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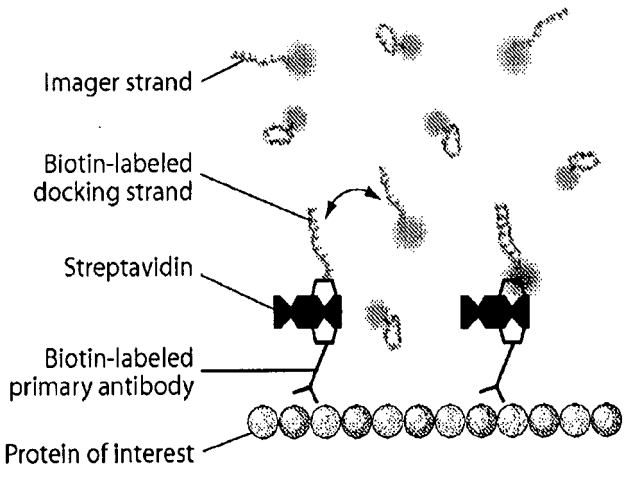

Fig. 2

(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 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 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, 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 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 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, 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 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.

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 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 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 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 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.

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 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 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 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.

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.

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 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 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 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 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.

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 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.

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.

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.

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 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 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.

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.

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.

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 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 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 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 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.

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.

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%.

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 at least one, 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 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.

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 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 a 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.

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 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 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.

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.

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, 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 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.

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 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.

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.

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.

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 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.

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, 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 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 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 ± 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 μm]. 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 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 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), 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.

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

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

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, thiacarbocyanine and merocyanine), naphthalene derivatives (*e.g.*, dansyl and prodan derivatives), coumarin derivatives, oxadiazole derivatives (*e.g.*, pyridyloxazole, nitrobenzoxadiazole and benzoxadiazole), pyrene derivatives (*e.g.*, cascade blue), oxazine derivatives (*e.g.*, Nile red, Nile blue, cresyl violet and oxazine 170), acridine derivatives (*e.g.*, proflavin, acridine orange and acridine yellow), arylmethine derivatives (*e.g.*, auramine, crystal violet and malachite green), and tetrapyrrole derivatives (*e.g.*, porphin,

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

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

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, 10^5, 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, 10^5, 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 additional 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.

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-labled 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-labled imager strand and the ATTO655-labled imager strand are present at the same time in solution with the sample, imaging is carried our 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 last 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). 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 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 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 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 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), 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, Adrenocorticotropic hormone (ACTH), Aequorin, Aflatoxin, Agar, Alamethicin, Alanine, Albumins, Aldosterone, Aleurone, Alpha-amanitin, Allantoin, Allethrin, α-Amanatin, Amino acid, Amylase, Anabolic steroid, Anethole, Angiotensinogen, Anisomycin, Antidiuretic

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

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

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

In some embodiments, a target 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 nicotinic acetylcholine receptors and GABAa receptors, and voltage-gated ion channels such as potassium, calcium and sodium channels), synport/antiport proteins (*e.g.*, glucose transporter); hormones and growth factors (*e.g.*, epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), peptide hormones such as insulin, insulin-like growth factor and oxytocin, and steroid hormones such as androgens, estrogens and progesterones); receptors such as transmembrane receptors (*e.g.*, G-protein-coupled receptor, rhodopsin) and intracellular receptors (*e.g.*, estrogen receptor); DNA-binding proteins (*e.g.*, histones, protamines, CI protein); transcription regulators (*e.g.*, c-myc, FOXP2, FOXP3, MyoD and P53); immune system proteins (*e.g.*, immunoglobulins, major histocompatibility antigens and T cell receptors); nutrient storage/transport proteins (*e.g.*, ferritin); chaperone proteins; and enzymes.

In some embodiments, a target 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}:

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

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

The kinetic parameters k_{on} and k_{off} are 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:

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

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.

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 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 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 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 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 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

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 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 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 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, 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 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 targes, 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.

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 FWHM ~= sigma*2.35 for a Gaussian distribution, where sigma 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 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 μm.

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.

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

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).

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 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 assessed by characterizing the Point Spread Function (PSF) of a single binding site. A statistical overlay of images of more than 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 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 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 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.

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 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.

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 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, 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 1μm in offset), which effectively removes interference between different drift markers and allows for incorporation of the next stage. As discussed above, in 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).

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.

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 (sigma) 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.

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

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 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 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

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 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 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 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 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 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 methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

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

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 a8, 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 $1 - 20\ s^{-1}$, 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 compromised 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 µl

- Rinse flow chamber with 100 µl 1 M KOH
- Rinse flow chamber with 100 µl buffer A twice
- Incubate for 5 min
- Rinse flow chamber with 100 µl buffer A
- Rinse flow chamber with 50 µl 1mg/ml BSA-Biotin in buffer A
- Incubate for 2 min
- Rinse flow chamber with 50 µl 1mg/ml BSA-Biotin in buffer A
- Incubate for 2 min
- Rinse flow chamber with 100 µl buffer A twice
- Rinse flow chamber with 50 µl 0.5 mg/ml Streptavidin in buffer A
- Incubate for 2 min
- Rinse flow chamber with 50 µl 0.5 mg/ml Streptavidin in buffer A
- Incubate for 2 min
- Rinse flow chamber with 100 µl buffer A twice
- Rinse flow chamber with 100 µl buffer B twice
- Incubate for 30 min
- Rinse flow chamber with 100 µl buffer B twice
- Rinse flow chamber with 50 µl 1nM origami in buffer B
- Incubate for 10 min
- Rinse flow chamber with 100 µl buffer B twice
- Attach tubing
- 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 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 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-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 Apo TIRF 100×, NA 1.49, Oil). For 2D imaging an additional 1.5 magnification was used to obtain a final magnification of ≈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, 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).

DNA origami self-assembly. The microtubule-like DNA origami structures were formed in a one-pot reaction with 40 µl total volume containing 10 nM scaffold strand (p8064), 500 nM folding staples and biotin handles, 750 nM biotin anti-handles and 1.1 µM DNA-PAINT docking strands in folding buffer (1×TAE Buffer with 20 mM $MgCl_2$). 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 $MgCl_2$, 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 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-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 (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 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, 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 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 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; 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 μg/ml in 5 % BSA); 3-times washing with PBS; post-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 glass slide (3×1 inch2, 1 mm thick) were sandwiched together by two strips of double-sided tape to form a flow chamber with inner volume of ≈20 μl. First, 20 μl of biotin-labeled bovine albumin (1 mg/ml, dissolved in buffer A) was flown into the chamber and incubated for 2 min. The chamber was then washed using 40 μl of buffer A. 20 μl of streptavidin (0.5 mg/ml, dissolved in buffer A) was then flown through the chamber and allowed to bind for 2 min. After washing with 40 μl of buffer A and subsequently with 40 μl of buffer B, 20 μl 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 μl of buffer B. The final imaging buffer solution contained 1.5 nM Cy3b-labeled imager strands in buffer B. The chamber was 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 bandwith 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).

Transmission electron microscopy imaging. For imaging, 3.5 µl 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 µm filter) uranyl formate solution containing 25 mM NaOH. Imaging was performed using a JEOL JEM-1400 operated at 80 kV.

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 µm, 0.38 N/m force constant cantilever. After self-assembly of the origami structure ≈20 µl 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 µl of the origami solution was deposited onto the mica surface. After another 30 s, 30 µl 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 ≈2, and proportional gains ≈4.

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 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

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–TTTCTTCATTA–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–TTTCTTCATTA–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 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.

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.

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 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.

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 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.

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.

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

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.

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

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.

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 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.

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.

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.

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

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

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.

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

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.

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.

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.

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.

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 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.

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

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.

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.

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.

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.

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

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 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;

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.

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;

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

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.

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.

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.

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.

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.

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.

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.

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.

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 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 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.

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 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 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.

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.

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.

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.

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.

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.

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 claim105, wherein the fluorescently-labeled imager strand is about 8 to about 10 nucleotides in length.

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

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.

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..

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.

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

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;

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:

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;

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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.

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;

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

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.

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.

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.

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.

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 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.

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 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.

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 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.

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.

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.

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.

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:

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 a 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.

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 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.

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 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 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.

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 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:

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 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.

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:

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.

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.

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

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 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 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

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 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.

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;

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.

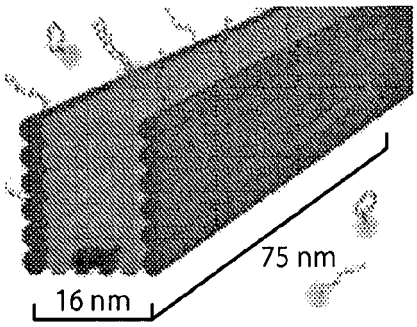


Fig. 1A

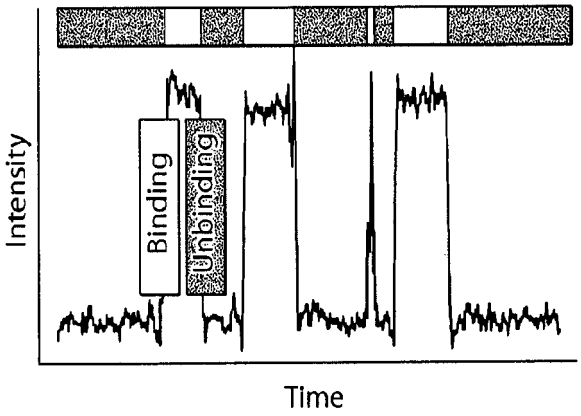


Fig. 1B

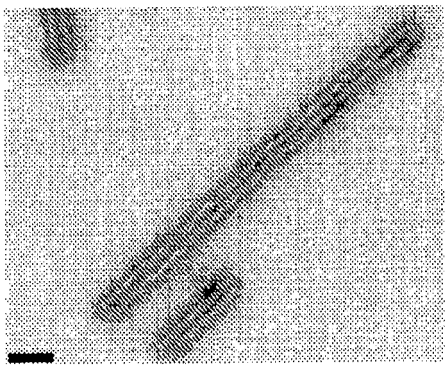


Fig. 1C

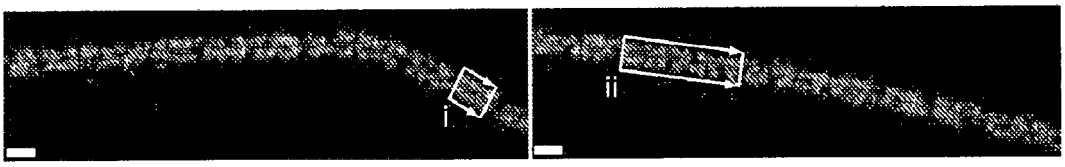

i
ii

Fig. 1D

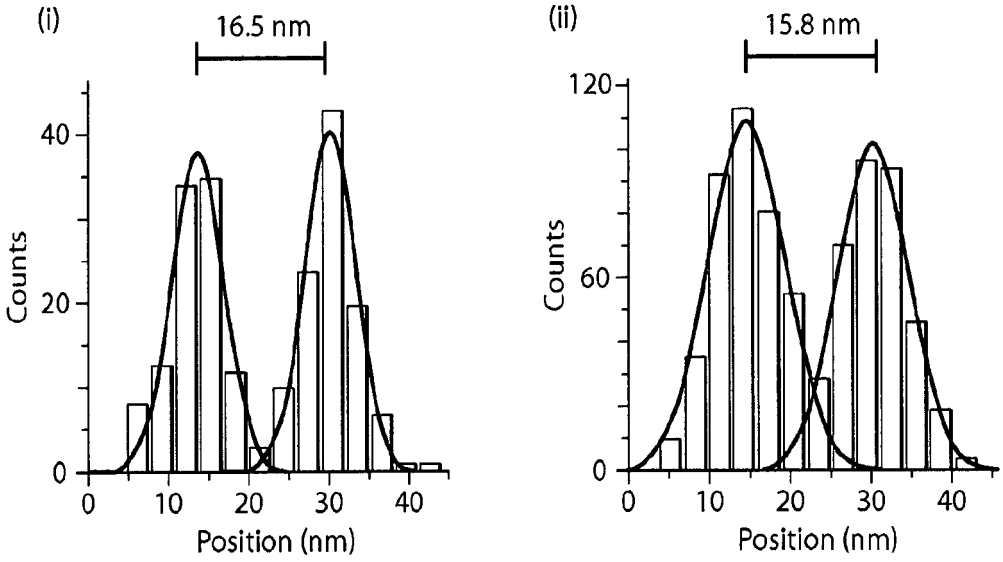

(i)
16.5 nm
40
20
0
Counts
0
10
20
30
40
Position (nm)
(ii)
15.8 nm
120
60
0
Counts
0
10
20
30
40
Position (nm)

Fig. 1E

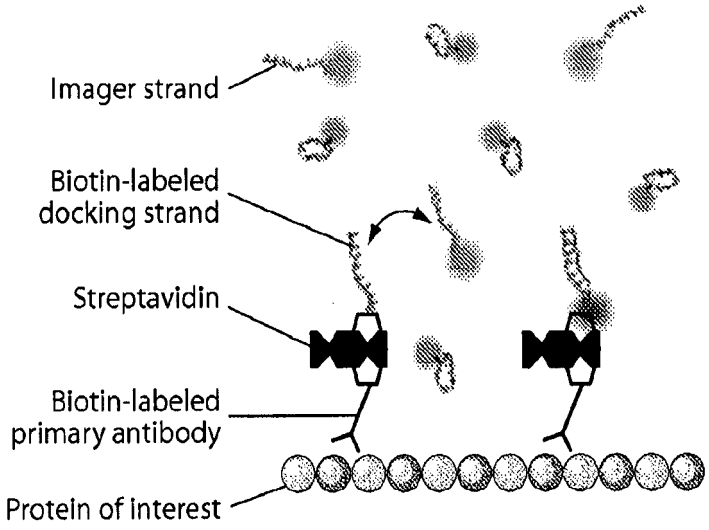

Imager strand
Biotin-labeled docking strand
Streptavidin
Biotin-labeled primary antibody
Protein of interest

Fig. 2

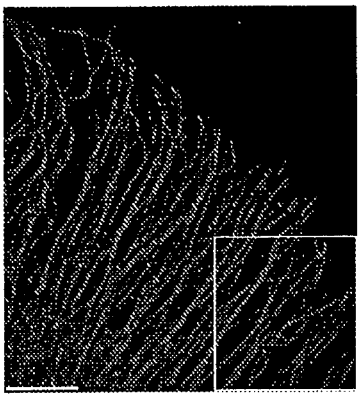


Fig. 3A

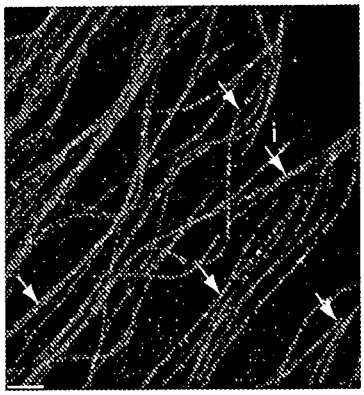


Fig. 3B

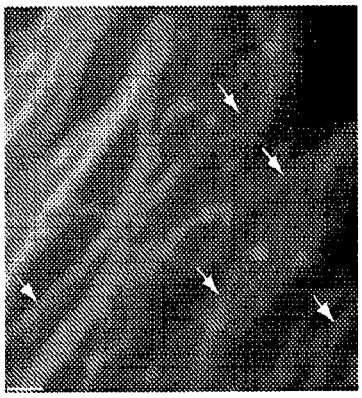


Fig. 3C

Fig. 3D

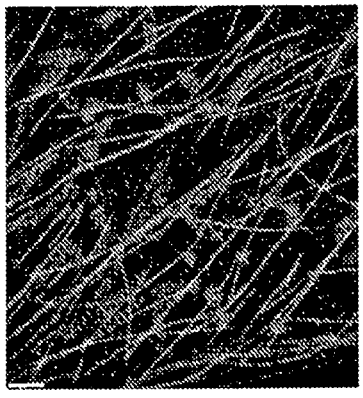


Fig. 3E

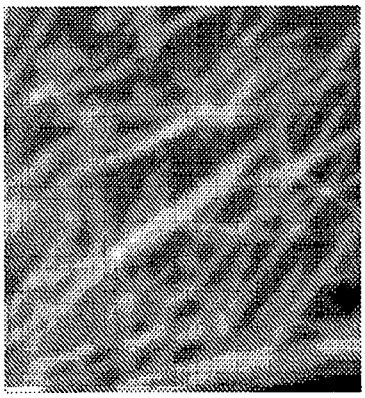


Fig. 3F

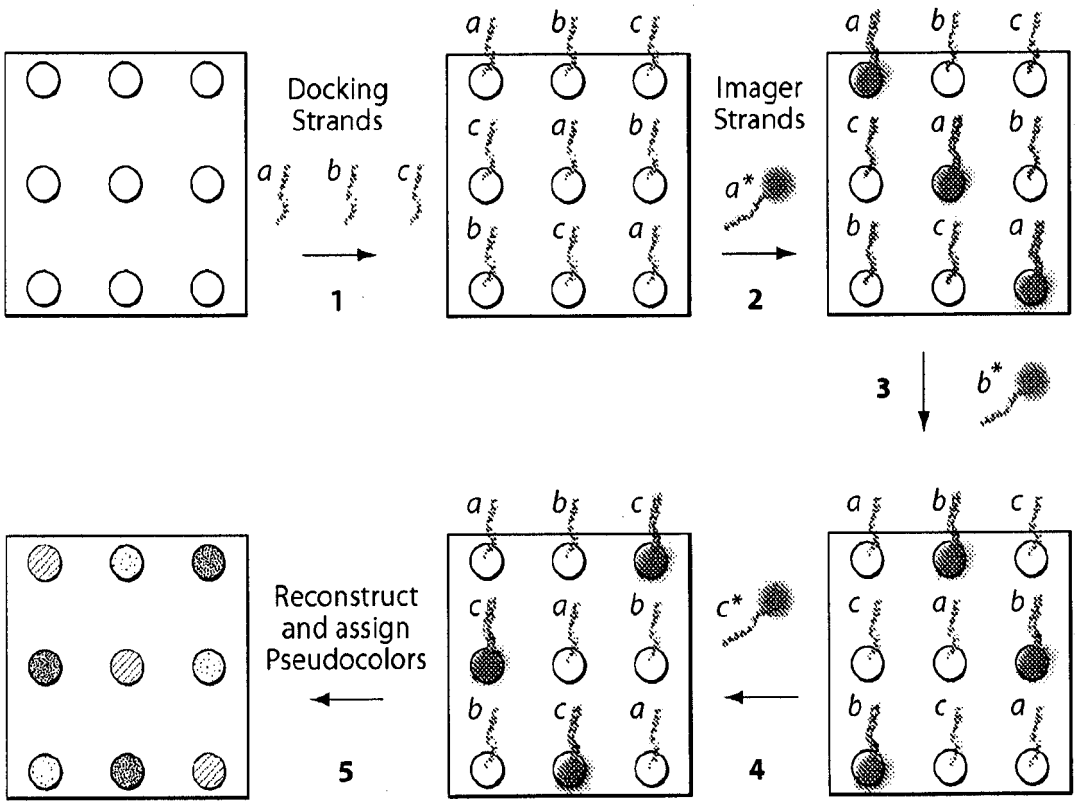

Docking Strands
a
b
c
1
Imager Strands
a*
2
3
b*
4
c*
Reconstruct and assign Pseudocolors
5

