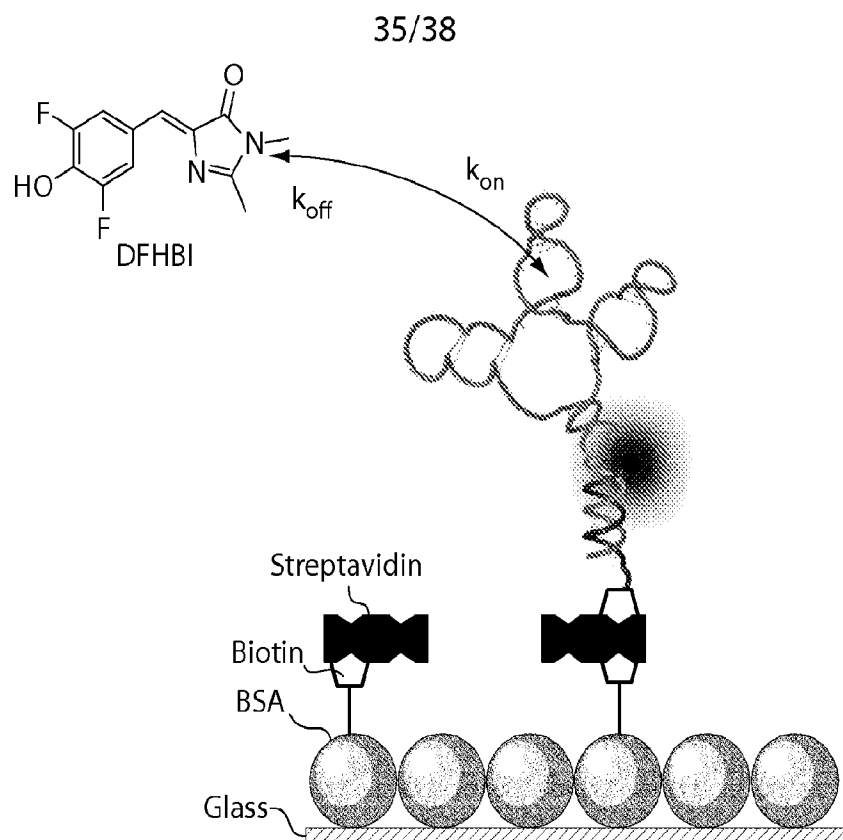


Fig. 20A

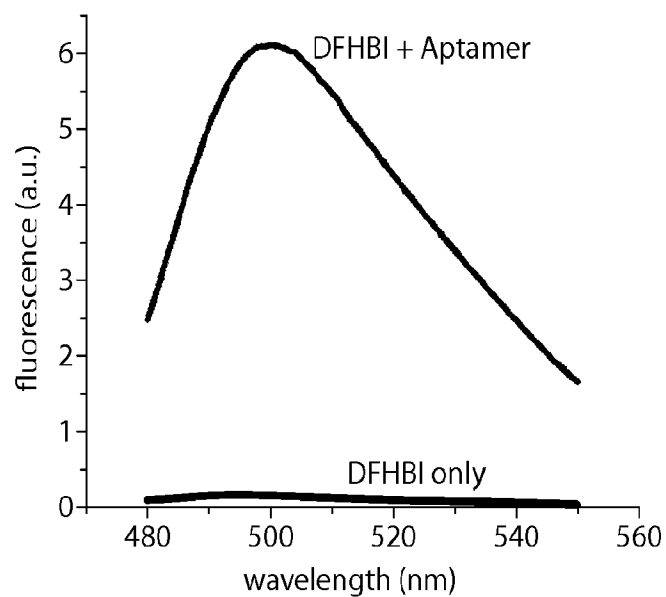


Fig. 20B

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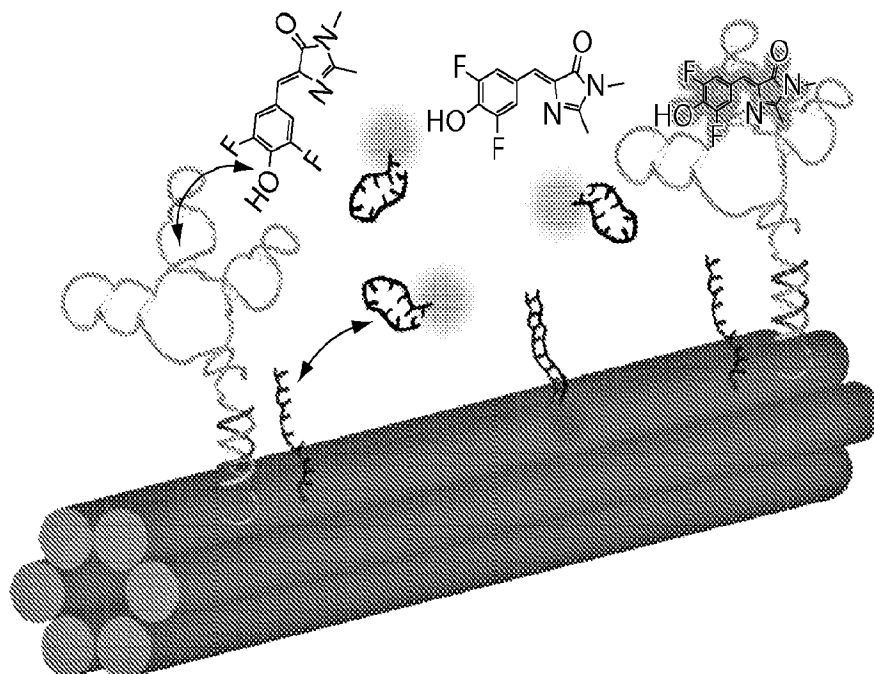