Fig. 4A

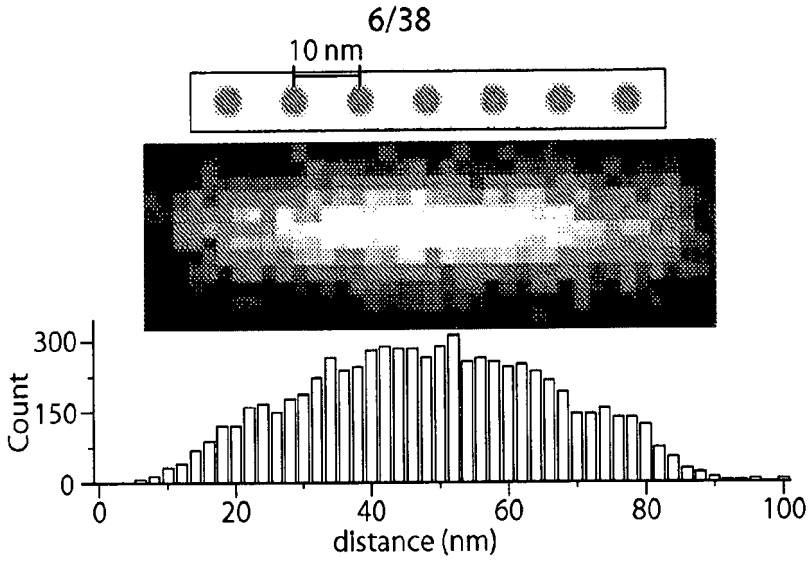


Fig. 4B1

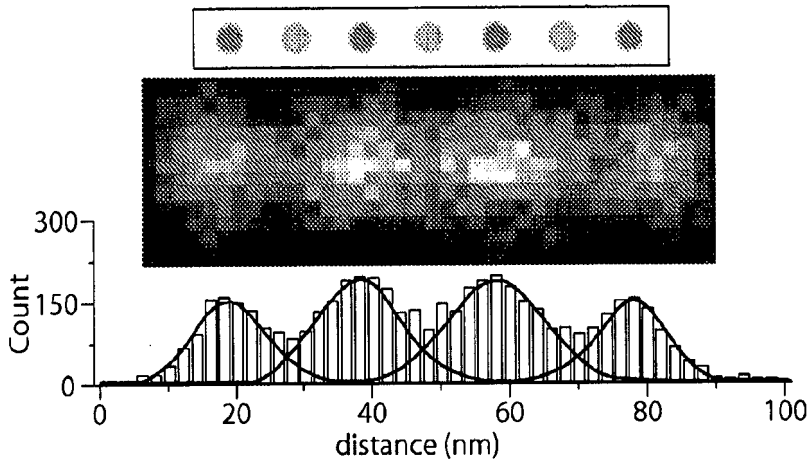


Fig. 4B2

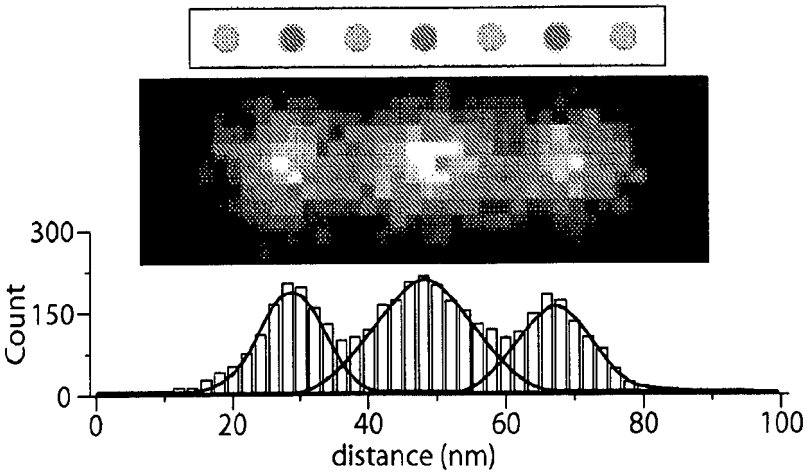
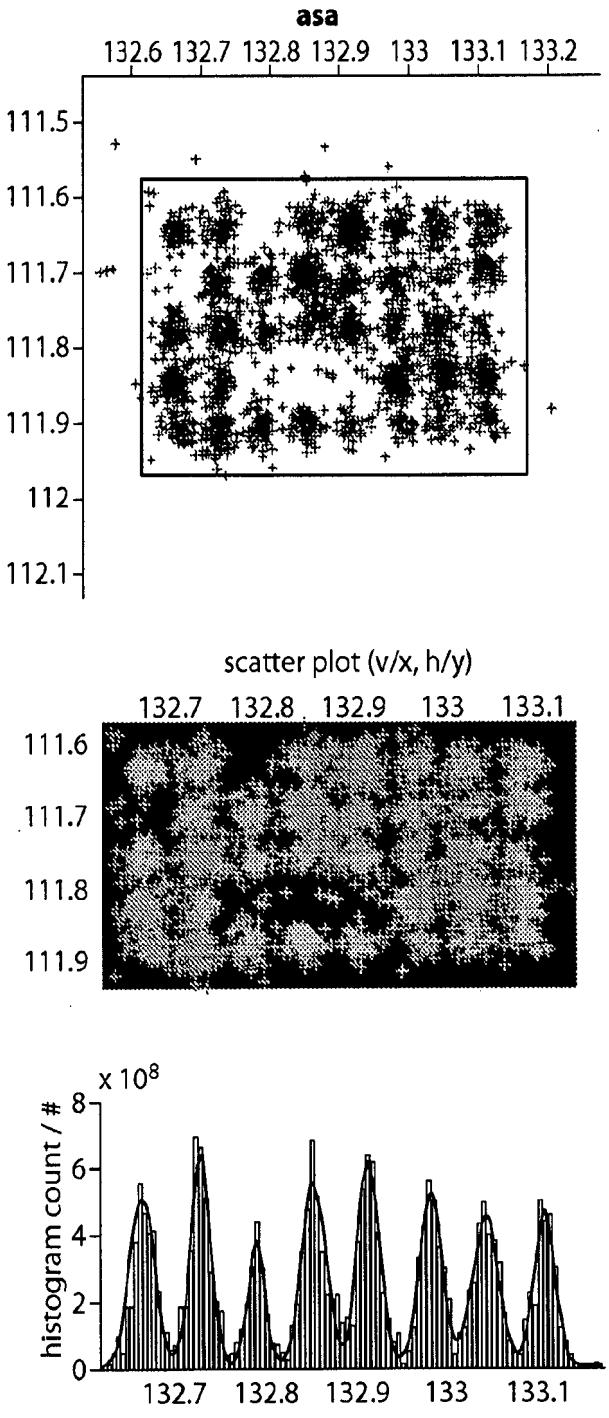


Fig. 4B3



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

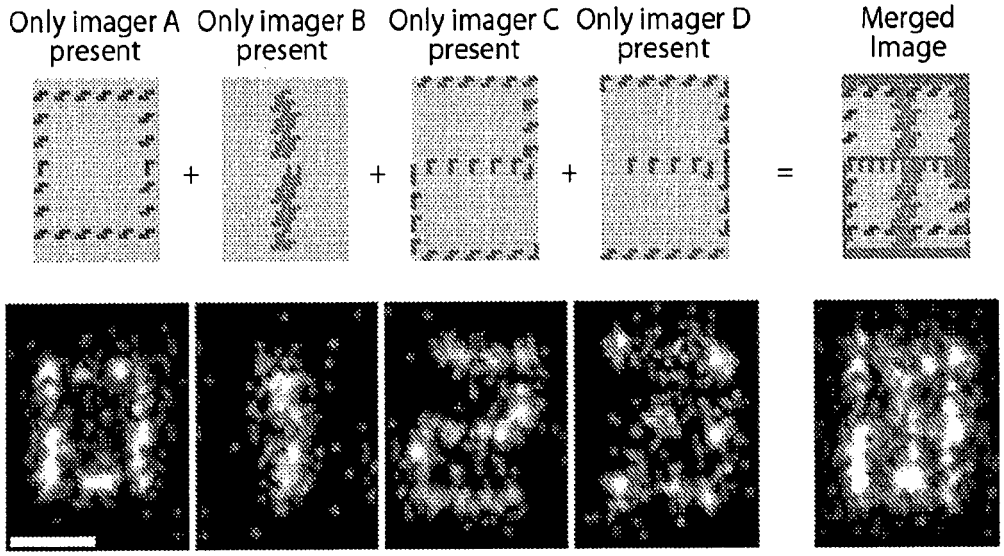

Only imager A present
Only imager B present
Only imager C present
Only imager D present
Merged Image
+
+
+
=

Fig. 5A

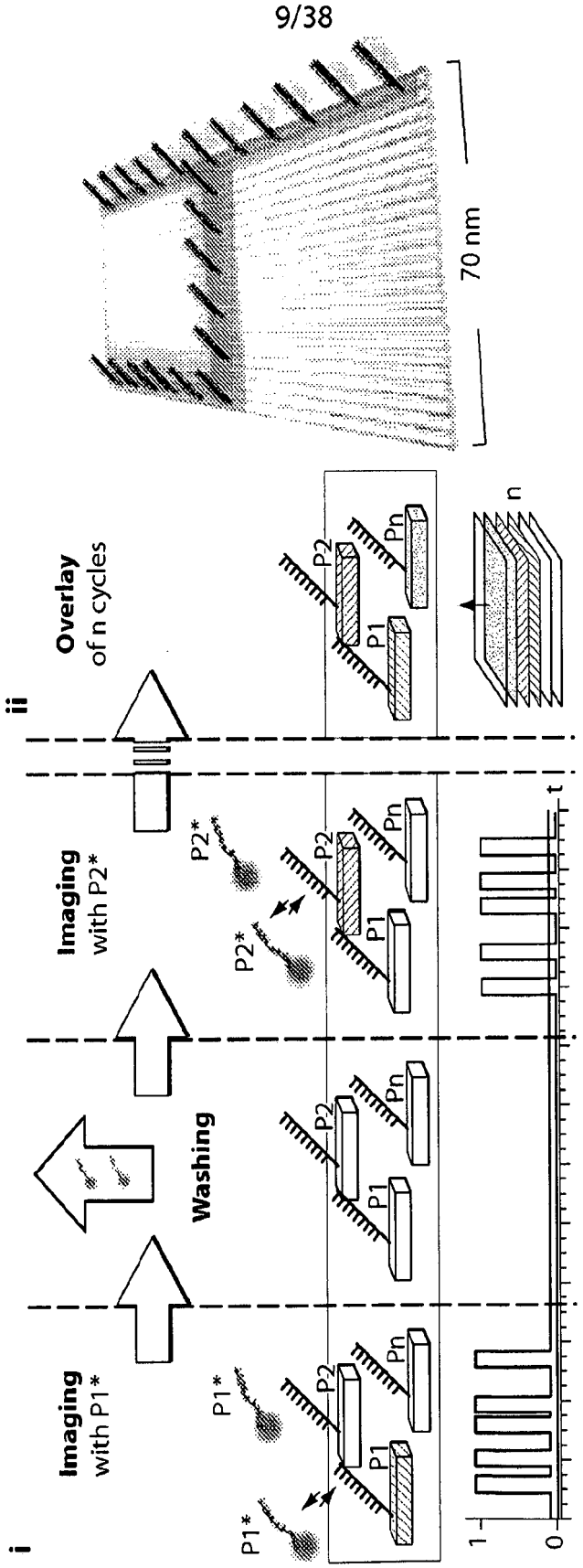

70 nm
i
ii
Imaging
with P1*
Washing
Imaging
with P2*
Overlay
of n cycles
P1
P2
Pn
P1*
P2*
n
t
1
0

Fig. 5B

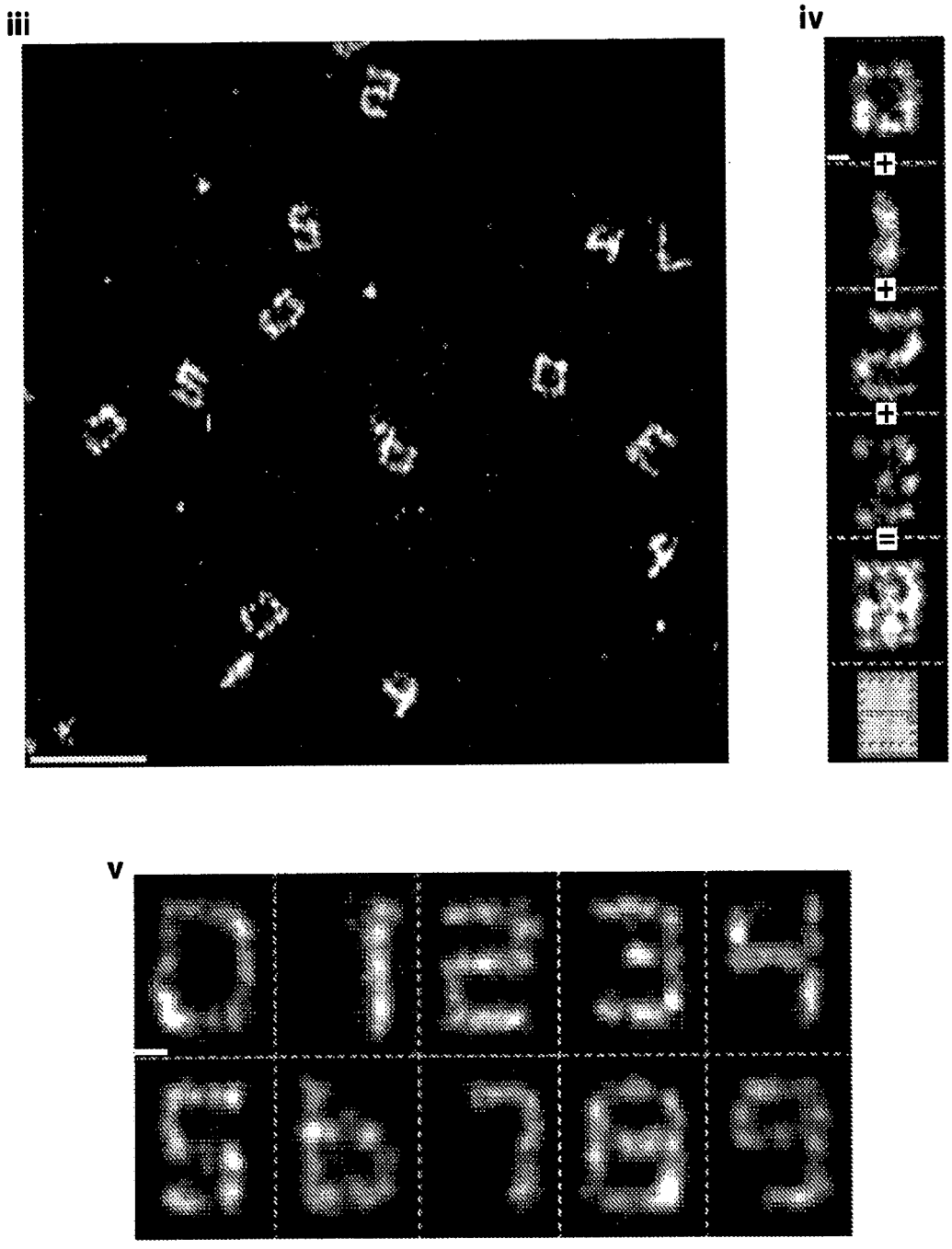


Fig. 5B (Continued)

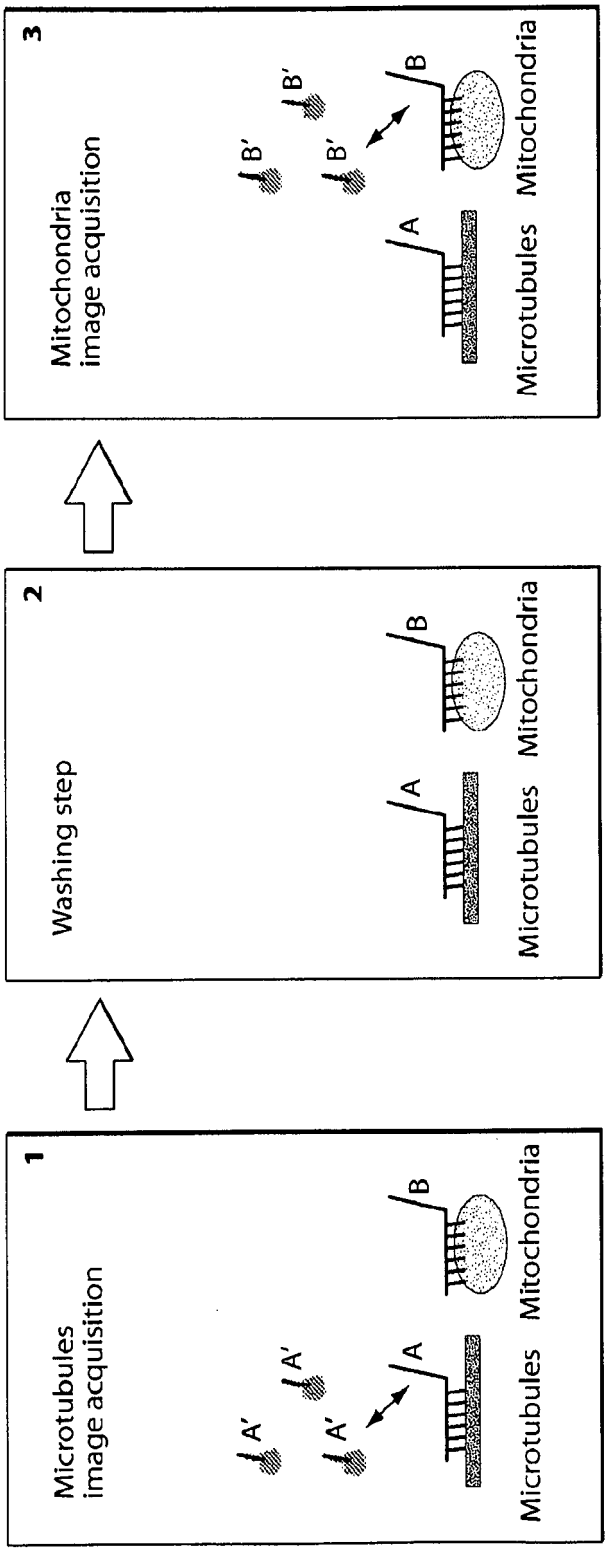

1
Microtubules
image acquisition
A'
A'
A'
A
B
Microtubules
Mitochondria
2
Washing step
A
B
Microtubules
Mitochondria
3
Mitochondria
image acquisition
B'
B'
B'
A
B
Microtubules
Mitochondria

Fig. 6A

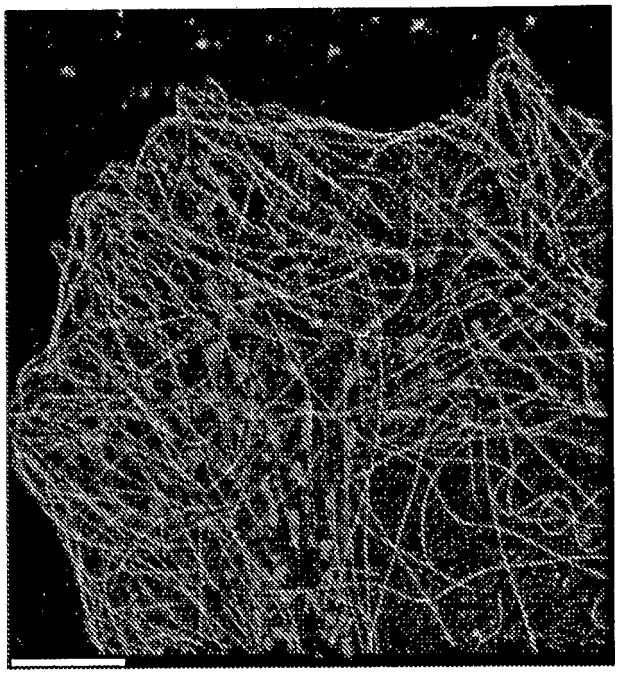


Fig. 6B

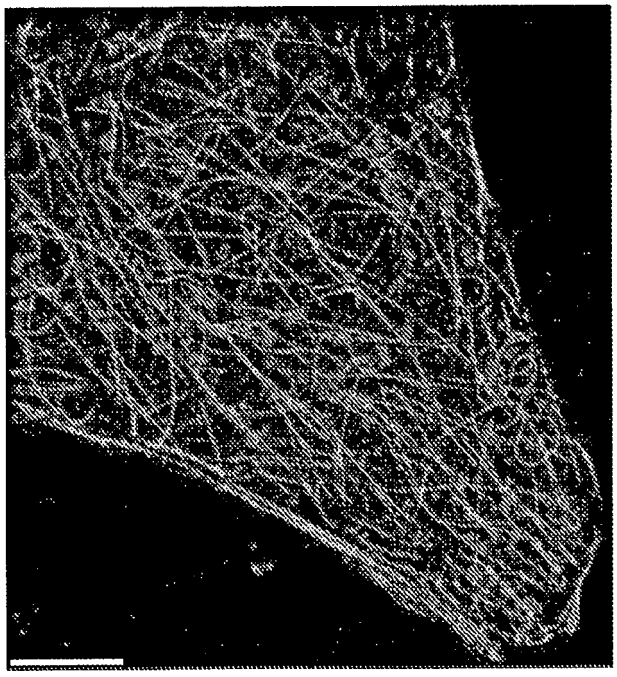


Fig. 6C

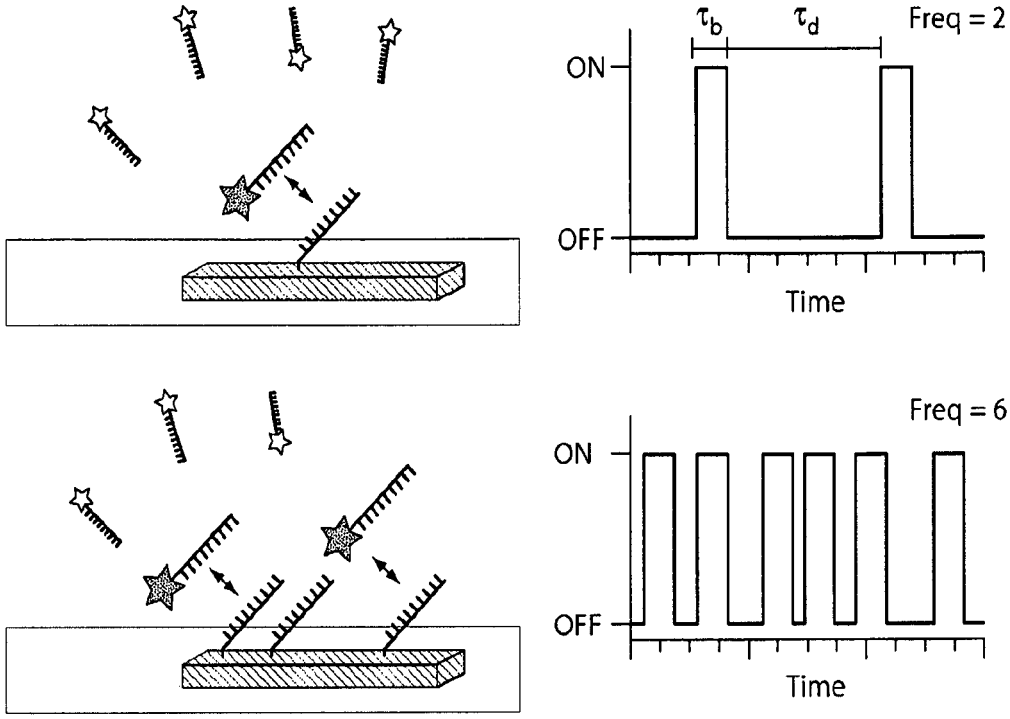

τ_b
τ_d
Freq = 2
ON
OFF
Time
Freq = 6
ON
OFF
Time

Fig. 7A

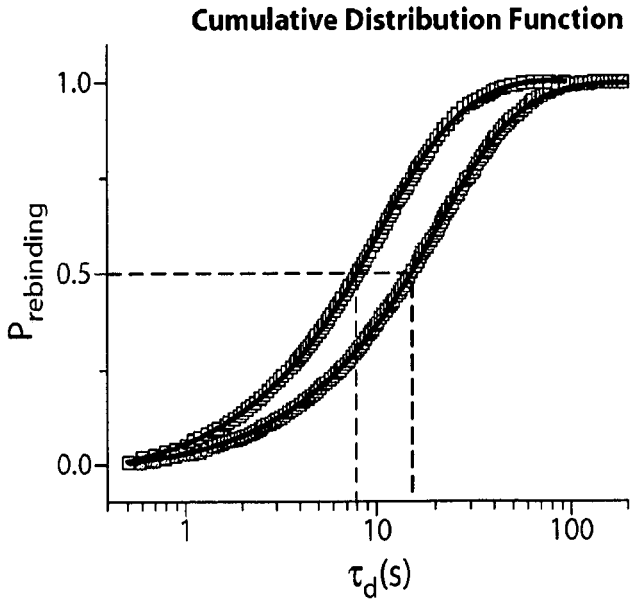

Cumulative Distribution Function
1.0
0.5
0.0
$P_{rebinding}$
1
10
100
τ_d(s)