Fig. 21A

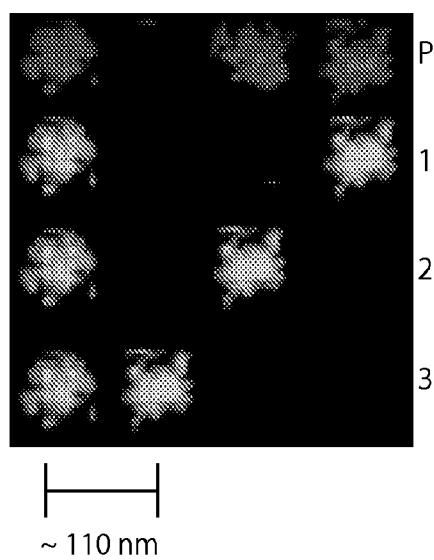


Fig. 21B

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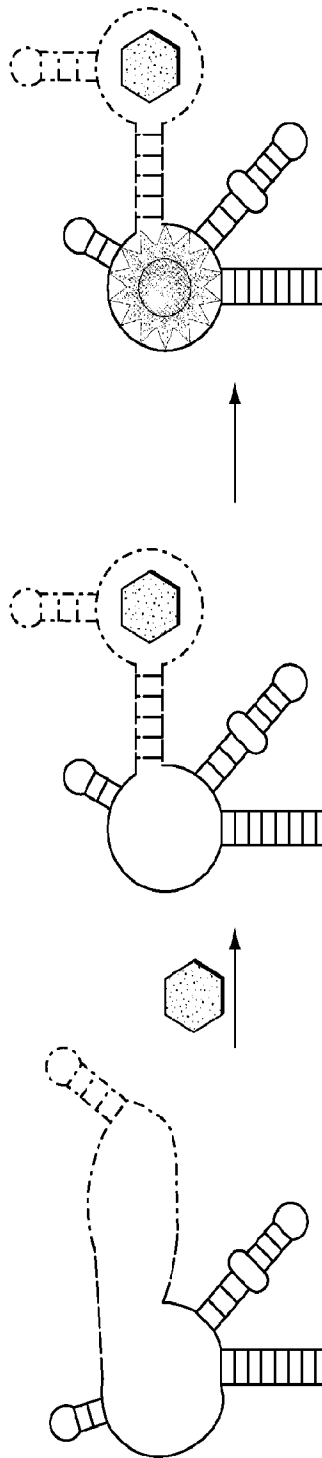