Fig. 7B

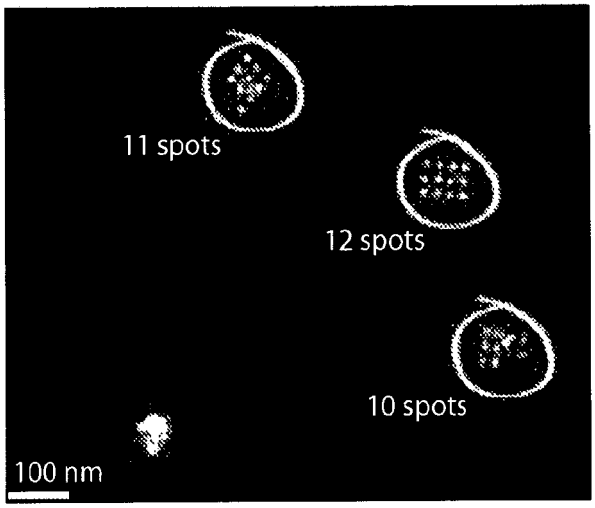


Fig. 7C

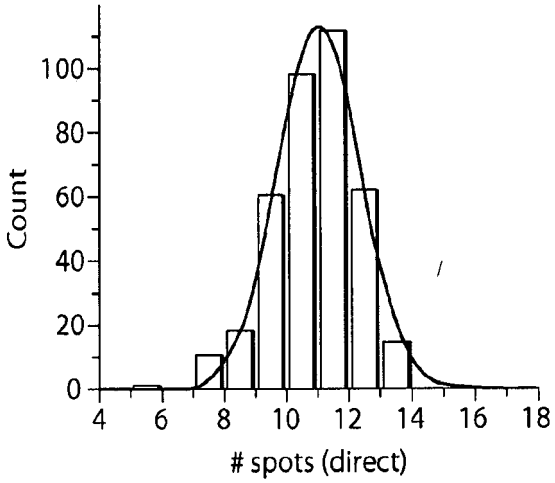


Fig. 7D1

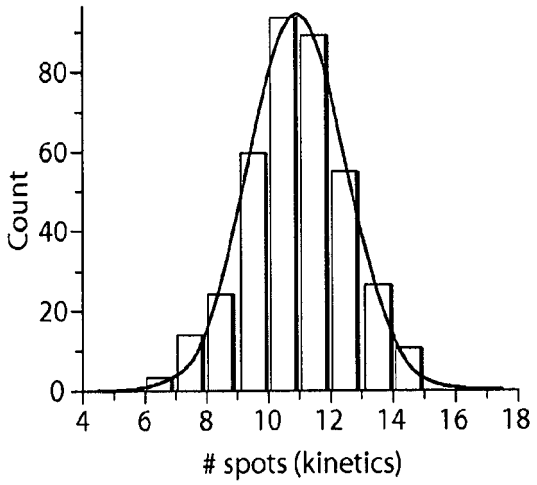


Fig. 7D2

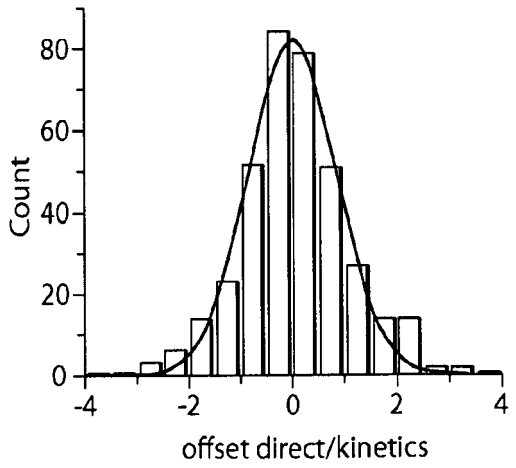


Fig. 7D3

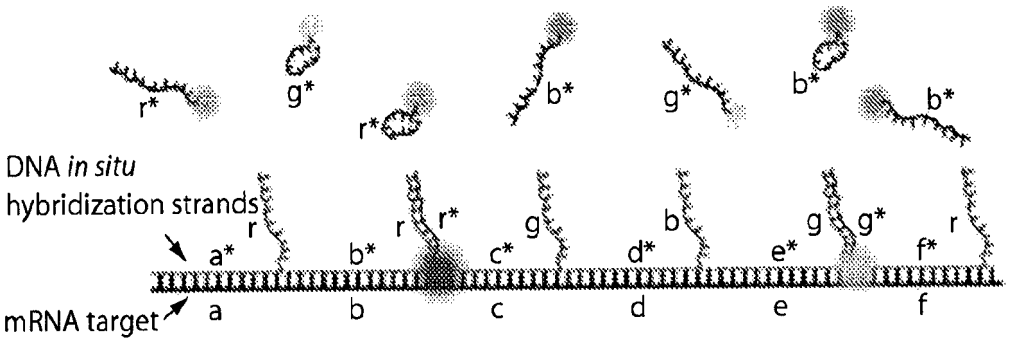

r*
g*
r*
b*
g*
b*
b*
DNA *in situ*
hybridization strands
r
r
r*
g
b
g
g*
r
a*
b*
c*
d*
e*
f*
mRNA target
a
b
c
d
e
f

Fig. 8A

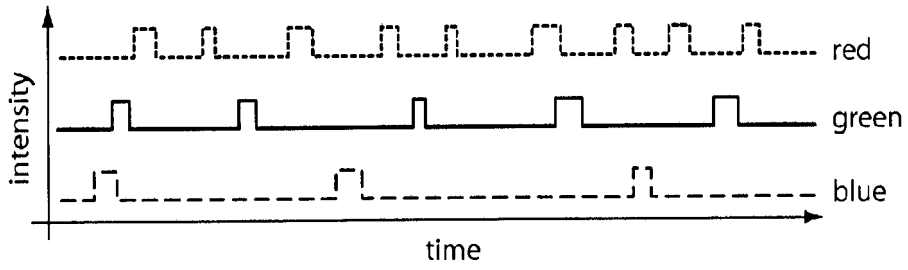

intensity
red
green
blue
time

Fig. 8B

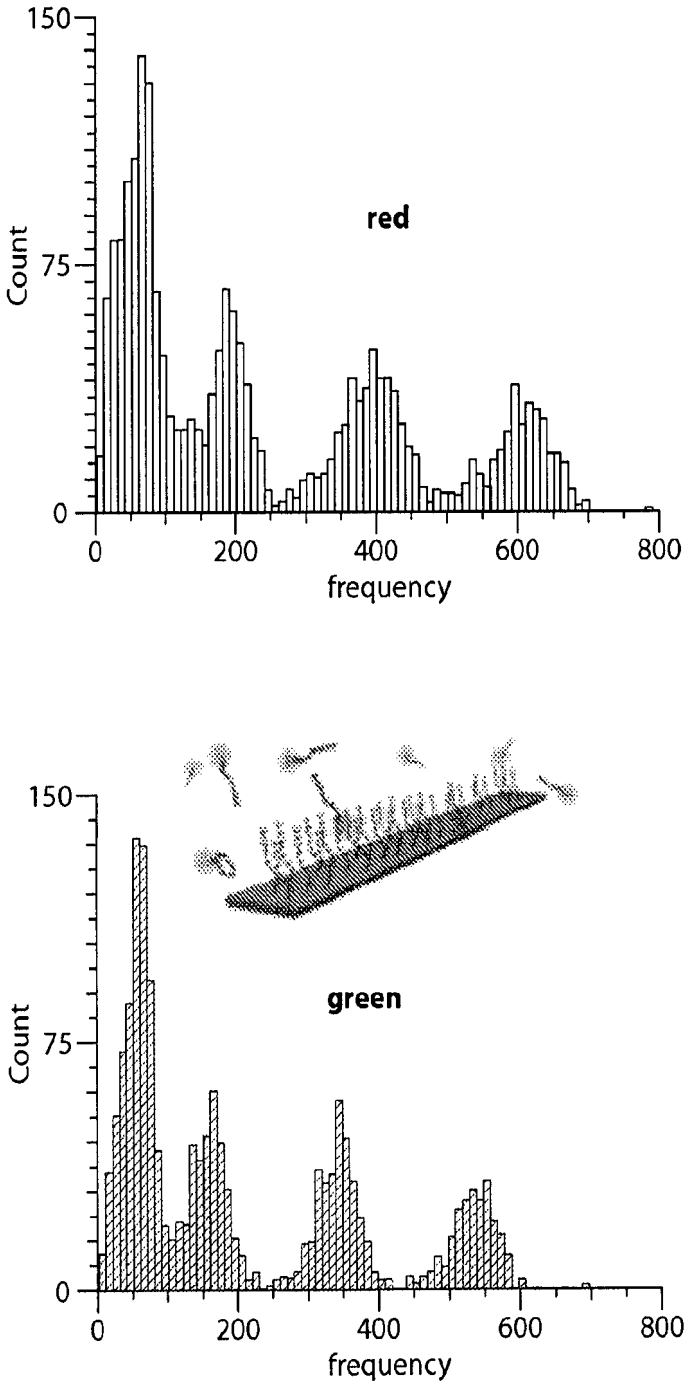

150
75
0
Count
red
0
200
400
600
800
frequency
150
75
0
Count
green
0
200
400
600
800
frequency

Fig. 8C

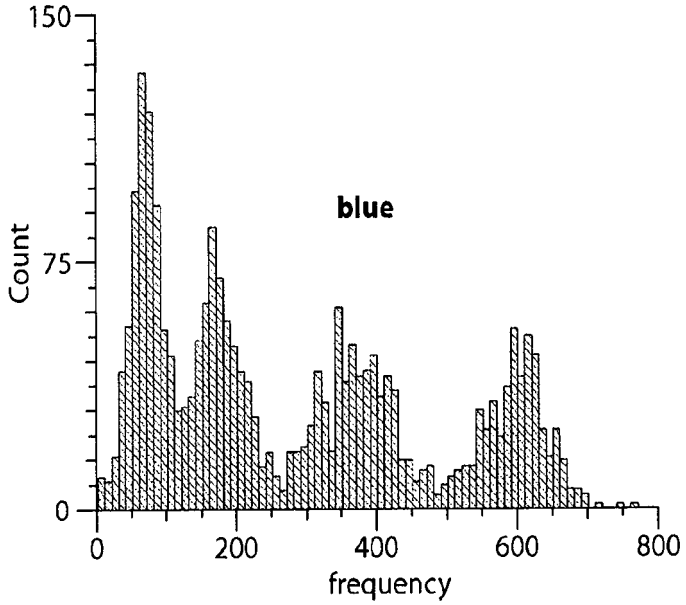


Fig. 8C (Continued)

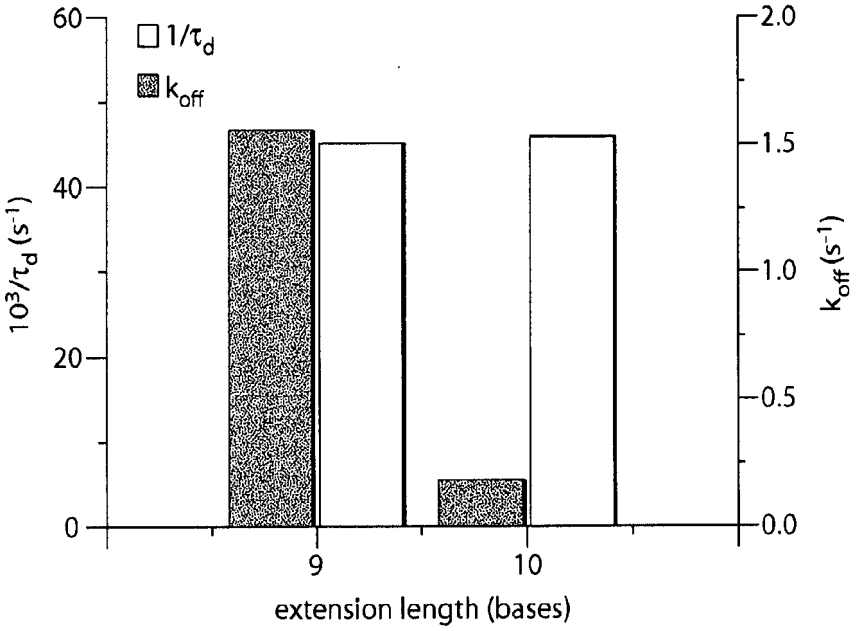

60
40
20
0
$10^3/\tau_d$ (s^{-1})
$1/\tau_d$
k_{off}
2.0
1.5
1.0
0.5
0.0
k_{off} (s^{-1})
9
10
extension length (bases)

Fig. 9

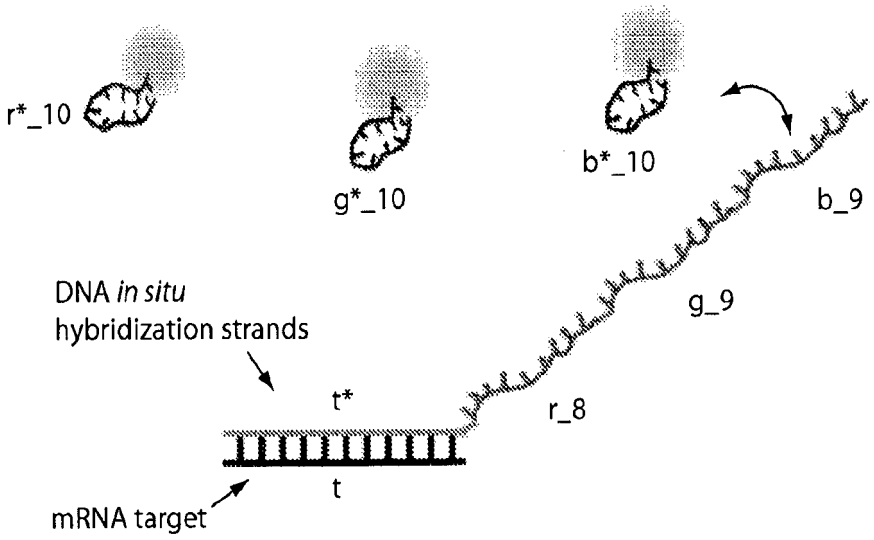


Fig. 10A

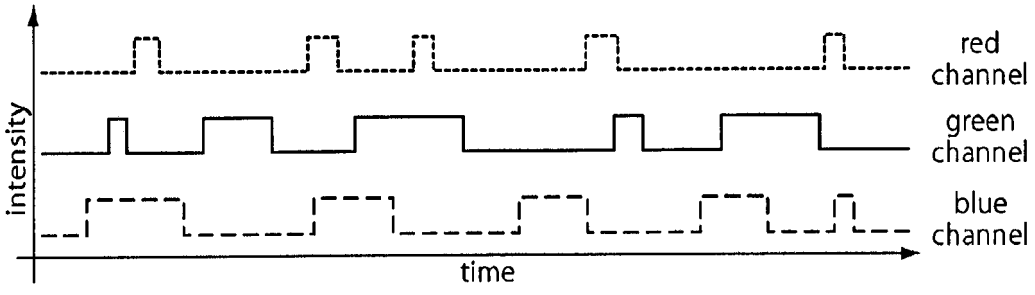


Fig. 10B

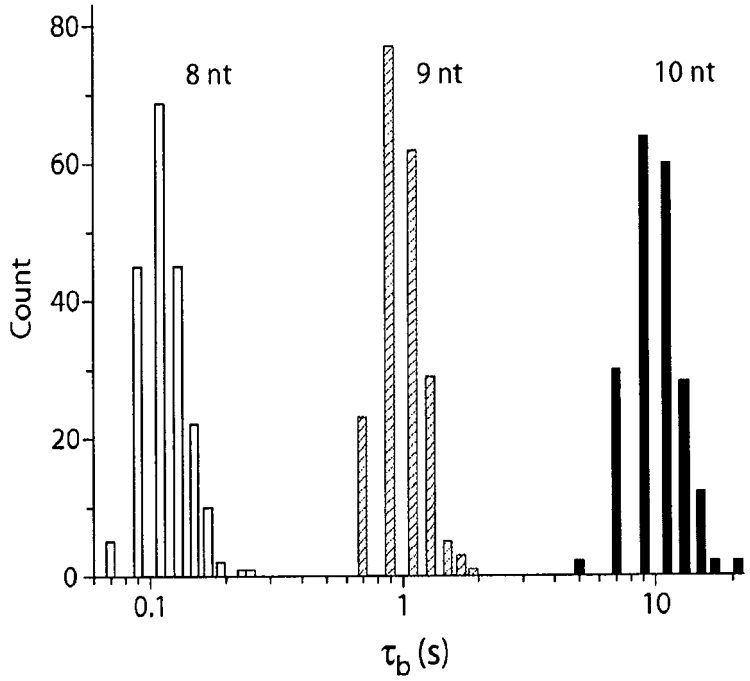


Fig. 10C

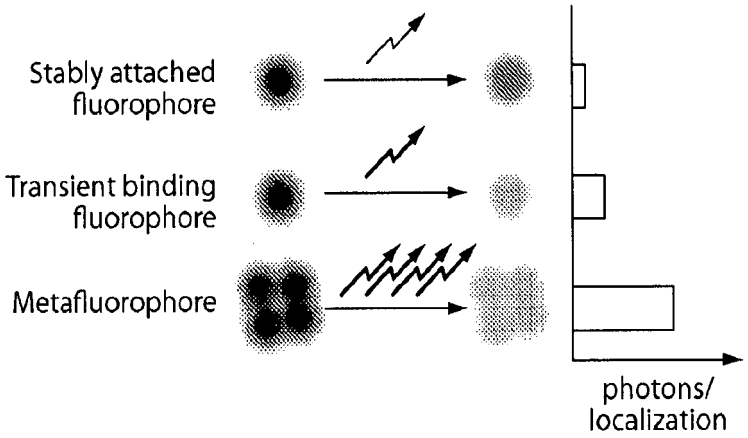


Fig. 11A

Fig. 11B1

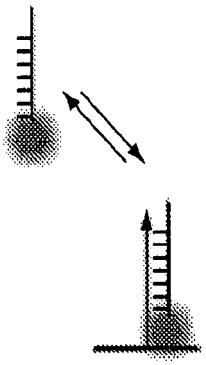


Fig. 11B2

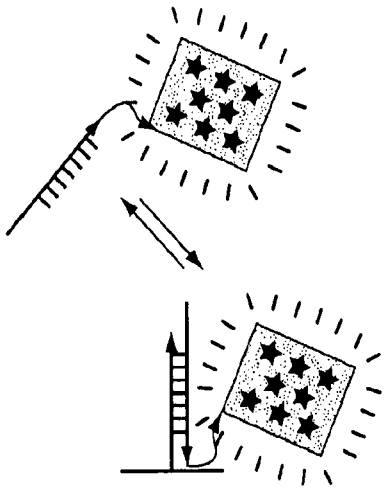


Fig. 11B3

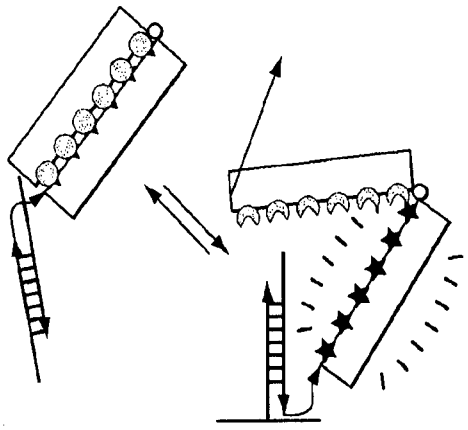


Fig. 11B4

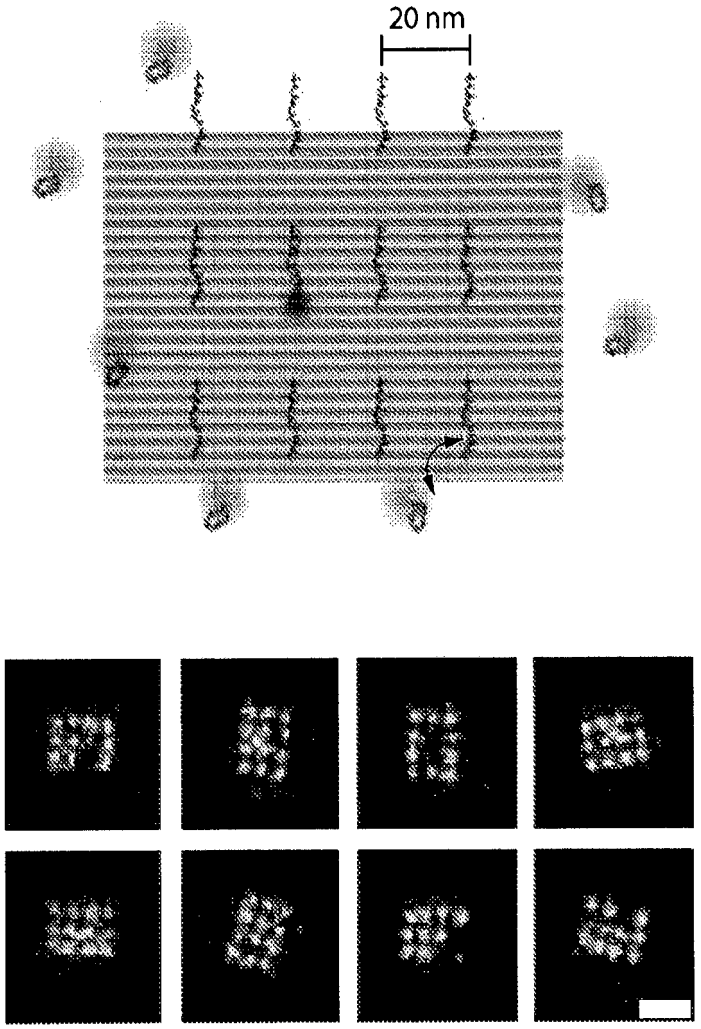


Fig. 11C

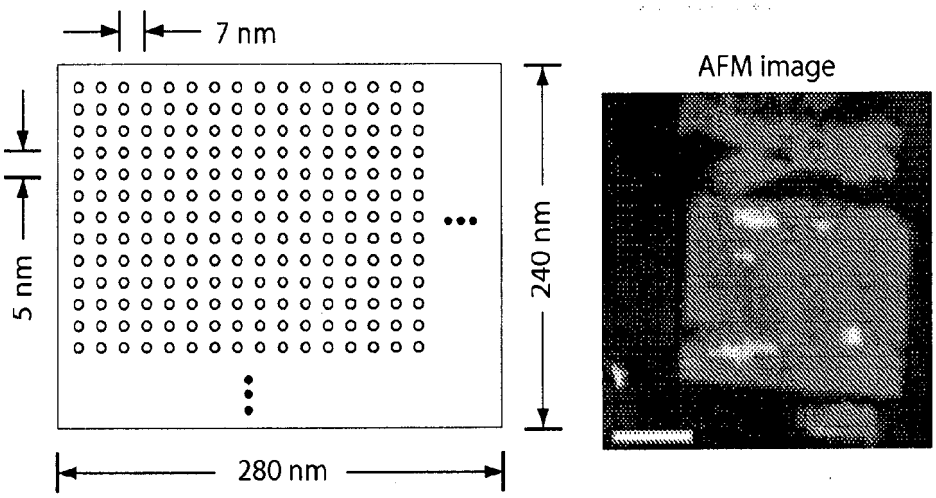


Fig. 11D

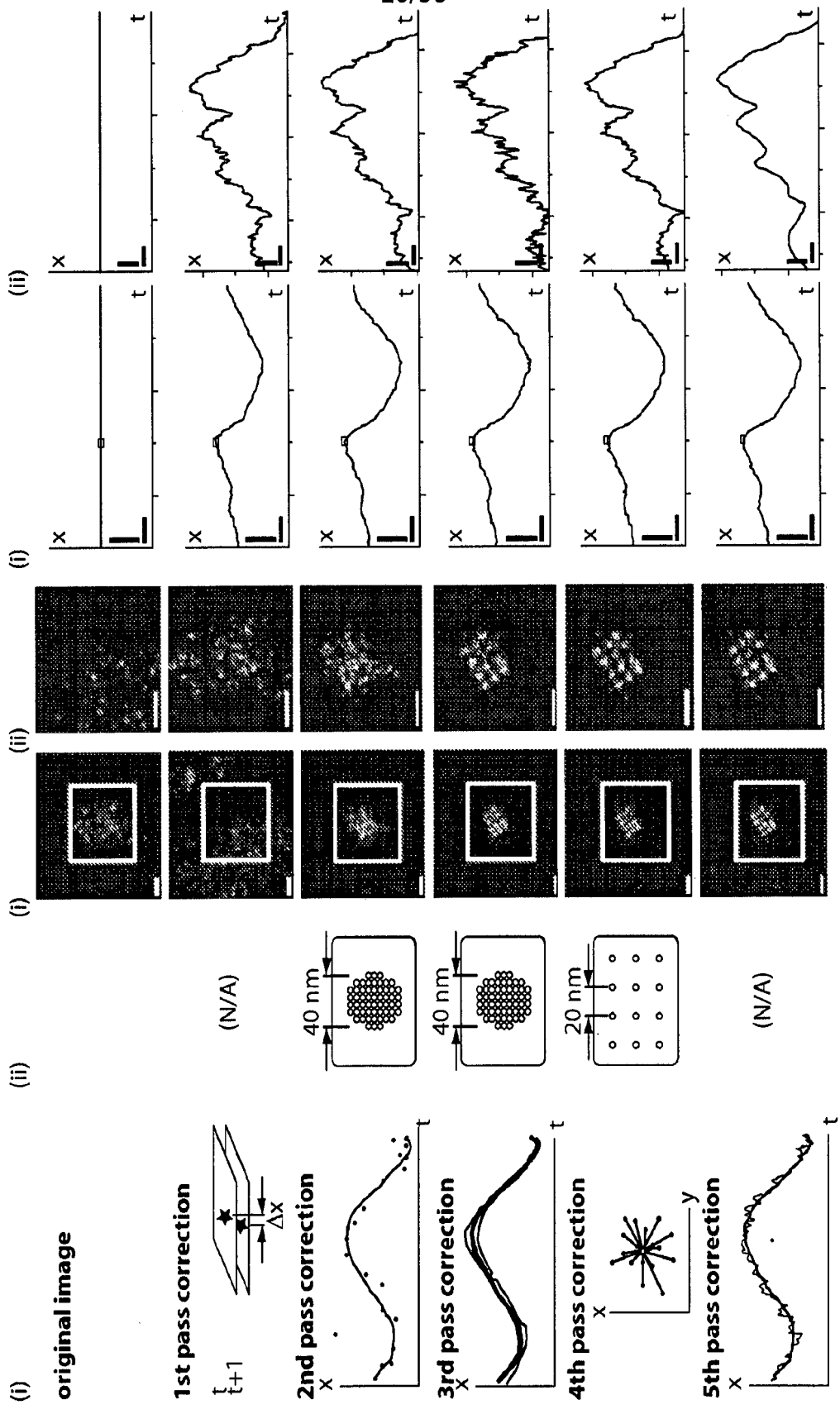


Fig. 12A

Fig. 12B

Fig. 12C

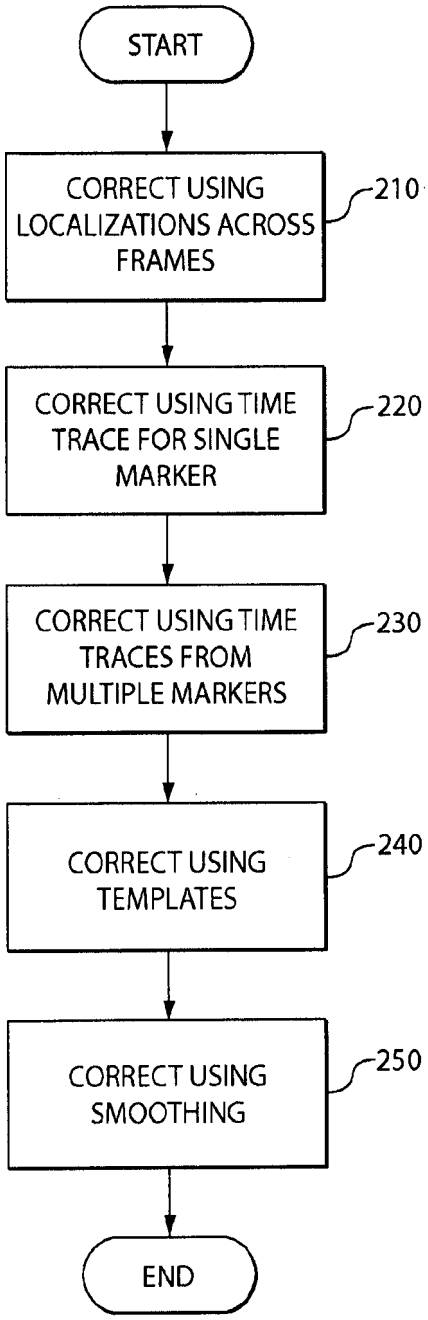

START
CORRECT USING LOCALIZATIONS ACROSS FRAMES
210
CORRECT USING TIME TRACE FOR SINGLE MARKER
220
CORRECT USING TIME TRACES FROM MULTIPLE MARKERS
230
CORRECT USING TEMPLATES
240
CORRECT USING SMOOTHING
250
END

Fig. 13

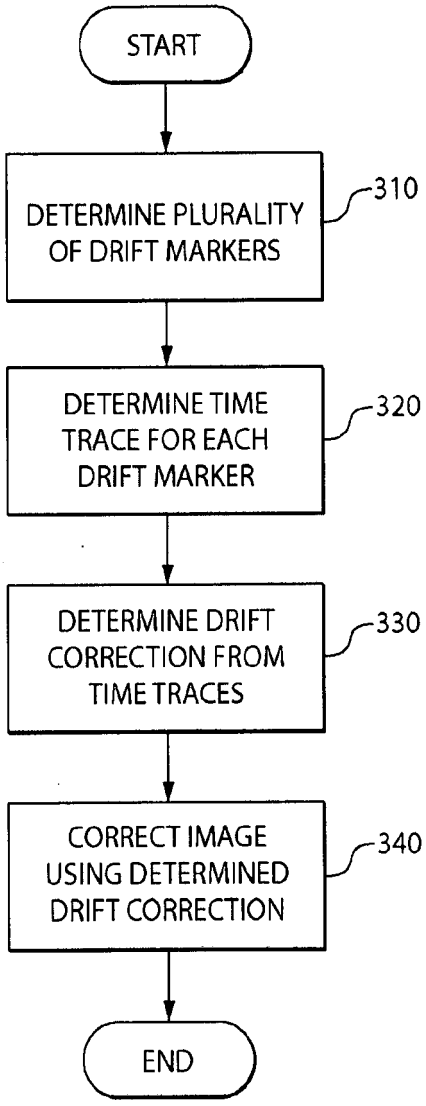

START
DETERMINE PLURALITY OF DRIFT MARKERS
310
DETERMINE TIME TRACE FOR EACH DRIFT MARKER
320
DETERMINE DRIFT CORRECTION FROM TIME TRACES
330
CORRECT IMAGE USING DETERMINED DRIFT CORRECTION
340
END

Fig. 14

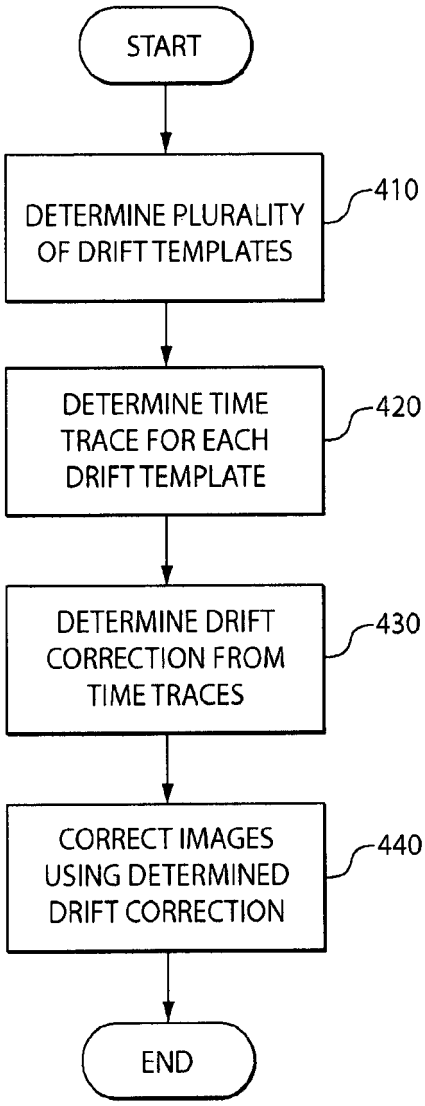

START
DETERMINE PLURALITY OF DRIFT TEMPLATES
410
DETERMINE TIME TRACE FOR EACH DRIFT TEMPLATE
420
DETERMINE DRIFT CORRECTION FROM TIME TRACES
430
CORRECT IMAGES USING DETERMINED DRIFT CORRECTION
440
END

Fig. 15

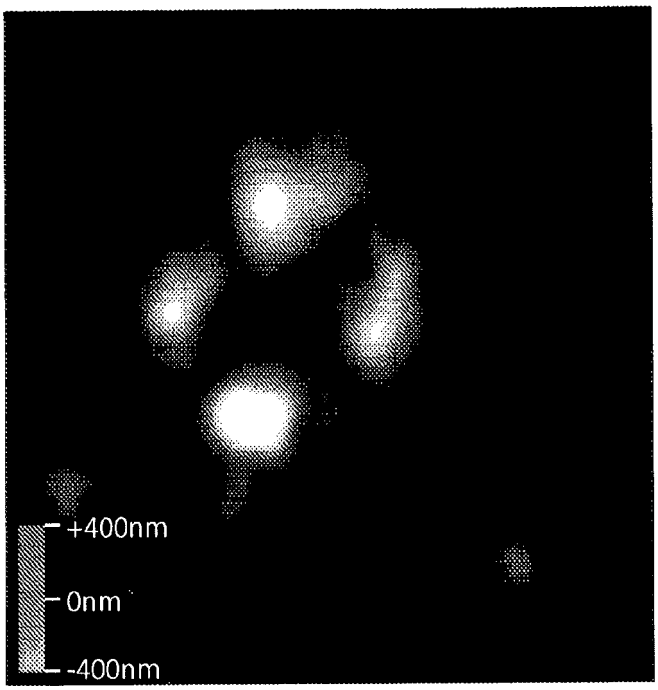


Fig. 16A

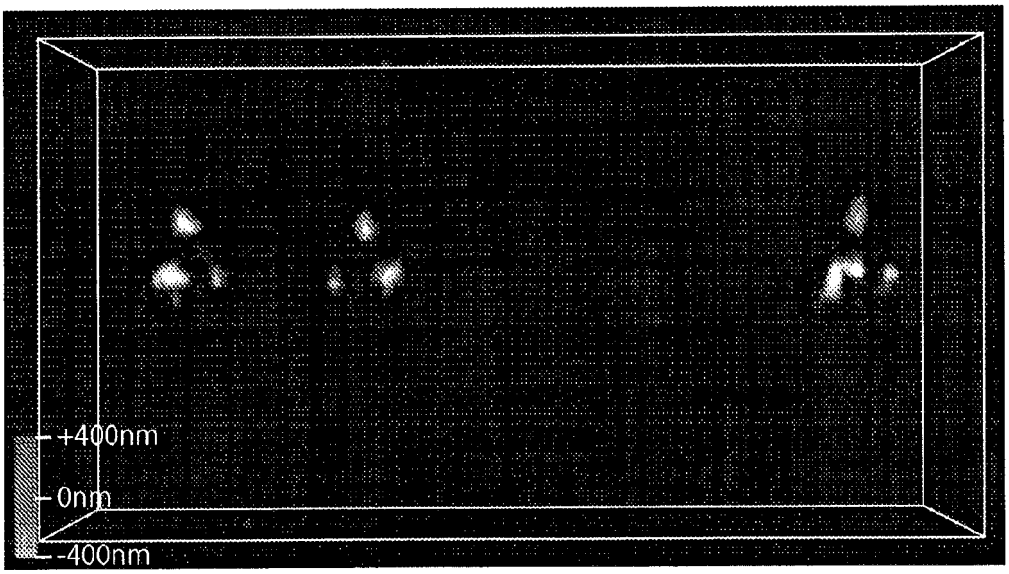


Fig. 16B

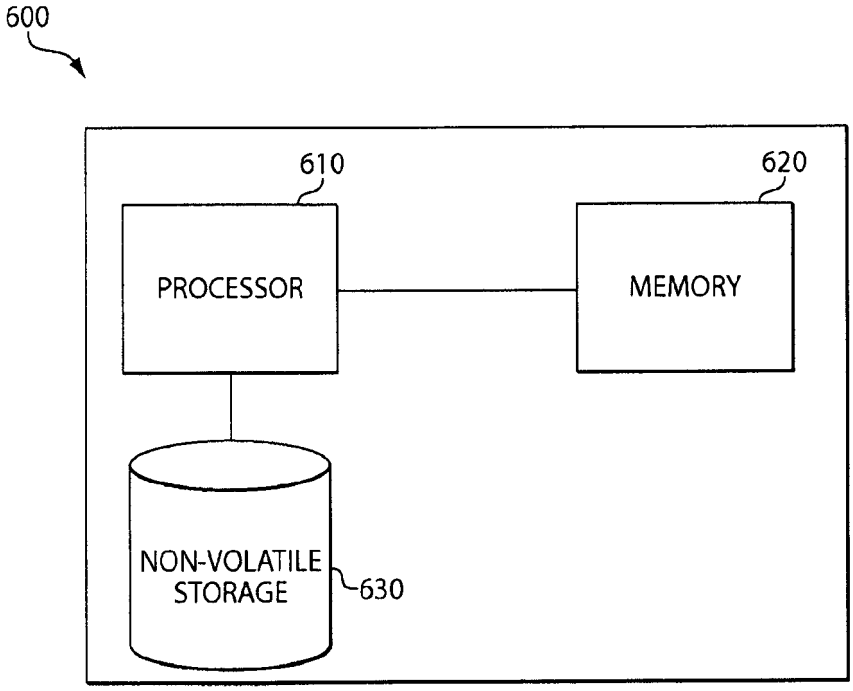


Fig. 17

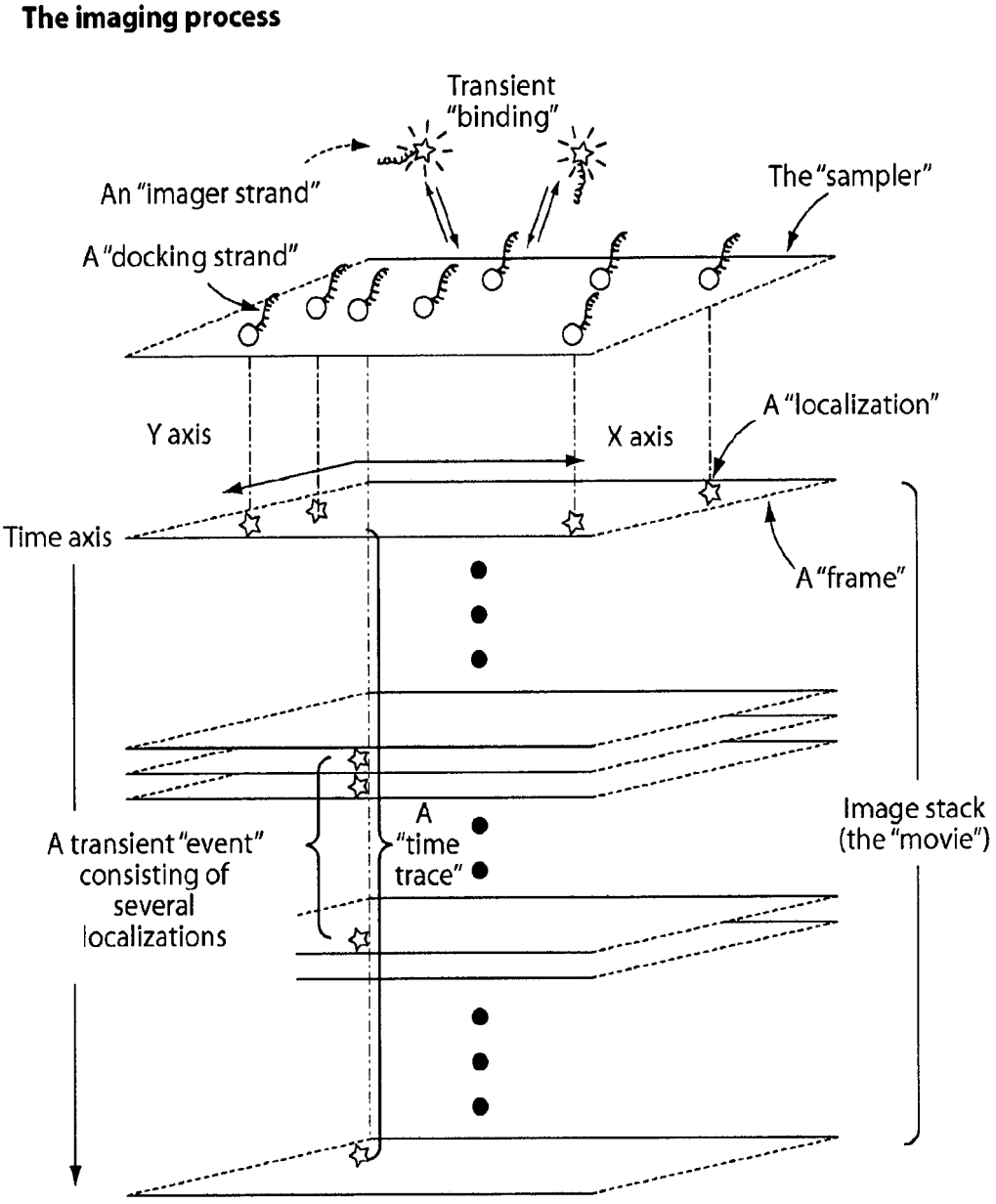

The imaging process
Transient "binding"
An "imager strand"
The "sampler"
A "docking strand"
A "localization"
Y axis
X axis
Time axis
A "frame"
A transient "event" consisting of several localizations
A "time trace"
Image stack (the "movie")