Fig. 22

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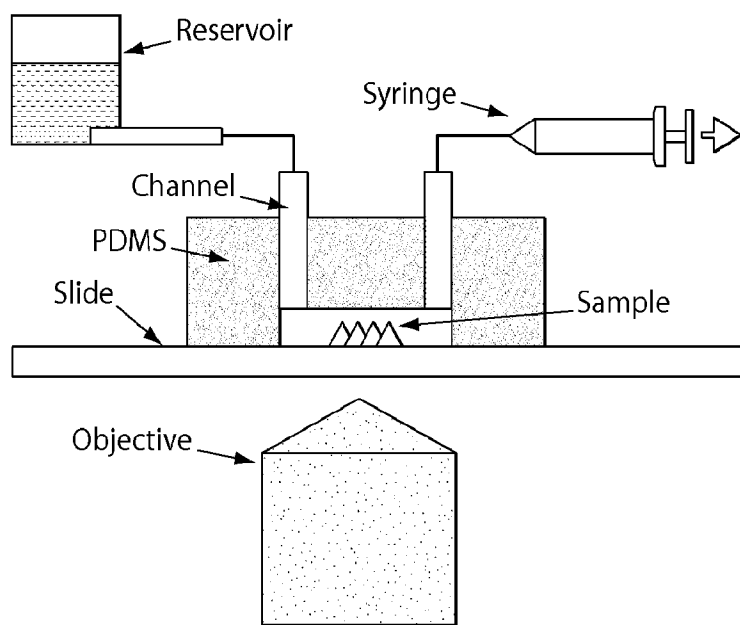


Fig. 23A

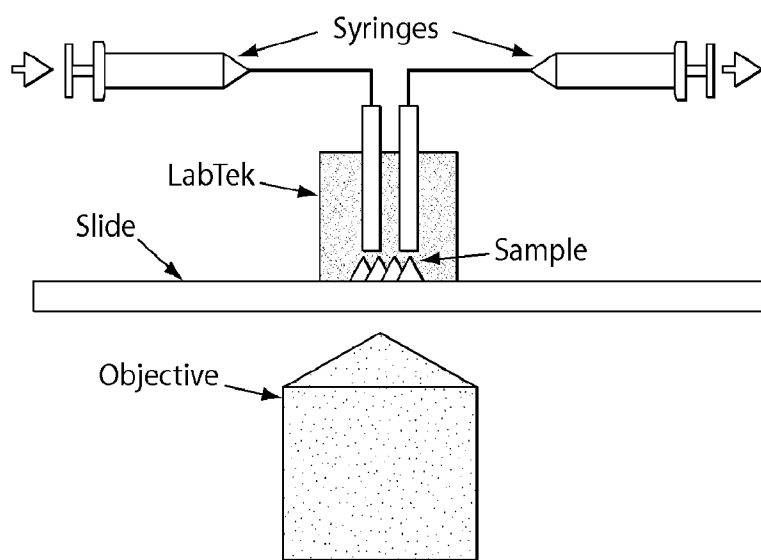


Fig. 23B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/48977

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68 (2014.01)

CPC - C12Q 1/6825

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)

IPC(8): C12Q 1/68; G01N 21/63, 21/64 (2014.01)

CPC: B82Y 15/00; C12Q 1/6825; G01N 21/6486; USPC: 506/9, 16

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

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

Patseer (EP, WO, US, JP, CN, KR, CA, DE, FR, GB, ES, AU, IN, CH, AT, BR, TH); Dialog ProQuest; IP.com; Google; Google Scholar; protein, 'docking strand,' 'imager strand,' 'nucleic acid,' 'transient, bind, 'drift correction,' 'time trace,' 'drift template'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	WO 2012/058638 A2 (HARVARD COLLEGE) May 3, 2012; abstract; page 2, lines 5-6, 19-21; page 4, lines 15-18; page 4, lines 24-25; page 5, lines 26-27; page 6, lines 8-11; page 9, lines 3-10; page 17, lines 9-10; page 17, lines 12-23; page 11, lines 24-25; page 12, lines 17-20; page 17, lines 9-11; page 18, lines 17-18; page 21, lines 1-14; page 22, lines 15-18; page 30, lines 2-4; page 30, lines 22-26; page 32, lines 25-27; figures. 5, 6	1-3, 16-19, 30-33, 35-38, 50-54, 69, 70-74, 80, 81, 91-93, 111-116 ----- 25, 26, 168-174, 178-181 ----- 137-143, 153-160
Y	US 2002/0015679 A1 (KOTOV, NA et al.) February 7, 2002; abstract; paragraph [0062]	25, 26
Y	WO 1998/18961 A (ZENECA LTD) May 7, 1998; abstract; page 4, lines 3-4; page 13, lines 26-27	168-174, 178-181
A	US 2012/0107798 A1 (SANTANGELO, PJ) May 3, 2012; abstract; paragraph [0015], [0108], [0112]	137-143, 153-160
A	US 2008/0182336 A1 (ZHUANG, X et al.) July 31, 2008; abstract; paragraphs [0055], [0078]	137-143, 153-160
A	US 2013/0027518 A1 (MACKAY, JF et al.) January 31, 2013; abstract; paragraphs [0004], [0006], [0017], [0042]	185-230
A	WO 2013/010023 A2 (APERIO TECHNOLOGIES INC) January 17, 2013; abstract; paragraphs [38], [78]	185-230