Fig. 18A

The drift markers

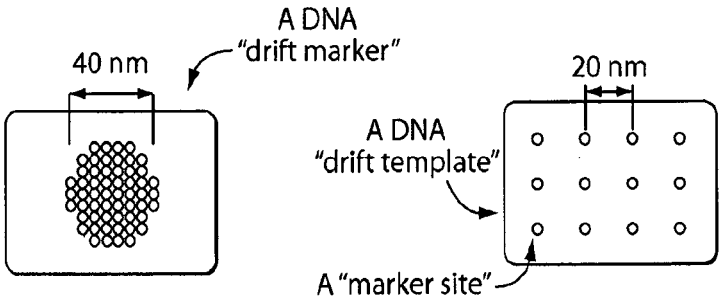


Fig. 18B

The drift correction principle

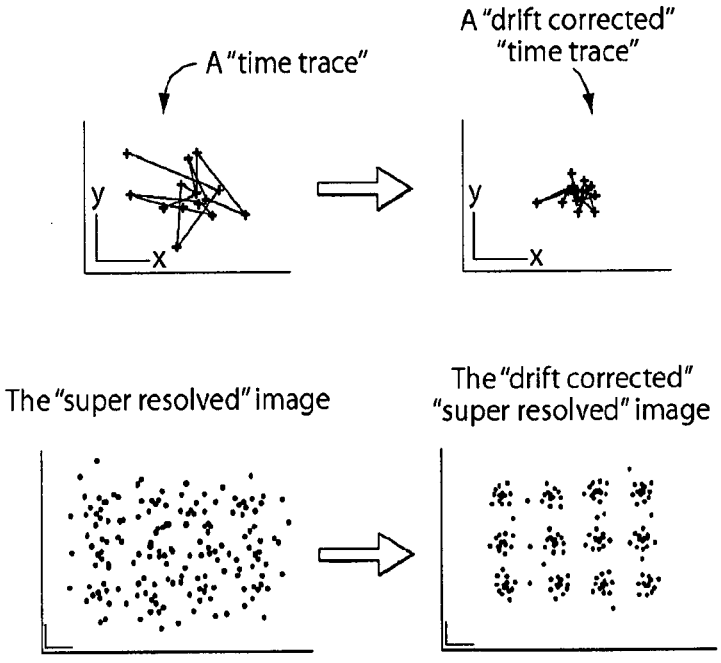


Fig. 18C

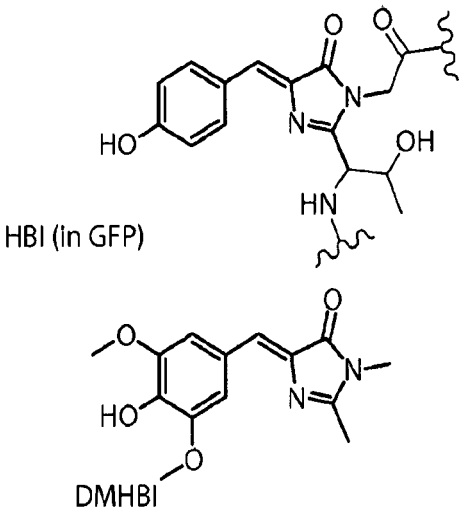


Fig. 19A

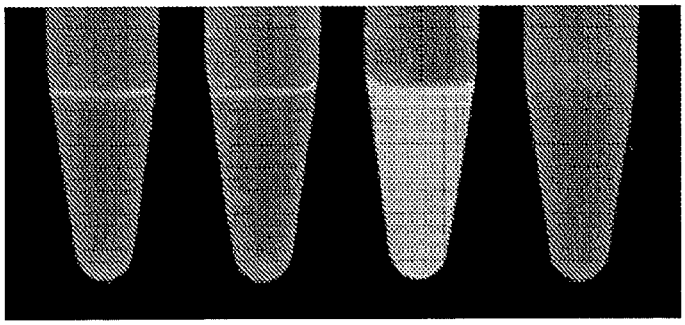


Fig. 19B

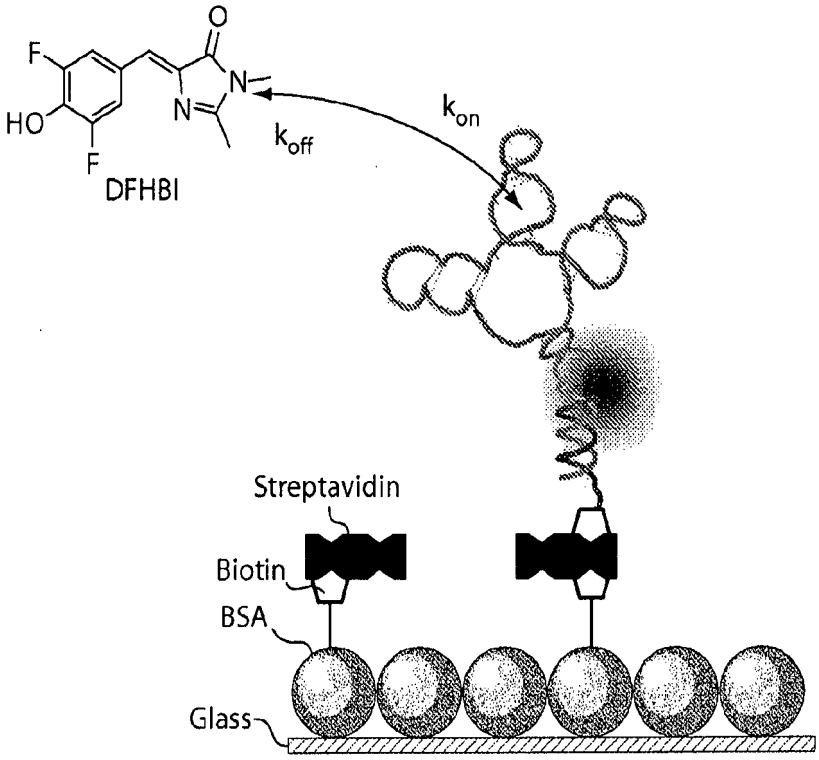


Fig. 20A

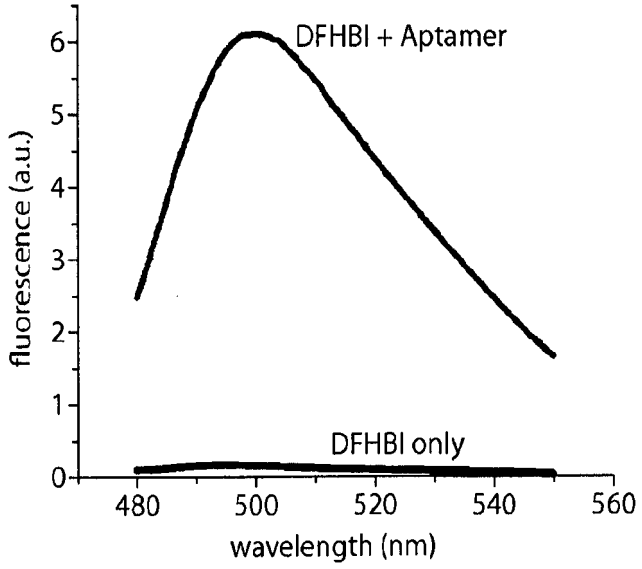


Fig. 20B

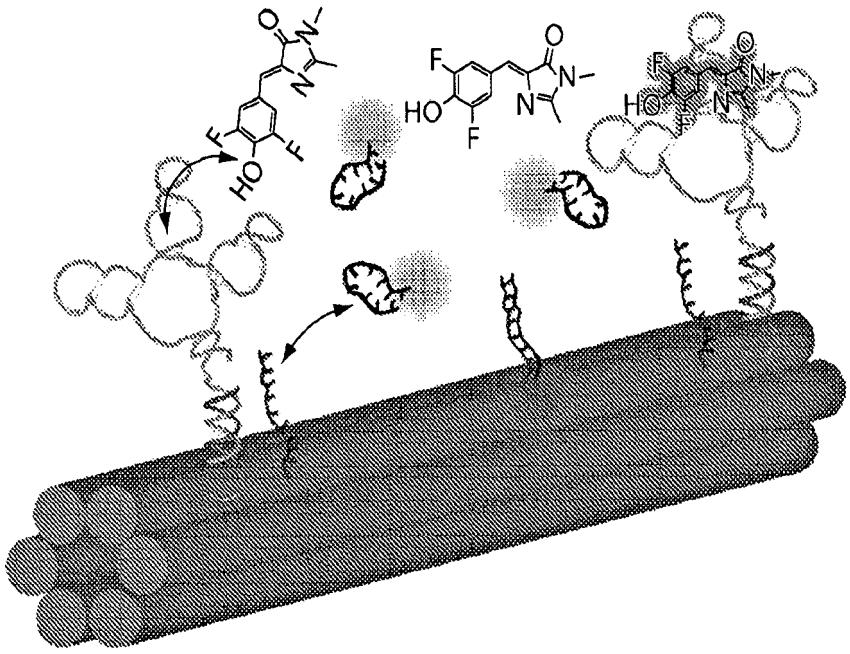


Fig. 21A

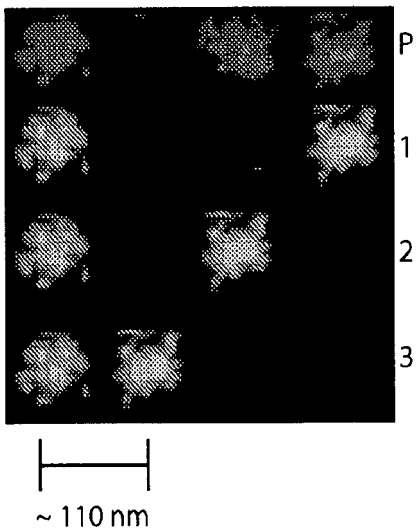


Fig. 21B

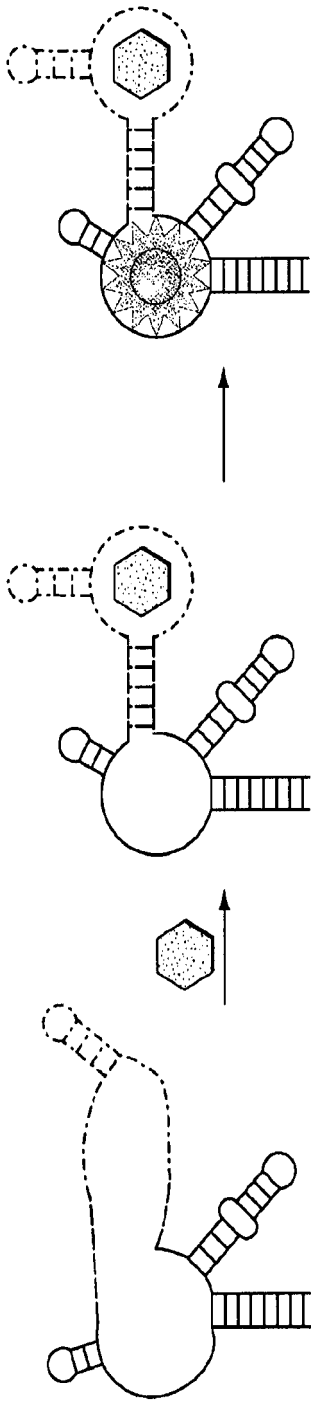


Fig. 22

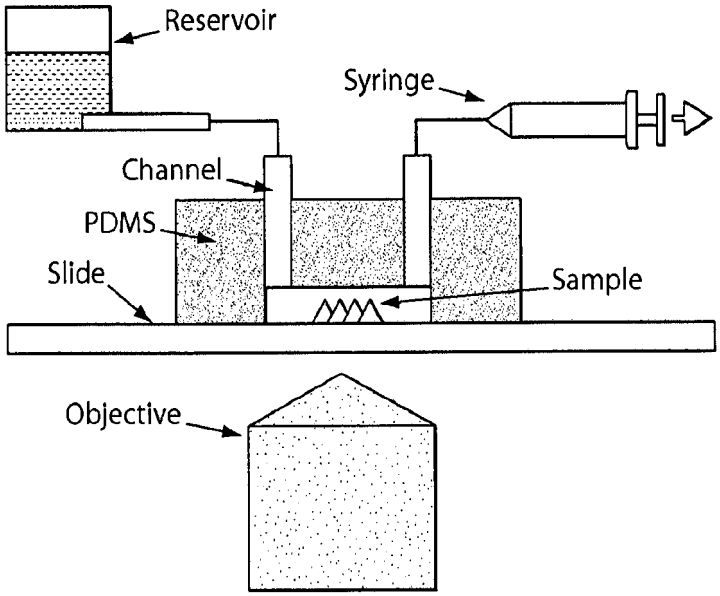


Fig. 23A

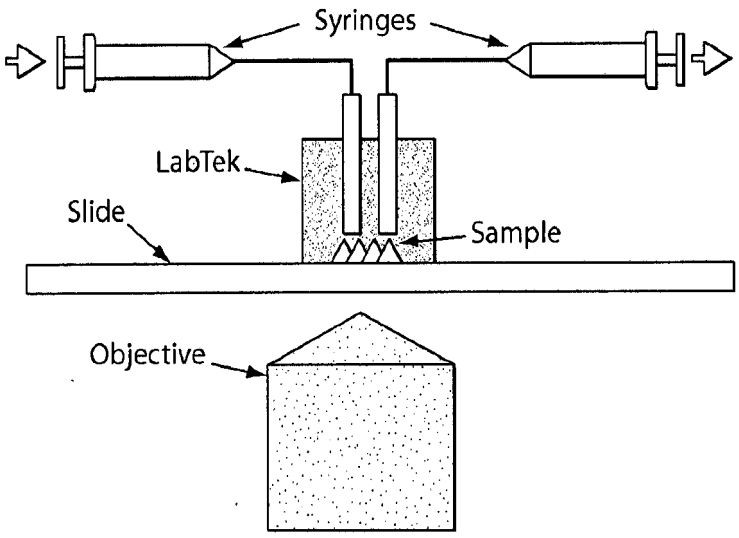


Fig. 23B

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	Date of mailing of the international search report
19 December 2014 (19.12.2014)	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

Form PCT/ISA/210 (second sheet) (July 2009)

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/48977

-***-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.

(19)中华人民共和国国家知识产权局

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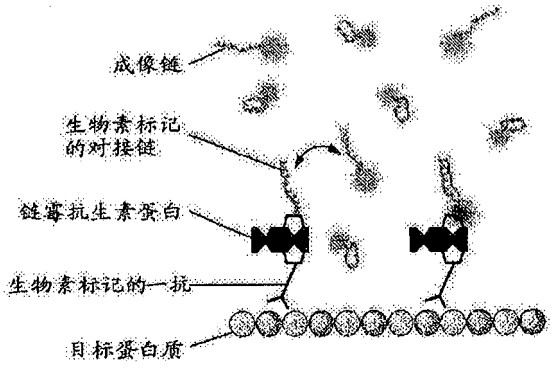
权利要求书15页 说明书60页
序列表112页 附图30页

(54)发明名称

基于DNA的定量成像和超分辨成像

(57)摘要

本公开内容提供了,除其它以外,用于以高空间分辨率对目标靶进行成像的方法和组合物(例如,缀合物)。



CN 105531377 A

1.一种蛋白质-核酸缀合物，其包含连接于对接链的蛋白质，所述对接链能够短暂地结合于互补的经标记的成像链。

2.权利要求1的蛋白质-核酸缀合物，其中所述对接链与所述互补的经标记的成像链短暂结合。

3.权利要求1或2的蛋白质-核酸缀合物，其中所述蛋白质为抗体、抗原结合抗体片段或肽适体。

4.权利要求1-3的任一项的蛋白质-核酸缀合物，其中所述蛋白质通过中间接头连接于所述对接链。

5.权利要求4的蛋白质-核酸缀合物，其中所述中间接头包含生物素和链霉抗生物素蛋白。

6.权利要求3-5的任一项的蛋白质-核酸缀合物，其中所述抗体为单克隆抗体。

7.权利要求1-6的任一项的蛋白质-核酸缀合物，其中所述互补的经标记的成像链是互补的荧光标记的成像链。

8.权利要求7的蛋白质-核酸缀合物，其中所述互补的荧光标记的成像链包含至少一个荧光团。

9.权利要求1-8的任一项的蛋白质-核酸缀合物，其中所述互补的经标记的成像链的长度为约4至约30个核苷酸。

10.权利要求9的蛋白质-核酸缀合物，其中所述互补的经标记的成像链的长度为约8至约10个核苷酸。

11.一种结合于至少一种蛋白质-核酸缀合物的靶，所述蛋白质-核酸缀合物为权利要求1-10的任一项的蛋白质-核酸缀合物。

12.权利要求11的靶，其中所述靶为蛋白质。

13.多个蛋白质-核酸缀合物，所述蛋白质-核酸缀合物为权利要求1-10的任一项的蛋白质-核酸缀合物。

14.权利要求13的多个蛋白质-核酸缀合物，其中所述多个蛋白质-核酸缀合物包含至少两个亚组的蛋白质-核酸缀合物，并且每个亚组的蛋白质-核酸缀合物结合于不同的靶。

15.一种包含权利要求13或14的多个蛋白质-核酸缀合物的组合物，其中所述蛋白质-核酸缀合物的至少一种结合于至少一种靶。

16.一种组合物，所述组合物包含：

包含连接于对接链的蛋白质的至少一种蛋白质-核酸缀合物，其中所述至少一种蛋白质-核酸缀合物结合于靶；和

短暂地结合于所述至少一种蛋白质-核酸缀合物的至少一种互补的经标记的成像链。

17.权利要求16的组合物，其包含至少两种互补的经标记的成像链，其中所述至少两种互补的经标记的成像链是相同的。

18.权利要求16的组合物，其包含至少两种互补的经标记的成像链，其中所述至少两种互补的经标记的成像链是不同的。

19.权利要求16-18的任一项的组合物，其中互补的经标记的成像链的数目少于、多于或等于蛋白质-核酸缀合物的数目。

20.权利要求13-19的任一项的组合物，其中所述组合物包含至少2种不同的互补的经

标记的成像链。

21.权利要求13-20的任一项的组合物，其中所述组合物包含至少5种不同的互补的经标记的成像链。

22.权利要求13-21的任一项的组合物，其中所述组合物包含至少10种不同的互补的经标记的成像链。

23.权利要求22的组合物，其中所述组合物包含至少100种不同的互补的经标记的成像链。

24.权利要求16-23的任一项的组合物，其中所述互补的经标记的成像链为互补的荧光标记的成像链。

25.一种抗体-DNA缀合物，其包含连接于对接链的单克隆抗体，所述对接链结合于互补的经标记的成像链，其中所述抗体和所述对接链各自为生物素化的并且通过生物素-链霉抗生物素蛋白接头彼此连接。

26.权利要求25的抗体-DNA缀合物，其中所述互补的经标记的成像链为互补的荧光标记的成像链。

27.权利要求1-26的任一项的抗体-DNA缀合物，其中所述对接链包含至少2个结构域，其中每个结构域结合各自的互补的经标记的成像链。

28.权利要求27的抗体-DNA缀合物，其中所述对接链包含至少3个结构域，其中每个结构域结合各自的互补的经标记的成像链。

29.权利要求21-28的任一项的抗体-DNA缀合物，其中短暂地结合于互补的经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

30.一种适体-核酸缀合物，其包含连接于短暂地结合于互补的经标记的成像链的对接链的核酸适体。

31.权利要求30的适体-核酸缀合物，其中所述互补的经标记的成像链为互补的荧光标记的成像链。

32.权利要求30或31的适体-核酸缀合物，其中所述对接链包含至少2个结构域，其中每个结构域结合各自的互补的经标记的成像链。

33.权利要求32的适体-核酸缀合物，其中所述对接链包含至少3个结构域，其中每个结构域结合各自的互补的经标记的成像链。

34.权利要求30-33的任一项的适体-核酸缀合物，其中短暂地结合于互补的经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

35.一种检测样品中的靶的方法，所述方法包括：

将样品与(a)至少一种包含连接于对接链的蛋白质的蛋白质-核酸缀合物和(b)至少一种与所述至少一种蛋白质-核酸缀合物互补并与其短暂结合的荧光标记的成像链接触；和

测定所述至少一种蛋白质-核酸缀合物是否结合于所述样品中的所述靶。

36.权利要求35的方法，其中所述测定步骤包括对所述至少一种荧光标记的成像链与所述至少一种蛋白质-核酸缀合物的对接链的短暂结合进行成像。

37.权利要求35或36的方法，其中所述蛋白质-核酸缀合物的蛋白质为抗体、抗原结合

抗体片段或肽适体。

38.权利要求36的方法，其中所述抗体为单克隆抗体。

39.权利要求35-38的任一项的方法，其中所述蛋白质-核酸缀合物的蛋白质通过中间接头连接于所述对接链。

40.权利要求39的方法，其中所述中间接头包含生物素和/或链霉抗生物素蛋白。

41.权利要求35-40的任一项的方法，其中所述互补的荧光标记的成像链包含至少一个荧光团。

42.权利要求35-41的任一项的方法，其中所述互补的荧光标记的成像链的长度为约4至约30个核苷酸。

43.权利要求42的方法，其中所述互补的荧光标记的成像链的长度为约8至约10个核苷酸。

44.权利要求35-43的任一项的方法，其中所述样品为细胞或细胞裂解物。

45.权利要求35-44的任一项的方法，其中所述靶为蛋白质。

46.权利要求35-45的任一项的方法，其中所述靶获自细胞或细胞裂解物。

47.权利要求35-46的任一项的方法，其中所述对接链包含至少2个结构域，其中每个结构域结合各自的互补的经标记的成像链。

48.权利要求47的方法，其中所述对接链包含至少3个结构域，其中每个结构域结合各自的互补的经标记的成像链。

49.权利要求35-48的任一项的方法，其中短暂地结合于经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

50.一种检测样品中的至少一种靶的方法，所述方法包括：

将样品与(a)各自包含连接于对接链的蛋白质的至少两种蛋白质-核酸缀合物和(b)与所述至少两种不同的蛋白质-核酸缀合物的各自对接链互补并与其结合的至少两种经标记的成像链接触；和

测定所述至少两种蛋白质-核酸缀合物是否结合于所述样品中的至少一种靶。

51.权利要求50的方法，其中所述测定步骤以下列顺序包括：

对所述至少两种经标记的成像链之一与所述至少两种蛋白质-核酸缀合物之一的对接链的短暂结合进行成像，以产生第一信号图像，和

对所述至少两种经标记的成像链之另一与所述至少两种蛋白质-核酸缀合物之另一的对接链的短暂结合进行成像，以产生至少一个其它的信号图像。

52.权利要求51的方法，其还包括将所述第一图像和所述至少一个其它的图像组合以产生合成信号图像，其中合成图像的信号代表所述至少一种靶。

53.权利要求50-52的任一项的方法，其中所述蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。

54.权利要求53的方法，其中所述抗体为单克隆抗体。

55.权利要求50-54的任一项的方法，其中所述蛋白质-核酸缀合物的蛋白质通过中间接头连接于所述对接链。

56.权利要求55的方法，其中所述中间接头包含生物素和链霉抗生物素蛋白。

57.权利要求50-56的任一项的方法，其中所述至少两种经标记的成像链的每一种为荧光标记的成像链。

58.权利要求57的方法，其中所述至少两种荧光标记的成像链为光谱上不同的荧光标记的成像链。

59.权利要求57或58的任一项的方法，其中所述至少两种经标记的成像链的每一种包含至少一个荧光团。

60.权利要求50-59的任一项的方法，其中所述至少两种经标记的成像链的每一种的长度为约4至约30个核苷酸。

61.权利要求60的方法，其中所述至少两种经标记的成像链的每一种的长度为约8至约10个核苷酸。

62.权利要求50-61的任一项的方法，其中所述样品为细胞或细胞裂解物。

63.权利要求50-62的任一项的方法，其中所述至少一种靶为蛋白质。

64.权利要求50-63的任一项的方法，其中所述至少一种靶获自细胞或细胞裂解物。

65.权利要求50-64的任一项的方法，其中测定所述至少两种蛋白质-核酸缀合物是否结合于所述样品中的至少一种靶的步骤包括测定所述至少两种蛋白质-核酸缀合物是否结合于所述样品中的至少两种靶。

66.权利要求50-65的任一项的方法，其中所述对接链包含至少两个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

67.权利要求66的方法，其中所述对接链包含至少3个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

68.权利要求50-67的任一项的方法，其中短暂地结合于经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