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

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 December 2014 (19.12.2014)

Date of mailing of the international search report

06 JAN 2015

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/48977

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☒

in the international application as filed

☐

together with the international application in electronic form

☐

subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/48977

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-15, 20-24, 27-29, 34, 39-49, 55-68, 75-79, 82-90, 94-110, 117-136, 144-152, 161-167, 175-177, 182-184 .
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

----Please See Supplemental Page----

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US14/48977

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AZCONA, J et al. Development And Clinical Evaluation Of Automatic Fiducial Detection For Tumor Tracking In Cine Megavoltage Images During Volumetric Modulated Arc Therapy. Medical Physics, Vol. 40, No. 3, March 2013; pages 031708-1-031708-11; abstract; figure 5 legend. http://dx.doi.org/10.1118/1.4791646 .	185-230

-----Continued from Box No. III: Observations Where Unity of Invention Is Lacking:

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

Group I: Claims 1-3, 16-19, 25, 26, 35-38, 50-54, 69-74, 80, 81, 91-93 and 111-116 are directed toward a methods of detecting at least one target in a sample with at least one, or at least two BP-NA conjugate(s), where the conjugate is a protein-nucleic acid conjugate linked to a docking strand that is capable of transiently binding to a complementary labeled imager strand; wherein the protein is an antibody.

Group II: Claims 30-33 are directed toward an aptamer-nucleic acid conjugate, comprising a nucleic acid aptamer linked to a docking strand that is transiently bound to a complementary labeled imager strand.

Group III: Claims 137-143 and 153-160 are directed toward a method of determining a number or relative amount of targets in a test sample that comprises targets transiently bound directly or indirectly to labeled imager strands, the method comprising determining variable Td; and determining the relative amount of two or more test targets in the sample based on Td.

Group IV: Claims 168-174 and 178-181 are directed toward a single-stranded DNA probe comprising a target binding domain of about 20 nucleotides in length linked to a docking domain comprising at least one subdomain complementary to at least one labeled imager strand of about 4 to 30 nucleotides in length, and wherein the target binding domain is bound to a complementary domain of a single-stranded mRNA target strand.

Group V: Claims 185-230 are directed toward a computer, a non-transitory computer medium encoded with a plurality of instructions to perform a method, and a method of performing drift correction for a plurality of images.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a protein-nucleic acid conjugate, which is not present in any other Group, the special technical features of Group II including a nucleic acid aptamer, which is not present in any other group; the special technical features of Group III including determining variable Td; and determining the relative amount of two or more test targets in the sample based on Td, which are not present in any other Group, the special technical features of Group IV including a single-stranded DNA probe bound to a complementary domain of a single-stranded mRNA target strand, which is not present in any other Group, the special technical features of Group V including drift correction for a plurality of images.

Groups I-V share the technical features including being related to images or imaging. Groups I-IV share the technical features including a labeled imager strand. Groups I-III share the technical features including a binding-partner nucleic acid (BP-NA) conjugate comprising a docking strand that is transiently bound to a complementary labeled imager strand. Groups II and IV share the technical features including nucleic acids linked to a docking strand or domain.

However, these shared technical features are previously disclosed by the publication entitled 'Single-Molecule Kinetics And Super-Resolution Microscopy By Fluorescence Imaging Of Transient Binding On DNA Origami' by Jungmann, et al. (hereinafter 'Jungmann').

Jungmann discloses images or imaging (imaging; abstract); a labeled imager strand (a red ATTO655 labeled imager strand (a labeled imager strand); figure 1 legend); a binding-partner nucleic acid (BP-NA) conjugate (a long rectangular DNA origami structure (LRO)(a binding partner nucleic acid (BP-NA) conjugate); figure 1, legend) comprising a docking strand (comprising a docking strand; figure 1, legend) that is transiently bound (transient binding events (transiently bound); figure 1, legend) to a complementary labeled imager strand (to a hybridizable red ATTO655 imager strand (to a complementary labeled imager strand); figure 1, legend); and nucleic acids (long rectangular DNA origami structures (LROs)(nucleic acids); figure 1, legend) linked to a docking strand or domain (comprising a docking strand (linked to a docking strand or domain); figure 1, legend).

Since none of the special technical features of the Groups I-V inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Jungmann reference, unity of invention is lacking.