69.一种检测样品中的至少一种蛋白质靶的方法，所述方法包括

(a)将样品与各自包含连接于对接链的蛋白质的至少两种蛋白质-核酸缀合物接触，和

(b)连续地将所述样品与至少两种经标记的成像链接触，所述至少两种经标记的成像链与所述至少两种蛋白质-核酸缀合物的各自对接链互补并与其结合；和

测定所述至少两种蛋白质-核酸缀合物是否结合于所述样品中的至少一种蛋白质靶。

70.权利要求69的方法，其按照以下顺序的步骤包括：

将所述样品与第一蛋白质-核酸缀合物和至少一种其它的蛋白质-核酸缀合物接触；

将所述样品与第一经标记的成像链接触，所述第一经标记的成像链与所述第一蛋白质-核酸缀合物的对接链互补并与其短暂结合；

任选地使用延时成像对所述样品进行成像以获得第一图像；

除去所述第一经标记的成像链；

将所述样品与至少一种其它的经标记的成像链接触，所述至少一种其它的经标记的成像链与所述至少一种其它的蛋白质-核酸缀合物的对接链互补并与其短暂结合；和

任选地使用延时成像对所述样品进行成像以获得至少一个其它的图像。

71.权利要求69的方法，其按照以下顺序的步骤包括：

将所述样品与第一蛋白质-核酸缀合物接触；

将所述样品与第一经标记的成像链接触，所述第一经标记的成像链与所述第一蛋白质-核酸缀合物的对接链互补并与其短暂结合；

任选地使用延时成像对所述样品进行成像以获得第一图像；

除去所述第一经标记的成像链；

将所述样品与至少一种其它的蛋白质-核酸缀合物接触；

将所述样品与至少一种其它的经标记的成像链接触，所述至少一种其它的经标记的成像链与所述至少一种其它的蛋白质-核酸缀合物的对接链互补并与其短暂结合；和

任选地使用延时成像对所述样品进行成像以获得至少一个其它的图像。

72.权利要求70或71的方法，其还包括测定所述第一蛋白质-DNA缀合物是否结合于第一靶和/或所述至少一种其它的蛋白质-DNA缀合物是否结合于至少一种其它的靶。

73.权利要求72的方法，其还包括将伪彩色赋予所述第一图像中的信号，和将至少一种其它的伪彩色赋予所述至少一个其它的图像中的信号。

74.权利要求73的方法，其还包括组合所述第一图像和所述至少一个其它的图像以产生伪彩色化信号的合成图像，其中所述合成图像的伪彩色化信号代表至少两种靶。

75.权利要求69-74的任一项的方法，其中所述蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。

76.权利要求75的方法，其中所述抗体为单克隆抗体。

77.权利要求69-76的任一项的方法，其中所述蛋白质-核酸缀合物的蛋白质通过中间接头连接于所述对接链。

78.权利要求77的方法，其中所述中间接头包含生物素和/或链霉抗生物素蛋白。

79.权利要求69-78的任一项的方法，其中所述经标记的成像链的每一种为荧光标记的成像链。

80.权利要求69的方法，其中所述经标记的成像链的每一种为光谱上不同的荧光标记的成像链。

81.权利要求69或70的方法，其中所述经标记的成像链的每一种包含至少一个荧光团。

82.权利要求69-81的任一项的方法，其中所述经标记的成像链的每一种的长度为约4至约30个核苷酸。

83.权利要求82的方法，其中所述经标记的成像链的每一种的长度为约8至约10个核苷酸。

84.权利要求69-83的任一项的方法，其中所述样品为细胞或细胞裂解物。

85.权利要求69-84的任一项的方法，其中所述靶为蛋白质。

86.权利要求69-85的任一项的方法，其中所述靶获自细胞或细胞裂解物。

87.权利要求69-86的任一项的方法，其中测定所述至少两种蛋白质-核酸缀合物是否结合于所述样品中的至少一种蛋白质靶的步骤包括测定所述至少两种蛋白质-核酸缀合物是否结合于所述样品中的至少两种蛋白质靶。

88.权利要求69-87的任一项的方法，其中所述对接链的每一种包含至少两个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

89.权利要求69-88的任一项的方法，其中所述对接链的每一种包含至少3个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

90.权利要求69-89的任一项的方法，其中短暂地结合于经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

91.一种检测分子的方法，其包括：

将包含至少一种靶的样品与(a)至少一种BP-NA缀合物和(b)至少一种经标记的成像链接触，所述BP-NA缀合物各自包含连接于对接链的结合伴侣，所述至少一种经标记的成像链与所述至少一种BP-NA缀合物的对接链互补并与其短暂结合；和

测定所述至少一种BP-NA缀合物是否结合于所述样品中的至少一种靶。

92.权利要求91的方法，其中所述测定步骤包括对所述至少一种经标记的成像链与所述至少一种BP-NA缀合物的对接链的短暂结合进行成像。

93.权利要求91或92的方法，其中所述样品为细胞或细胞裂解物。

94.权利要求91-93的任一项的方法，其中所述至少一种靶获自细胞或细胞裂解物。

95.权利要求91-94的任一项的方法，其中所述靶为天然存在的生物分子。

96.权利要求95的方法，其中所述天然存在的生物分子为蛋白质。

97.权利要求96的方法，其中所述蛋白质为抗体、抗原结合抗体片段或肽适体。

98.权利要求97的方法，其中所述抗体为单克隆抗体。

99.权利要求91-98的任一项的方法，其中所述靶通过中间接头连接于所述对接链。

100.权利要求99的方法，其中所述中间接头包含生物素和/或链霉抗生物素蛋白。

101.权利要求95的方法，其中所述天然存在的生物分子为核酸。

102.权利要求101的方法，其中所述核酸为核酸适体。

103.权利要求91-103的任一项的方法，其中所述经标记的成像链为荧光标记的成像链。

104.权利要求103的方法，其中所述荧光标记的成像链包含至少一个荧光团。

105.权利要求91-104的任一项的方法，其中所述荧光标记的成像链的长度为约4至约30个核苷酸。

106.权利要求105的方法，其中所述荧光标记的成像链的长度为约8至约10个核苷酸。

107.权利要求91-106的任一项的方法，其中所述结合伴侣为蛋白质或核酸。

108.权利要求91-107的任一项的方法，其中所述对接链包含至少两个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

109.权利要求108的方法，其中所述对接链包含至少3个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

110.权利要求91-109的任一项的方法，其中短暂地结合于经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

111.一种检测天然存在的生物分子的方法，其包括：

将包含至少一种靶的样品与(a)至少两种BP-NA缀合物和(b)至少两种经标记的成像链接触，所述BP-NA缀合物各自包含连接于对接链的结合伴侣，所述至少两种经标记的成像链与所述至少两种BP-NA缀合物的各自的对接链互补并与其短暂结合；和

测定所述至少两种BP-NA缀合物是否结合于所述样品中的至少一种靶。

112.权利要求111的方法，其按照以下顺序的步骤包括：

将所述样品与第一BP-NA缀合物和至少一种其它的BP-NA缀合物接触；和

将所述样品与第一经标记的成像链接触，所述第一经标记的成像链与所述第一BP-NA缀合物的对接链互补并与其短暂结合；

任选地使用延时成像对所述样品进行成像以获得第一图像；

除去第一荧光标记的成像链；

将所述样品与至少一种其它的经标记的成像链接触，所述至少一种其它的经标记的成像链接触与所述至少一种其它的BP-NA缀合物的对接链互补并与其短暂结合；和

任选地使用延时成像对所述样品进行成像以获得至少一个其它的图像。

113.权利要求111的方法，其按照以下顺序的步骤包括：

将所述样品与第一BP-NA缀合物接触；

将所述样品与第一经标记的成像链接触，所述第一经标记的成像链与所述第一BP-NA缀合物的对接链互补并与其短暂结合；

任选地使用延时成像对所述样品进行成像以获得第一图像；

除去所述第一经标记的成像链；

将所述样品与至少一种其它的BP-NA缀合物接触；

将所述样品与至少一种其它的经标记的成像链接触，所述至少一种其它的经标记的成像链与所述至少一种其它的BP-NA缀合物的对接链互补并与其短暂结合；和

任选地使用延时成像对所述样品进行成像以获得至少一个其它的图像。

114.权利要求112或113的方法，其还包括测定所述第一蛋白质DNA缀合物是否结合于第一靶和/或所述至少一种其它的蛋白质-DNA缀合物是否结合于至少一种其它的靶。

115.权利要求114的方法，其还包括将伪彩色赋予所述第一图像中的信号，和将至少一种其它的伪彩色赋予所述至少一个其它的图像中的信号。

116.权利要求115的方法，其还包括组合所述第一图像和所述至少一个其它的图像以产生伪彩色化信号的合成图像，其中所述合成图像的伪彩色化信号代表至少一种靶。

117.权利要求111-116的任一项的方法，其中所述样品为细胞或细胞裂解物。

118.权利要求111-117的任一项的方法，其中所述至少一种靶获自细胞或细胞裂解物。

119.权利要求111-118的任一项的方法，其中所述靶为天然存在的生物分子。

120.权利要求119的方法，其中所述天然存在的生物分子为蛋白质。

121.权利要求120的方法，其中所述蛋白质为抗体、抗原结合抗体片段或肽适体。

122.权利要求121的方法，其中所述抗体为单克隆抗体。

123.权利要求111-122的任一项的方法，其中所述靶通过中间接头连接于所述对接链。

124.权利要求123的方法，其中所述中间接头包含生物素和/或链霉抗生物素蛋白。

125.权利要求119的方法，其中所述天然存在的生物分子为核酸。

126.权利要求125的方法，其中所述核酸为核酸适体。

127.权利要求111-126的任一项的方法，其中所述第一经标记的成像链为荧光标记的成像链。

128.权利要求111-126的任一项的方法，其中所述至少一种其它的经标记的成像链为荧光标记的成像链。

129.权利要求127或128的方法，其中所述第一经标记的成像链和/或所述至少一种其它的经标记的成像链包含至少一个荧光团。

130.权利要求111-129的任一项的方法，其中所述至少一种其它的经标记的成像链的长度为约4至约30个核苷酸。

131.权利要求130的方法，其中所述至少一种其它的经标记的成像链的长度为约8至约10个核苷酸。

132.权利要求111-131的任一项的方法，其中所述结合伴侣为蛋白质或核酸。

133.权利要求111-132的任一项的方法，其中所述对接链为DNA对接链。

134.权利要求111-133的任一项的方法，其中所述对接链包含至少2个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

135.权利要求134的方法，其中所述对接链包含至少3个结构域，其中每个结构域结合于各自的互补的经标记的成像链。

136.权利要求111-135的任一项的方法，其中短暂地结合于经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

137.一种测定测试样品中的靶的数目的方法，其包括：

获得包含直接或间接地短暂结合于经标记的成像链的靶的样品；

获得所述样品的延时图像；

对所述图像进行斑点检测和定位以获得所述样品的高分辨率图像；

校准$k_{on} \cdot c_{imager}$，其中k_{on}为二阶缔合常数，并且c_{imager}为测试样品中的经标记的成像链的浓度；

测定变量τ_d；和

基于如下方程式测定样品中测试靶的数目：测试靶的数目$=(k_{on} \cdot c_{imager} \cdot \tau_d)^{-1}$。

138.权利要求137的方法，其中所述测试靶为蛋白质靶。

139.权利要求138的方法，其中所述蛋白质靶结合于包含连接于对接链的蛋白质的蛋白质-核酸缀合物，并且所述经标记的成像链与所述蛋白质-核酸缀合物的各自对接链互补并与其短暂结合。

140.权利要求139的方法，其中所述蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。

141.权利要求137的方法，其中所述测试靶为单链核酸。

142.权利要求141的方法，其中所述单链核酸为DNA或RNA。

143.权利要求137-142的任一项的方法，其中所述经标记的成像链的每一种为荧光标记的成像链。

144.权利要求137-143的任一项的方法，其中荧光标记的成像链的每一种包含至少一个荧光团。

145.权利要求137-144的任一项的方法，其中荧光标记的成像链的每一种的长度为约3至约30个核苷酸。

146.权利要求144的方法，其中所述荧光标记的成像链的每一种的长度为约8至约10个核苷酸。

147.权利要求137-146的任一项的方法，其中在约24分钟的时期内获得所述延时图像。

148.权利要求137-147的任一项的方法，其中所述延时图像为延时衍射极限荧光图像。

149.权利要求137-148的任一项的方法，其中对所述图像进行定位的步骤包括对图像进行高斯拟合。

150.权利要求137-149的任一项的方法，其中校准$k_{on} \cdot c_{imager}$的步骤包括利用具有已知数目的靶的对照样品校准$k_{on} \cdot c_{imager}$。

151.权利要求137-150的任一项的方法，其中测定变量τ_d的步骤包括通过将信号关断时间分布拟合至累积分布函数来测定变量τ_d。

152.权利要求137-151的任一项的方法，其中以大于90％的精确度测定测试靶的数目。

153.一种测定测试样品中的靶的相对量的方法，其包括：

获得包含直接或间接地短暂结合于经标记的成像链的靶的样品；

获得所述样品的延时图像；

对所述图像进行斑点检测和定位以获得所述样品的高分辨率图像；

测定变量τ_d；和

基于τ_d测定所述样品中两种或多种测试靶的相对量。

154.权利要求153的方法，其中所述测试靶为蛋白质靶。

155.权利要求154的方法，其中所述蛋白质靶结合于包含连接于对接链的蛋白质的蛋白质-核酸缀合物，并且所述经标记的成像链与所述蛋白质-核酸缀合物的各自对接链互补并与其短暂结合。

156.权利要求155的方法，其中所述蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。

157.权利要求153的方法，其中所述测试靶为单链核酸。

158.权利要求157的方法，其中所述单链核酸为DNA或RNA。

159.权利要求153-158的任一项的方法，其中所述经标记的成像链的每一种为荧光标记的成像链。

160.权利要求159的方法，其中所述经标记的成像链的每一种包含至少一个荧光团。

161.权利要求153-160的任一项的方法，其中所述经标记的成像链的每一种的长度为约4至约30个核苷酸。

162.权利要求161的方法，其中所述经标记的成像链的每一种的长度为约8至约10个核苷酸。

163.权利要求153-162的任一项的方法，其中在约25分钟的时期内获得所述延时图像。

164.权利要求153-163的任一项的方法，其中所述延时图像为延时衍射极限荧光图像。

165.权利要求153-164的任一项的方法，其中对所述图像进行定位的步骤包括对图像进行高斯拟合。

166.权利要求153-165的任一项的方法，其中测定变量τ_d的步骤包括通过将信号关断时间分布拟合至累积分布函数来测定变量τ_d。

167.权利要求153-166的任一项的方法，其中以大于90％的精确度测定测试靶的数目。

168.一种单链DNA探针，所述探针包含连接于对接结构域的长度为约20个核苷酸的靶结合结构域，所述对接结构域包含与至少一种长度为约4至30个核苷酸的经标记的成像链

互补的至少一个子结构域，并且其中所述靶结合结构域结合于单链mRNA靶链的互补结构域。

169.权利要求168的单链DNA探针，其中所述至少一个子结构域短暂地结合于所述至少一种经标记的成像链。

170.权利要求169的单链DNA探针，其中所述至少一种经标记的成像链是荧光标记的。

171.权利要求170的单链DNA探针，其中所述至少一种经标记的成像链包含荧光团。

172.权利要求168的单链DNA探针，其中所述对接结构域包含至少两个子结构域，其中所述至少两个子结构域各自与至少两个长度为约4至30个核苷酸的经标记的成像链互补。

173.权利要求172的单链DNA探针，其中所述至少两个子结构域各自与至少两个长度为约8至10个核苷酸的经标记的成像链互补。

174.权利要求172或173的单链DNA探针，其中所述至少两个子结构域短暂地结合于各自的互补的经标记的成像链。

175.权利要求172-174的任一项的单链DNA探针，其中所述各自的互补的经标记的成像链为不同标记的成像链。

176.权利要求172-175的任一项的单链DNA探针，其中所述各自的互补的经标记的成像链为各自的互补的荧光标记的成像链。

177.权利要求176的单链DNA探针，所述各自的互补的经标记的成像链各自包含荧光团。

178.权利要求168的单链DNA探针，其中所述对接结构域包含至少3个子结构域，其中所述至少3个子结构域各自与至少3个长度为约4至30个核苷酸的经标记的成像链互补。

179.权利要求178的单链DNA探针，其中所述至少3个子结构域短暂地结合于各自的互补的经标记的成像链。

180.权利要求179的单链DNA探针，其中所述各自的互补的经标记的成像链是不同标记的成像链。

181.权利要求179或180的单链DNA探针，其中所述各自的互补的经标记的成像链为各自的互补的荧光标记的成像链。

182.权利要求180或181的单链DNA探针，其中所述各自的互补的经标记的成像链各自包含荧光团。

183.权利要求168-182的任一项的单链DNA探针，其中所述长度为约20个核苷酸的靶结合结构域在其3'末端连接于对接结构域。

184.权利要求168-182的任一项的单链DNA探针，其中所述长度为约20个核苷酸的靶结合结构域在其5'末端连接于对接结构域。

185.一种进行多个图像的漂移校正的方法，其中所述多个图像的每一个包含图像的时间序列的帧，其中所述图像的时间序列捕捉多个短暂事件，所述方法包括：

测定在所述多个图像中鉴定的多个漂移标记的每一个的时间轨迹，其中每个漂移标记的时间轨迹对应于图像中的物体在图像的时间序列上的运动；

利用至少一个计算机处理器，至少部分地基于多个漂移标记的至少一个的时间轨迹测定来自多个漂移标记的至少一个的第一漂移校正；

测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一

个的时间轨迹，其中多个漂移模板中的每个漂移模板描述了漂移模板中短暂事件的多个可几何寻址的标记位点之间的几何关系；

至少部分地基于来自所述多个漂移模板的多个可几何寻址的标记位点的时间轨迹测定第二漂移校正；

至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像；和

基于所校正的多个图像输出最终图像。

186.权利要求185的方法，其还包括：

鉴定所述多个图像的每一个中的多个定位；

产生所述多个定位的二维直方图；和

至少部分地基于所述二维直方图鉴定多个漂移标记的位置；

其中测定多个漂移标记的每一个的时间轨迹包括至少部分地基于所述多个漂移标记的位置测定时间轨迹。

187.权利要求186的方法，其中鉴定多个定位包括：

鉴定所述多个图像的每一个上的多个斑点；和

使用局部高斯拟合算法测定所述多个斑点的每一个的拟合中心位置；

其中所述多个定位的每一个包括在图像上鉴定的斑点及其相关拟合的中心位置。

188.权利要求187的方法，其中所述多个定位的每一个还包括所检测的对应于所述定位的光子计数。

189.权利要求186的方法，其中产生多个定位的二维直方图包括将所有定位区间化在二维网格中并使用每个区间中的定位的总数作为直方图计数。

190.权利要求186的方法，其中产生多个定位的二维直方图包括将所有定位区间化在二维网格中并使用每个区间中的多个定位的光子计数的总数作为直方图计数。

191.权利要求186的方法，其中至少部分地基于所述二维直方图鉴定多个漂移标记的位置包括下述的至少一种：

使用一种或多种选择标准二值化所述二维直方图，其中所述一种或多种选择标准包括直方图值的下限阈值或直方图值的上限阈值；

将二值化图像划分成分区并基于一种或多种选择标准过滤所述分区，其中一种或多种选择标准包括分区区域的面积的下限阈值、面积的上限阈值、最长分区的最长或最短线性尺度的下限或上限、以及分区的偏心率的下限或上限中的一种或多种；和

使用一种或多种二值图像运算扩大和缩小二值化图像，其中所述一种或多种二值图像运算包括以下的一种或多种：膨胀运算、腐蚀运算、桥接运算、闭运算、开运算、填充运算、清理运算、顶帽运算、底帽运算、增厚运算、减薄运算等。

192.权利要求185的方法，其中至少部分地基于多个漂移标记的时间轨迹测定第一漂移校正包括：

测定多个漂移标记的每一个的相对时间轨迹，其中通过将所述漂移标记的时间轨迹与相同轨迹的平均位置进行比较来测定相对时间轨迹；和

基于多个漂移标记的每一个的相对时间轨迹测定组合时间轨迹，

其中至少部分地基于多个漂移标记的时间轨迹测定第一漂移校正包括至少部分地基于多个漂移标记的每一个的相对时间轨迹测定第一漂移校正。

193.权利要求192的方法，其中至少部分地基于多个漂移标记的每一个的相对时间轨迹测定所述第一漂移校正包括进行多个漂移标记的每一个的相对时间轨迹的加权平均。

194.权利要求193的方法，其中进行加权平均包括：

测定相对时间轨迹的每一个的质量评分，其中至少部分地基于与所述时间轨迹相关的随时间过去的变化性的测量或所述时间轨迹内个体定位的定位不确定性的测量来测定所述质量评分。

195.权利要求194的方法，其中随时间过去的变化性的测量包括随时间过去的时间轨迹的标准差。

196.权利要求194的方法，其中个体定位的定位不确定性的测量至少部分地包括来自高斯拟合的不确定性评估或与其它同时定位的比较，其中所述其它同时定位来自相同的图像内且来自多个漂移标记的其它时间轨迹，其中所述比较包括所有同时定位的平均值和标准差。

197.权利要求185的方法，其还包括：

测定多个漂移标记的第一漂移标记不存在于图像的时间序列的至少一个帧中；和

针对所述至少一个帧线性地内插所述第一漂移标记的时间轨迹，以产生所述第一漂移标记的平滑时间轨迹。

198.权利要求185的方法，其中测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹包括：

鉴定所述多个图像的每一个中的多个定位；

产生多个定位的二维直方图；和

至少部分地基于所述二维直方图鉴定多个漂移模板；

其中鉴定多个漂移模板包括使用直方图计数的下限域值和/或上限域值评估二维直方图。

199.权利要求185的方法，其中测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹包括测定多个漂移模板的每一个内的多个可几何寻址的标记位点的每一个的时间轨迹，并且其中测定所述第二漂移校正包括至少部分地基于多个漂移模板的每一个内的多个标记位点的每一个的时间轨迹测定所述第二漂移校正。

200.权利要求185的方法，其中至少部分地基于来自多个漂移模板的每一个的多个可几何寻址的标记位点的每一个的时间轨迹测定所述第二漂移校正包括：

鉴定所述多个漂移模板的每一个内的多个可几何寻址的标记位点；和

测定多个漂移模板的每一个的多个可几何寻址的漂移标记的每一个的相对时间轨迹；

其中至少部分地基于多个漂移模板的时间轨迹测定所述第二漂移校正包括至少部分地基于多个漂移模板的每一个内的多个漂移标记的每一个的相对时间轨迹测定所述第二漂移校正。

201.权利要求200的方法，其中鉴定来自多个漂移模板的每一个的多个可几何寻址的标记位点包括至少部分地基于对应的漂移模板中多个定位的二维直方图和/或一种或多种选择标准测定多个标记位点，其中所述一种或多种选择标准包括定位的总数、定位的表面密度和定位的标准差的一种或多种。

202.权利要求200的方法，其中至少部分地基于多个漂移模板的每一个内的多个漂移标记的每一个的相对时间轨迹测定所述第二漂移校正包括进行所述漂移模板的每一个内的多个漂移标记的每一个的相对时间轨迹的加权平均。

203.权利要求202的方法，其中进行加权平均包括：

测定所述相对时间轨迹的每一个的质量评分，其中至少部分地基于与时间轨迹相关的随时间过去的变化性和/或所述时间轨迹内的定位不确定性的测量来测定所述质量评分。

204.权利要求203的方法，其中随时间过去的变化性的测量包括随时间过去的时间轨迹的标准差。

205.权利要求203的方法，其中个体定位的定位不确定性的测量包括来自高斯拟合的不确定性评估或与其它同时定位的比较，其中所述其它同时定位来自相同的图像内并来自多个漂移模板的多个标记位点的其它时间轨迹，其中所述比较包括所有同时定位的平均值和标准差。

206.权利要求185的方法，其中至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像包括使用所述第一漂移校正来校正所述多个图像以产生第一校正的多个图像，并且其中测定从所述多个图像鉴定的多个漂移模板的每一个的时间轨迹包括测定从所述第一校正的多个图像鉴定的多个漂移模板的每一个的时间轨迹。

207.权利要求185的方法，其还包括：

在使用所述第一漂移校正来校正所述多个图像之前使所述第一漂移校正平滑。

208.权利要求207的方法，其中使所述第一漂移校正平滑包括使用具有通过所述第一漂移校正的特征性漂移时间尺度测定的窗口的局部回归处理所述第一漂移校正。

209.权利要求185的方法，其还包括在使用所述第二漂移校正来校正所述多个图像之前使所述第二漂移校正平滑。

210.权利要求209的方法，其中使所述第二漂移校正平滑包括使用具有通过所述第二漂移校正的特征性漂移时间尺度测定的窗口的局部回归处理所述第二漂移校正。

211.权利要求185的方法，其还包括：

选择所述多个漂移标记的单个漂移标记；和

至少部分地基于所选择的单个漂移标记测定第三漂移校正；

其中校正所述多个图像包括至少部分地基于所述第三漂移校正来校正所述多个图像。

212.权利要求211的方法，其中至少部分地基于所述第三漂移校正来校正所述多个图像在至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像之前进行。

213.权利要求185和211的任一项的方法，其还包括：

鉴定多个帧的第一图像中第一多个点的位置；

鉴定多个图像的第二图像中第二多个点的位置，其中所述第二图像对应于图像的时间序列中第一图像的相邻的帧；和

至少部分地基于所述第一多个点和所述第二多个点的位置之间的差异测定第四漂移校正；

其中校正所述多个图像包括至少部分地基于所述第四漂移校正来校正所述多个图像。

214.权利要求213的方法，其中所述第二图像对应于紧接在对应于图像的时间序列中

的第一图像的帧之后的帧。

215.权利要求213的方法，其中至少部分地基于所述第一多个点和所述第二多个点的位置之间的差异测定第四漂移校正包括：

产生所述第一多个点和所述第二多个点的位置之间的距离的直方图；

至少部分地基于所述直方图测定所述第一图像与所述第二图像之间对应于相同短暂事件的成对的点；和

测定所测定的成对的点的每一对之间的位置偏移；

其中基于所测定的成对的点的每一对的位置偏移的矢量平均值测定所述第四漂移校正。

216.权利要求185的方法，其中所述多个图像对应于基于DNA的图像，并且其中所述多个短暂事件是成像链与DNA对接链之间的结合事件。

217.权利要求216的方法，其中所述成像链为荧光成像探针，其被配置为当与DNA对接链结合时发荧光。

218.权利要求185的方法，其中所述漂移标记的至少一种为基于DNA的纳米结构。

219.权利要求218的方法，其中所述基于DNA的纳米结构为具有对接链的DNA折纸纳米结构。

220.权利要求185的方法，其中所述漂移模板的至少一个为基于DNA的纳米结构。

221.权利要求220的方法，其中所述基于DNA的纳米结构为具有对接链的DNA折纸纳米结构。

222.权利要求185的方法，其中所述漂移模板的至少一个为三维漂移模板。

223.权利要求222的方法，其中所述三维漂移模板为四面体。

224.权利要求185的方法，其中所述漂移模板的至少一个包含对应于不同类型的短暂事件的多个颜色。

225.权利要求224的方法，其中所述不同类型的短暂事件包括第一成像链与第一类型的DNA对接链的第一结合事件和第二成像链与第二类型的DNA对接链的第二结合事件。

226.权利要求185的方法，其中输出最终图像包括在显示器上显示所述最终图像。

227.权利要求185的方法，其中输出最终图像包括通过至少一个网络将所述最终图像发送至计算机。

228.权利要求185的方法，其中输出最终图像包括将所述最终图像存储在至少一个存储设备上。

229.一种利用多个指令编码的非暂时性计算机可读介质，所述指令在通过至少一个计算机处理器执行时，执行针对多个图像进行漂移校正的方法，其中所述多个图像的每一个包含图像的时间序列的帧，其中所述图像的时间序列捕捉多个短暂事件，所述方法包括：

测定在所述多个图像中鉴定的多个漂移标记的每一个的时间轨迹，其中每个漂移标记的时间轨迹对应于图像中的物体在图像的时间序列上的运动；

至少部分地基于所述多个漂移标记的至少一个的时间轨迹测定来自所述多个漂移标记的至少一个的第一漂移校正；

测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹，其中所述多个漂移模板中的每个漂移模板描述了漂移模板中短暂事件的多

个可几何寻址的标记位点之间的几何关系；

至少部分地基于来自所述多个漂移模板的多个可几何寻址的标记位点的时间轨迹测定第二漂移校正；

至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像；和

基于校正的多个图像输出最终图像。

230.一种计算机，其包含：

被配置为接受多个图像的输入接口，其中所述多个图像的每一个包含图像的时间序列的帧，其中所述图像的时间序列捕捉多个短暂事件；

至少一个处理器，其被编程来：

测定在多个图像中鉴定的多个漂移标记的每一个的时间轨迹，其中每个漂移标记的时间轨迹对应于图像中的物体在图像的时间序列上的运动；

至少部分地基于所述多个漂移标记的至少一个的时间轨迹测定来自所述多个漂移标记的至少一个的第一漂移校正；

测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹，其中所述多个漂移模板中的每个漂移模板描述了漂移模板中短暂事件的多个可几何寻址的标记位点之间的几何关系；

至少部分地基于来自所述多个漂移模板的多个可几何寻址的标记位点的时间轨迹测定第二漂移校正；

至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像；和

基于校正的多个图像测定最终图像；和

被配置为输出所述最终图像的输出接口。

基于DNA的定量成像和超分辨成像

[0001] 相关申请

[0002] 本申请依据35U.S.C.§119(e)，要求2014年2月1日提交的美国临时申请号61/934,759，2013年9月29日提交的美国临时申请号61/884,126和2013年7月30日提交的美国临时申请号61/859,891的权益，所述美国临时申请号的每一篇通过引用整体并入本文。

发明领域

[0003] 本公开内容总体上涉及靶的检测和定量的领域。

[0004] 发明背景

[0005] 自从出现了规避经典衍射极限的方法例如超分辨率显微镜(参考文献1,2)，远场荧光显微镜已经得到了重大进展。大多数工具将分子在荧光接通和关断状态之间进行开关以允许个体分子的连续定位。开关(switching)传统地以两种方式之一来获得："靶向"开关主动地将荧光激发限制于小于光的衍射的区域(例如，受激发射损耗显微术，或STED(参考文献3))，而"随机"开关使用光开关蛋白(光敏定位显微术，或PALM(参考文献4))或光开关有机染料(随机光学重建显微术，或STORM(参考文献1))。虽然这些方法提供了增强的空间分辨率，但它们倾向于需要昂贵的仪器或高度专业化的实验条件下，从而还未被开发成普通生物学实验室技术。

[0006] 发明概述

[0007] 本公开内容提供，除其它以外，用于以高或低空间分辨率对例如细胞环境中的目标靶(例如，生物分子)进行成像的方法、组合物(例如，缀合物)和试剂盒。本公开内容的方法、组合物和试剂盒利用短的经标记的(例如，荧光标记的)寡核苷酸(例如，DNA寡核苷酸)或"成像"链(imager strand)与互补的"对接"链(docking strand)(其在一些实施方案中通过中间分子诸如抗体(诸如第一抗体或第二抗体)附接于目标靶)的重复、短暂结合，以获得荧光接通(ON)和关断(OFF)状态之间的随机开关(图1A和1B)。在未结合的状态下，只观察到来自部分淬灭(参考文献8)的成象链的本底荧光(由图1A中未结合的成像链的暗弱荧光描绘的)。这被认为是"关断"状态。在结合和固定成像链后，使用例如全内反射(TIR)或高度倾斜和层叠光学片(HILO)显微术(参考文献9)检测荧光发射。这被认为是"接通"的状态。一般地，如本文中提供的方法、组合物和试剂盒提高成像分辨率和从而检测的灵敏度。在一些方面中，它们还提高特异性以及增加可用于检测目标靶(包括但不限于例如天然存在的生物分子)的可利用的荧光团的数目。

[0008] 通过将短的对接链连接于结合伴侣(例如，蛋白质结合部分或核酸结合部分，无论一级还是二级)诸如抗体(包括第一和第二抗体)，可以标记不同种类的靶(例如，生物分子，任选在细胞环境中)并随后通过引入与对接链互补并通过短暂的Watson-Crick相互作用与其结合的荧光标记的成像链来检测其。与现有的检测方法不同，本公开内容的方法不受可用于检测不同靶(例如，生物分子)的光谱上不同的荧光团的数目限制。相反地，核酸(例如，DNA和/或RNA)分子和连续延时成像的可编程性在本文中用于在一些实施方案中仅使用单一优化荧光团来提供高达数百个不同种类的靶的图像。另外，可使用单一荧光标记的成像

链与它们的互补靶对接链的可预测的结合动力学来定量这些不同种类的靶(例如,生物分子)。

[0009] 在一些情况下,所述方法可用于产生超分辨率图像,值得注意地甚至无需超分辨率显微镜。应当理解,虽然将如本文中提供的方法、组合物和试剂盒描述用于超分辨率成像,但在一些实施方案中,还可将它们用于不要求超分辨率的成像。因此,在一些实施方案中,通常可将本公开内容的方法、组合物和试剂盒用于成像。

[0010] 在一些方面,本文中提供了蛋白质-核酸缀合物,其包含连接于能够短暂结合于互补的经标记的成像链的对接链的蛋白质。在一些方面,本文中提供了蛋白质-核酸缀合物,其包含连接于短暂结合于互补的经标记的成像链的对接链的蛋白质。在一些实施方案中,用可检测标记物标记成像链。可检测标记物可以是例如荧光标记物或其它可检测标记物,例如,金纳米颗粒。虽然本文中的各个方面和实施方案提及荧光标记的成像链,但应理解在许多情况下,可将这样的荧光标记物与其它可检测标记物互换。因此,在一些实施方案中,可将荧光标记的成像链(其可通过例如荧光显微术来检测)与利用例如金纳米颗粒标记的成像链(其可通过例如暗视野显微术来检测)互换。还应理解,对接链可以能够短暂结合多个互补的标记链(例如,对接链可包含针对互补的标记链的多个结合位点)。

[0011] 在一些实施方案中,可进行牵涉多个对接链和成像链对的方法。这样的方法可用于检测多个靶(例如,每个对接链-成像链对对应于一个靶)。多个对接链-成像链对必须共有几乎相等的在单个环境或条件(如例如由温度、盐浓度、链摩尔浓度等定义的)下杂交的概率,以使得如果一组成像链的结合(和从而检测)水平之间存在观察到的差异,则终端用户可得出这样的差异是对接链的量和从而最终地靶的量的函数。在一些实施方案中,通常选择对接链和成像链,以使得它们的结合状态具有在约+/-0.5kcal/mol的范围内的热稳定性。对于该范围的热稳定性,有可能选择至少200个正交(例如,不同的)序列来用于这些复用方法。

[0012] 在一些实施方案中,蛋白质是抗体诸如第一抗体或第二抗体、抗原结合抗体片段或肽适体。

[0013] 在一些实施方案中,将蛋白质通过中间接头连接于对接链。在一些实施方案中,所述中间接头包含生物素和链霉抗生物素蛋白。

[0014] 在一些实施方案中,抗体为单克隆抗体。

[0015] 在一些实施方案中,互补的荧光标记的成像链包含至少一个荧光团。

[0016] 在一些实施方案中,互补的经标记的(任选地荧光标记的)成像链的长度为约4个至约30个核苷酸,或约8个至约10个核苷酸。在一些实施方案中,互补的经标记的成像链长于30个核苷酸。

[0017] 在本文所述的这方面和其它方面及实施方案中,对接链可包含多个结构域,其每一个与经标记的成像链互补。所述结构域在序列上可以是相同的(从而将结合于相同的成像链)或它们可具有不同的序列(从而可结合于被不同地标记的成像链)。这样的结构域在本文中还可被称为成像链的结合位点。

[0018] 在一些实施方案中,对接链包括至少2个或至少3个结构域,其每一个各自与经标记的成像链互补。

[0019] 在一些方面,本文中提供了结合于至少一个蛋白质-核酸缀合物的靶。

[0020] 在一些实施方案中，所述靶为蛋白质。在一些实施方案中，所述靶为核酸(例如，DNA或RNA)。

[0021] 在一些方面，本文中提供了多个蛋白质-核酸缀合物。在一些实施方案中，所述多个蛋白质-核酸缀合物包含至少2个亚组的蛋白质-核酸缀合物，并且每一个亚组的蛋白质-核酸缀合物结合不同的靶。

[0022] 在一些方面，本文中提供了包含多个蛋白质-核酸缀合物的组合物或试剂盒，任选地其中蛋白质-核酸缀合物的至少一种结合于至少一种靶。

[0023] 在一些方面，本文中提供了包含至少一种含有连接于对接链的蛋白质的蛋白质-核酸缀合物的组合物或试剂盒，任选地其中所述至少一种蛋白质-核酸缀合物结合于靶，和至少一种互补的经标记的(任选地荧光标记的)成像链，所述成像链短暂地结合于(或能够短暂地结合于)所述至少一种蛋白质-核酸缀合物。

[0024] 在一些实施方案中，组合物或试剂盒包含至少两种互补的经标记的(任选地荧光标记的)成像链，其中所述至少两种互补的经标记的成像链是相同的。在一些实施方案中，所述组合物或试剂盒包含至少两种互补的经标记的成像链，其中所述至少两种互补的经标记的成像链是不同的。

[0025] 在一些实施方案中，互补的经标记的(任选地荧光标记的)成像链的数目少于、多于或等于蛋白质-核酸缀合物的数目。

[0026] 在一些实施方案中，组合物或试剂盒包含至少2、3、4、5、6、7、9或10种不同的互补的经标记的(任选地荧光标记的)成像链。在一些实施方案中，所述组合物或试剂盒包含至少50种或至少100种不同的互补的荧光标记的的成像链。

[0027] 在一些方面，本文中提供了包含(例如，一种或多种)对接链和(例如，一种或多种)成像链的组合物或试剂盒。可修饰对接链以包括亲和标记物，从而促进其随后与一个或多个结合伴侣诸如抗体的附着。例如，可将所述对接链生物素化或可将其附接于抗生物素蛋白或链霉抗生物素蛋白。可替换使用其它亲和标记物。可标记(诸如荧光标记)成像链。所述成像链可以是多个相同的成像链(例如，在序列和标记方面)或它们可以是多个不同的成像链(例如，在序列和标记方面)。所述组合物或试剂盒还可包含靶特异性结合伴侣，诸如抗体。应理解，这样的组合物和试剂盒中的组分可彼此结合或它们可以是未结合的，包括彼此物理分开。这些和其它组合物以及试剂盒还可包含一种或多种缓冲剂，包括氧清除剂。

[0028] 在一些方面，本文中提供了包含抗体-核酸缀合物的组合物或试剂盒，其中所述抗体为具有针对抗体的特异性，通常地针对来自特定物种的抗体的特定同种型或Fc结构域的特异性的“第二抗体”(例如针对人IgG1抗体特异的小鼠抗体)。缀合物中的核酸为如本文所述的对接链。所述组合物或试剂盒还可包含如本文中描述的一种或多种成像链(一个或多个亚组或群体的成像链)。这些和其它组合物及试剂盒还可包含一种或多种缓冲剂，包括氧清除剂。

[0029] 在一些方面，本公开内容提供了抗体-DNA缀合物，其包含连接于对接链的单克隆抗体，所述对接链结合于互补的经标记的(任选地荧光标记的)成像链，其中所述抗体和所述对接链各自被生物素化并且通过抗生物素蛋白或链霉抗生物素蛋白接头或生物素-链霉抗生物素蛋白接头彼此连接。

[0030] 在一些方面，本文中提供了适体-核酸缀合物，其包含连接于短暂地结合于互补的

经标记的(任选地荧光标记的)成像链的对接链的核酸适体。

[0031] 在一些方面,本文中提供了检测样品中的靶的方法,所述方法包括将样品与如下物质接触:(a)至少一种包含连接于对接链的蛋白质的蛋白质-核酸缀合物和(b)至少一种与所述至少一种蛋白质-核酸缀合物的对接链互补并短暂结合的荧光标记的成像链;以及测定所述至少一种蛋白质-核酸缀合物是否结合于样品中的靶。在一些实施方案中,所述测定步骤包括对所述至少一种荧光标记的成像链与所述至少一种蛋白质-核酸缀合物的对接链的短暂结合进行成像。

[0032] 在一些方面,本文中提供了用于检测样品中的靶的方法,所述方法包括将样品与如下物质接触:(a)至少一种包含连接于对接链的蛋白质的蛋白质-核酸缀合物和(b)至少一种与所述至少一种蛋白质-核酸缀合物的对接链互补并短暂结合的荧光标记的成像链;以及任选地使用延时成像对所述至少一种荧光标记的的成像链与所述至少一种蛋白质-核酸缀合物的对接链的短暂结合进行成像。

[0033] 在一些实施方案中,所述蛋白质-核酸缀合物的蛋白质是抗体、抗原结合抗体片段或肽适体。在一些实施方案中,抗体为单克隆抗体。

[0034] 在一些实施方案中,所述蛋白质-核酸缀合物的蛋白质通过中间接头连接于对接链。在一些实施方案中,中间接头包含生物素和/或链霉抗生物素蛋白。

[0035] 在一些实施方案中,互补的荧光标记的成像链包含至少一个荧光团。

[0036] 在一些实施方案中,互补的经标记的(任选地荧光标记的)成像链的长度为约4至约10个核苷酸,或约8至约10个核苷酸。

[0037] 在一些实施方案中,样品为细胞或细胞裂解物。

[0038] 在一些实施方案中,靶为蛋白质。在一些实施方案中,靶为核酸(例如,DNA或RNA)。

[0039] 在一些实施方案中,从细胞或细胞裂解物获得靶。

[0040] 在一些方面,本文中提供了检测样品中的至少一种或至少两种靶的方法,所述方法包括将样品与如下物质接触:(a)至少两种蛋白质-核酸缀合物,其各自包含连接于对接链的蛋白质,和(b)至少两种经标记的(任选地光谱上不同的、或荧光标记的、或光谱上不同的且荧光标记的)成像链,所述成像链与至少一种或至少两种不同的蛋白质-核酸缀合物的各自的对接链互补并与其短暂结合;以及测定所述至少两种蛋白质-核酸缀合物是否结合于样品中的至少两种靶。在一些实施方案中,所述测定步骤按以下顺序包括:对至少两种经标记的成像链之一与所述至少两种蛋白质-核酸缀合物之一的对接链的短暂结合进行成像以产生第一图像(例如,荧光信号的),和对所述至少两种经标记的成像链的另一种与所述至少两种蛋白质-核酸缀合物的另一种的对接链的短暂结合进行成像以产生至少一个其它的图像(例如,荧光信号的)。在一些实施方案中,所述方法还包括将所述第一图像与所述至少一个其它的图像组合以产生信号(例如,荧光信号)的合成图像,其中所述合成图像的信号代表至少两种靶。

[0041] 在一些实施方案中,蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。在一些实施方案中,抗体为单克隆抗体。

[0042] 在一些实施方案中,蛋白质-核酸缀合物的蛋白质通过中间接头连接于对接链。在一些实施方案中,中间接头包含生物素和链霉抗生物素蛋白。

[0043] 在一些实施方案中,至少两种光谱上不同的荧光标记的成像链的每一种包含至少

一个荧光团。

[0044] 在一些实施方案中，所述至少两种经标记的(任选地光谱上不同的、荧光标记的)成像链的每一种的长度为约4至约10个核苷酸，或约8至约10个核苷酸。

[0045] 在一些实施方案中，样品为细胞或细胞裂解物。

[0046] 在一些实施方案中，至少两种靶为蛋白质。在一些实施方案中，至少两种靶为核酸(例如，DNA或RNA)。

[0047] 在一些实施方案中，至少两种靶获自细胞或细胞裂解物。

[0048] 在一些方面，本文中提供了检测样品中的至少一种或至少两种蛋白质靶的方法，所述方法包括(a)将样品与至少两种蛋白质-核酸缀合物接触，所述缀合物各自包含连接于对接链的蛋白质，和(b)随后将所述样品与至少两种经标记的(例如，任选地光谱上无差别的，或荧光标记的，或光谱上不同的且荧光标记的)成像链接触，所述成像链与所述至少两种蛋白质-核酸缀合物的各自的对接链互补并与其短暂结合，以及测定所述至少一种或至少两种蛋白质-核酸缀合物是否结合于样品中的至少两种靶。在一些实施方案中，所述方法按照以下顺序的步骤包括：将所述样品与第一蛋白质-核酸缀合物和至少一种其它的蛋白质-核酸缀合物接触，将所述样品与第一经标记(任选地荧光标记的)成像链接触，所述成像链与所述第一蛋白质-核酸缀合物的对接链互补并与其短暂结合，任选地使用延时成像对样品进行成像以获得第一图像，除去所述第一经标记的成像链，将样品与至少一种其它的经标记的成像链接触，所述成像链与所述至少一种其它的蛋白质-核酸缀合物的对接链互补并与其短暂结合，以及任选地使用延时成像对样品进行成像以获得至少一个其它的图像。

[0049] 在一些实施方案中，方法按照下列顺序步骤包括：将样品与第一蛋白质-核酸缀合物接触，将所述样品与第一经标记的(任选地荧光标记的)成像链接触，所述成像链与第一蛋白质-核酸缀合物的对接链互补并与其短暂结合，任选地使用延时成像对样品进行成像以获得第一图像，除去所述第一经标记的成像链，将样品与至少一种其它的蛋白质-核酸缀合物接触，将样品与至少一种其它经标记的(任选地荧光标记的)成像链接触，所述成像链与所述至少一种其它蛋白质-核酸缀合物的对接链互补并与其短暂结合结合，以及任选地使用延时成像对样品成像以获得至少一个其它的图像。

[0050] 在一些实施方案中，方法还包括测定第一蛋白质-DNA缀合物是否结合于第一靶和/或至少一种其它的蛋白质-DNA缀合物是否结合于至少一种其它的靶。

[0051] 在一些实施方案中，方法还包括将伪彩色赋予第一图像中的信号(例如，荧光信号)，和将至少一种其它的伪彩色赋予所述至少一个其它的图像中的荧光信号。

[0052] 在一些实施方案中，方法还包括将第一图像和所述至少一个其它的图像组合以产生伪彩色化信号的合成图像，其中所述合成图像的伪彩色化信号代表至少两种靶。

[0053] 在一些实施方案中，蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。在一些实施方案中，抗体为单克隆抗体。

[0054] 在一些实施方案中，蛋白质-核酸缀合物的蛋白质通过中间接头连接于对接链。在一些实施方案中，所述中间接头包含生物素和/或链霉抗生物素蛋白。

[0055] 在一些实施方案中，荧光标记的成像链的每一个包含至少一个荧光基团。

[0056] 在一些实施方案中，荧光标记的成像链的每一个的长度为约4至约30个核苷酸，或

约8至约10个核苷酸。

[0057] 在一些实施方案中，样品为细胞或细胞裂解物。

[0058] 在一些实施方案中，靶为蛋白质。在一些实施方案中，靶为核酸(例如，DNA或RNA)。

[0059] 在一些实施方案中，从细胞或细胞裂解物获得靶。

[0060] 在一些方面，本文中提供了检测靶(任选地天然存在的生物分子)的方法，所述方法包括将含有至少一种靶(任选地天然存在的生物分子)的样品与如下物质接触：(a)至少一种BP-NA缀合物，任选地每种BP-NA缀合物包含连接于对接链的蛋白质或核酸，和(b)至少一种经标记的(任选地荧光标记的)成像链，所述成像链与所述至少一种BP-NA缀合物的对接链互补并与其短暂结合；以及测定所述至少一种BP-NA缀合物是否结合于样品中的至少一种靶(任选地天然存在的生物分子)。在本文中描述的此方面和其它方面或实施方案中，应理解，所述方法可使用怀疑含有至少一种靶的样品或终端用户希望就至少一种靶的存在进行分析的样品来进行而无需样品的关于其含有所述靶的可能性的任何先验知识。

[0061] 在一些实施方案中，所述测定步骤包括对所述至少一种经标记的(任选地荧光标记的)成像链与所述至少一种BP-NA缀合物的对接链的短暂结合进行成像。

[0062] 在一些实施方案中，样品为细胞或细胞裂解物。

[0063] 在一些实施方案中，至少一种靶(任选地天然存在的生物分子)获自细胞或细胞裂解物。

[0064] 在一些实施方案中，蛋白质为抗体、抗原结合抗体片段或肽适体。在一些实施方案中，抗体为单克隆抗体。

[0065] 在一些实施方案中，蛋白质通过中间接头连接于对接链。在一些实施方案中，中间接头包含生物素和/或链霉抗生物素蛋白。

[0066] 在一些实施方案中，核酸为核酸适体。

[0067] 在一些实施方案中，荧光标记的成像链包含至少一个荧光团。

[0068] 在一些实施方案中，成像链(任选地荧光标记的成像链)的长度为约4至约30个核苷酸，或约8至约10个核苷酸。

[0069] 在一些方面，本文中提供了检测靶(任选地天然存在的生物分子)的方法，所述方法包括将含有至少两种靶(任选地天然存在的生物分子)的样品与如下物质接触：(a)至少两种不同的BP-NA缀合物，任选地各BP-NA缀合物包含连接于DNA对接链的蛋白质或核酸，和(b)至少两种经标记的(任选地光谱上无差别的、或荧光标记的、或光谱上不同的且荧光标记的)成像链，所述成像链与所述至少两种BP-NA缀合物的各自的对接链互补并与其短暂结合；以及测定所述至少两种BP-NA缀合物是否结合于样品中的至少一种或至少两种天然存在的生物分子。

[0070] 在一些实施方案中，方法按照以下顺序的步骤包括：将样品与第一BP-NA缀合物和至少一种其它的BP-NA缀合物接触，将样品与第一经标记的(任选地荧光标记的)成像链接触，所述成像链与所述第一BP-NA缀合物的对接链互补并与其短暂结合，任选地使用延时成像对样品成像以获得第一图像，除去所述第一经标记的成像链，将样品与至少一种其它的经标记的(任选地荧光标记的)成像链接触，所述成像链与所述至少一种其它的BP-NA缀合物的对接链互补并与其短暂结合，以及任选地使用延时成像对样品成像以获得至少一个其它的图像。

[0071] 在一些实施方案中，方法按照以下顺序的步骤包括：将样品与第一BP-NA缀合物接触，将样品与第一经标记的（任选地荧光标记的）成像链接触，所述成像链与所述第一BP-NA缀合物的对接链互补并与其短暂结合，任选地使用延时成像对样品成像以获得第一图像，除去所述第一经标记的成像链，将样品与至少一种其它的BP-NA缀合物接触，将样品与至少一种其它的经标记的（任选地荧光标记的）成像链接触，所述成像链与所述至少一种其它的BP-NA缀合物的对接链互补并与其短暂结合，以及任选地使用延时成像对样品成像以获得至少一个其它的图像。

[0072] 在一些实施方案中，方法还包括测定第一蛋白质DNA缀合物是否结合于第一靶（任选地天然存在的生物分子），和/或至少一种其它的蛋白质-DNA缀合物是否结合于至少一种其它的靶（任选地天然存在的生物分子）。

[0073] 在一些实施方案中，方法还包括将伪彩色赋予第一图像中的信号（例如，荧光信号），和将至少一种其它的伪彩色赋予至少一个其它的图像中的信号（例如，荧光信号）。

[0074] 在一些实施方案中，方法还包括将第一图像和至少一个其它的图像组合以产生伪彩色化信号的合成图像，其中所述合成图像的伪彩色化信号代表至少一种或至少两种靶（例如，天然存在的生物分子）。

[0075] 在一些方面，本文中提供了测定测试样品中的靶的数目的方法，所述方法包括获得包含直接或间接地短暂结合于经标记的（任选地荧光标记的）成像链的靶的样品，获得样品的延时图像，任选地延时衍射极限荧光图像，在衍射极限图像上进行斑点检测（例如，荧斑点检测）和定位（例如，通过使用高斯拟合）以获得样品的高分辨率图像，任选地使用具有已知数目的靶的对照样品校准$k_{on} \cdot c_{imager}$，其中k_{on}为二阶缔合常数，c_{imager}为测试样品中经标记的（例如，荧光标记的）成像链的浓度，任选地通过将荧光关断时间分布拟合至累积分布函数来测定变量τ_d，和基于下述方程测定样品中测试靶的数目：测试靶的数目$=(k_{on} \cdot c_{imager} \cdot \tau_d)^{-1}$。

[0076] 在一些方面，本文中提供了测定测试样品中的靶的相对量的方法，所述方法包括获得包含直接或间接地短暂结合于经标记的成像链的靶的样品，获得样品的延时图像，在图像上进行斑点检测和定位，以获得样品的高分辨率图像，测定变量τ_d，和基于τ_d测定样品中的两种或更多种测试靶的相对量。

[0077] 在一些实施方案中，测试靶为蛋白质靶。

[0078] 在一些实施方案中，蛋白质靶结合于蛋白质-核酸缀合物，所述蛋白质-核酸缀合物包含连接于对接链的蛋白质，并且经标记的（例如，荧光标记的）成像链与所述蛋白质-核酸缀合物的各自的对接链互补并与其短暂地结合。

[0079] 在一些实施方案中，所述蛋白质-核酸缀合物的蛋白质为抗体、抗原结合抗体片段或肽适体。

[0080] 在一些实施方案中，测试靶为单链核酸。

[0081] 在一些实施方案中，单链核酸为DNA或RNA。

[0082] 在一些实施方案中，荧光标记的成像链的每一个包含至少一个荧光团。

[0083] 在一些实施方案中，经标记的（任选地荧光标记的）成像链的每一个的长度为约4至约30个核苷酸，或约8至约10个核苷酸。

[0084] 在一些实施方案中，在约25分钟的时期内获得延时荧光图像。

[0085] 在一些实施方案中，以大于90％的精确度测定测试靶的数目。

[0086] 在一些方面，本文中提供了单链DNA探针，所述单链DNA探针包含任选地在其3'末端连接于至少1个、至少2个或至少3个子结构域的对接结构域的长度为约20个核苷酸的靶结合结构域，其中所述至少1个、至少2个或至少3个子结构域各自与至少1个、至少2个或至少3个长度为约4至约30个核苷酸，或约8至约10个核苷酸的经标记的(任选地荧光标记的)成像链互补，并且其中所述靶结合结构域结合于单链mRNA靶链的互补结构域。

[0087] 在一些实施方案中，至少1个、至少2个或至少3个子结构域的至少一个短暂地结合于至少一个经标记的(任选地荧光标记的)成像链。

[0088] 在一些方面，本文中提供了针对多个图像进行漂移校正的方法，其中多个图像的每一个包含图像的时间序列的帧，其中图像的时间序列捕捉多个短暂事件，所述方法包括测定在多个图像中鉴定的多个漂移标记(drift marker)的每一个的时间轨迹，其中每一个漂移标记的时间轨迹对应于图像中的物体在图像的时间序列上的运动，利用至少一个计算机处理器，至少部分基于多个漂移标记的时间轨迹测定来自多个漂移标记的至少一个的第一漂移校正，测定来自从多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹，其中多个漂移模板中的每个漂移模板描述漂移模板中的短暂事件的多个可几何寻址的标记位点之间的几何关系，至少部分地基于来自多个漂移模板的多个可几何寻址的标记位点的时间轨迹测定第二漂移校正，至少部分地基于所述第一漂移校正和所述第二漂移校正来校正多个图像，基于校正的多个图像输出最终图像。

[0089] 在一些实施方案中，方法还包括鉴定多个图像的每一个中的多个定位，产生多个定位的二维直方图，以及至少部分地基于所述二维直方图鉴定多个漂移标记的位置，其中测定多个漂移标记的每一个的时间轨迹包括至少部分地基于多个漂移标记的位置测定时间轨迹。

[0090] 在一些实施方案中，鉴定多个定位包括鉴定多个图像的每一个上的多个斑点，和使用局部高斯拟合算法测定多个斑点的每一个的拟合中心位置，其中多个定位的每一个包含在图像上鉴定的斑点及其相关拟合的中心位置。

[0091] 在一些实施方案中，多个定位的每一个还包含所检测的对应于所述定位的光子计数。

[0092] 在一些实施方案中，产生多个定位的二维直方图包括将所有定位区间化(binning)在二维网格中并使用每个区间(bin)中的定位的总数作为直方图计数。

[0093] 在一些实施方案中，产生多个定位的二维直方图包括将所有定位区间化在二维网格中并使用每个区间中的多个定位的光子计数的总数作为直方图计数。

[0094] 在一些实施方案中，至少部分地基于二维直方图鉴定多个漂移标记的位置包括下述的至少一种：使用一种或多种选择标准二值化所述二维直方图，其中所述一种或多种选择标准包括直方图值的下限阈值或直方图值的上限阈值；将二值化图像划分成分区并基于一种或多种选择标准过滤所述分区，其中一种或多种选择标准包括分区区域的面积的下限阈值、面积的上限阈值、最长分区的最长或最短线性尺度的下限或上限、以及分区的偏心率的下限或上限中的一种或多种；和使用一种或多种二值图像运算扩大和缩小二值化图像，其中所述一种或多种二值图像运算包括以下的一种或多种：膨胀运算、腐蚀运算、桥接运算、闭运算、开运算、填充运算、清理运算、顶帽运算、底帽运算、增厚运算、减薄运算等。

[0095] 在一些实施方案中，至少部分地基于多个漂移标记的时间轨迹测定第一漂移校正包括：测定多个漂移标记的每一个的相对时间轨迹，其中通过将所述漂移标记的时间轨迹与相同轨迹的平均位置进行比较来测定相对时间轨迹；和基于多个漂移标记的每一个的相对时间轨迹测定组合时间轨迹，其中至少部分地基于多个漂移标记的时间轨迹测定第一漂移校正包括至少部分地基于多个漂移标记的每一个的相对时间轨迹测定第一漂移校正。

[0096] 在一些实施方案中，至少部分地基于多个漂移标记的每一个的相对时间轨迹测定第一漂移校正包括进行多个漂移标记的每一个的相对时间轨迹的加权平均。

[0097] 在一些实施方案中，进行加权平均包括：测定相对时间轨迹的每一个的质量评分，其中至少部分地基于与所述时间轨迹相关的随时间过去的变化性的测量和/或所述时间轨迹内个体定位的定位不确定性的测量来测定所述质量评分。

[0098] 在一些实施方案中，随时间过去的变化性的测量包括随时间过去的时间轨迹的标准差。

[0099] 在一些实施方案中，个体定位的定位不确定性的测量至少部分地包括来自高斯拟合的不确定性评估或与其它同时定位的比较，其中所述其它同时定位来自相同的图像内且来自多个漂移标记的其它时间轨迹，其中所述比较包括所有同时定位的平均值和标准差。

[0100] 在一些实施方案中，所述方法还包括测定多个漂移标记的第一漂移标记不存在于图像的时间序列的至少一个帧中，和针对所述至少一个帧线性地内插所述第一漂移标记的时间轨迹，以产生所述第一漂移标记的平滑时间轨迹。

[0101] 在一些实施方案中，测定来自从多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹包括：鉴定多个图像的每一个中的多个定位；产生多个定位的二维直方图；和至少部分地基于所述二维直方图鉴定多个漂移模板，其中鉴定多个漂移模板包括使用直方图计数的下限域值和/或上限域值评估二维直方图。

[0102] 在一些实施方案中，测定来自从多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹包括测定多个漂移模板的每一个内的多个可几何寻址的标记位点的每一个的时间轨迹，并且其中测定第二漂移校正包括至少部分地基于多个漂移模板的每一个内的多个标记位点的每一个的时间轨迹测定第二漂移校正。

[0103] 在一些实施方案中，至少部分地基于来自多个漂移模板的每一个的多个可几何寻址的标记位点的每一个的时间轨迹测定第二漂移校正包括：鉴定所述多个漂移模板的每一个内的多个可几何寻址的标记位点；和测定多个漂移模板的每一个的多个可几何寻址的漂移标记的每一个的相对时间轨迹；其中至少部分地基于多个漂移模板的时间轨迹测定第二漂移校正包括至少部分地基于多个漂移模板的每一个内的多个漂移标记的每一个的相对时间轨迹测定第二漂移校正。

[0104] 在一些实施方案中，鉴定来自多个漂移模板的每一个的多个可几何寻址的标记位点包括至少部分地基于对应的漂移模板中多个定位的二维直方图和/或一种或多种选择标准测定多个标记位点，其中所述一种或多种选择标准包括定位的总数、定位的表面密度和定位的标准差的一种或多种。

[0105] 在一些实施方案中，至少部分地基于多个漂移模板的每一个内的多个漂移标记的每一个的相对时间轨迹测定第二漂移校正包括进行所述漂移模板的每一个内的多个漂移标记的每一个的相对时间轨迹的加权平均。

[0106] 在一些实施方案中，进行加权平均包括：

[0107] 测定相对时间轨迹的每一个的质量评分，其中至少部分地基于与时间轨迹相关的随时间过去的变化性和/或时间轨迹内的定位不确定性的测量来测定所述质量评分。

[0108] 在一些实施方案中，随时间过去的变化性的测量包括随时间过去的时间轨迹的标准差。

[0109] 在一些实施方案中，个体定位的定位不确定性的测量包括来自高斯拟合的不确定性评估或与其它同时定位的比较，其中所述其它同时定位来自相同的图像内并来自多个漂移模板的多个标记位点的其它时间轨迹，其中所述比较包括所有同时定位的平均值和标准差。

[0110] 在一些实施方案中，至少部分地基于第一漂移校正和第二漂移校正来校正多个图像包括使用第一漂移校正来校正多个图像以产生第一校正的多个图像，并且其中测定从所述多个图像鉴定的多个漂移模板的每一个的时间轨迹包括测定从所述第一校正的多个图像鉴定的多个漂移模板的每一个的时间轨迹。

[0111] 在一些实施方案中，方法还包括在使用第一漂移校正来校正多个图像之前使第一漂移校正平滑。

[0112] 在一些实施方案中，使第一漂移校正平滑包括使用具有通过所述第一漂移校正的特征性漂移时间尺度测定的窗口的局部回归处理所述第一漂移校正。

[0113] 在一些实施方案中，方法还包括在使用第二漂移校正来校正多个图像之前使第二漂移校正平滑。

[0114] 在一些实施方案中，使第二漂移校正平滑包括使用具有通过第二漂移校正的特征性漂移时间尺度测定的窗口的局部回归处理所述第二漂移校正。

[0115] 在一些实施方案中，方法还包括选择多个漂移标记的单个漂移标记；和至少部分地基于所选择的单个漂移标记测定第三漂移校正；其中校正多个图像包括至少部分地基于所述第三漂移校正来校正所述多个图像。

[0116] 在一些实施方案中，至少部分地基于第三漂移校正来校正所述多个图像在至少部分地基于第一漂移校正和第二漂移校正来校正所述多个图像之前进行。

[0117] 在一些实施方案中，方法还包括鉴定多个帧的第一图像中第一多个点的位置；鉴定多个图像的第二图像中第二多个点的位置，其中所述第二图像对应于图像的时间序列中第一图像的相邻的帧；和至少部分地基于所述第一多个点和所述第二多个点的位置之间的差异测定第四漂移校正；其中校正多个图像包括至少部分地基于所述第四漂移校正来校正所述多个图像。

[0118] 在一些实施方案中，所述第二图像对应于紧接在对应于图像的时间序列中的第一图像的帧之后的帧。

[0119] 在一些实施方案中，至少部分地基于所述第一多个点和所述第二多个点的位置之间的差异测定第四漂移校正包括：产生所述第一多个点和所述第二多个点的位置之间的距离的直方图；至少部分地基于所述直方图测定所述第一图像与所述第二图像之间对应于相同短暂事件的成对的点；和测定所测定的成对的点的每一对之间的位置偏移，其中基于所测定的成对的点的每一对的位置偏移的矢量平均值测定所述第四漂移校正。

[0120] 在一些实施方案中，多个图像对应于基于DNA的图像并且其中多个短暂事件是成

像链与DNA对接链之间的结合事件。

[0121] 在一些实施方案中，所述成像链为被配置为当与DNA对接链结合时发荧光的荧光成像探针。

[0122] 在一些实施方案中，漂移标记的至少一种为基于DNA的纳米结构。

[0123] 在一些实施方案中，基于DNA的纳米结构为具有对接链的DNA折纸纳米结构。

[0124] 在一些实施方案中，漂移模板的至少一个为基于DNA的纳米结构。

[0125] 在一些实施方案中，基于DNA的纳米结构为具有对接链的DNA折纸纳米结构。

[0126] 在一些实施方案中，漂移模板的至少一个为三维漂移模板。

[0127] 在一些实施方案中，三维漂移模板为四面体。

[0128] 在一些实施方案中，漂移模板的至少一个包含对应于不同类型的短暂事件的多个颜色。

[0129] 在一些实施方案中，所述不同类型的短暂事件包括第一成像链与第一类型的DNA对接链的第一结合事件和第二成像链与第二类型的DNA对接链的第二结合事件。

[0130] 在一些实施方案中，输出最终图像包括在显示器上显示所述最终图像。

[0131] 在一些实施方案中，输出最终图像包括通过至少一个网络将最终图像发送至计算机。

[0132] 在一些实施方案中，输出最终图像包括将最终图像存储在至少一个存储设备上。

[0133] 在一些方面，本文中提供了利用多个指令编码的非暂时性计算机可读介质，所述指令在通过至少一个计算机处理器执行时，执行针对多个图像进行漂移校正的方法，其中所述多个图像的每一个包含图像的时间序列的帧，其中所述图像的时间序列捕捉多个短暂事件，所述方法包括：测定在所述多个图像中鉴定的多个漂移标记的每一个的时间轨迹，其中每个漂移标记的时间轨迹对应于图像中的物体在图像的时间序列上的运动；至少部分地基于所述多个漂移标记的时间轨迹测定来自所述多个漂移标记的至少一个的第一漂移校正；测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹，其中所述多个漂移模板中的每个漂移模板描述了漂移模板中短暂事件的多个可几何寻址的标记位点之间的几何关系；至少部分地基于来自所述多个漂移模板的多个可几何寻址的标记位点的时间轨迹测定第二漂移校正；至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像；和基于校正的多个图像输出最终图像。

[0134] 在一些方面，本文中提供了计算机，其包含：被配置为接受多个图像的输入接口，其中所述多个图像的每一个包含图像的时间序列的帧，其中所述图像的时间序列捕捉多个短暂事件；至少一个处理器，其被编程来：测定在多个图像中鉴定的多个漂移标记的每一个的时间轨迹，其中每个漂移标记的时间轨迹对应于图像中的物体在图像的时间序列上的运动；至少部分地基于所述多个漂移标记的时间轨迹测定来自所述多个漂移标记的至少一个的第一漂移校正；测定来自从所述多个图像鉴定的多个漂移模板的多个可几何寻址的标记位点的每一个的时间轨迹，其中所述多个漂移模板中的每个漂移模板描述了漂移模板中短暂事件的多个可几何寻址的标记位点之间的几何关系；至少部分地基于来自所述多个漂移模板的多个可几何寻址的标记位点的时间轨迹测定第二漂移校正；至少部分地基于所述第一漂移校正和所述第二漂移校正来校正所述多个图像；和基于校正的多个图像测定最终图像；以及被配置为输出所述最终图像的输出接口。

[0135] 附图概述

[0136] 图1A显示在一对间隔约16纳米(nm)的相对面(以深灰色着色的)上用单链DNA对接链"标记的"微管样DNA折纸聚合物。互补的荧光标记的成像链从溶液短暂地结合于对接链。生物素化的DNA链(存在于底部两个中央螺旋上)用于将微管样DNA结构结合于玻璃表面以用于荧光成像。图1B显示荧光标记的成像链与对接链的短暂结合产生荧光"闪烁"(荧光强度相对于时间轨迹)。此闪烁用于连续定位衍射极限之下的点。图1C显示具有测量的16±1nm(平均值±stdv)[比例尺:40nm]的宽度的DNA折纸聚合物的透射电子显微镜(TEM)图像。图1D显示使用Cy3b-标记的成像链获得的超分辨率荧光图像(15,000帧,5Hz帧率)。两个不同的线是可见的[比例尺:40nm]。图1E显示图1D中的突出显示的区域<i>和<ii>的横截面直方图(箭头表示直方图方向),其显示清楚地解析了约16nm的设计距离(观察到每个分布的半峰全宽(FWHM)为约9nm)。

[0137] 图2显示本公开内容的生物分子标记方案的实例,其中利用本公开内容的抗体-DNA缀合物和互补的荧光标记的成像链标记蛋白质(例如,蛋白质靶)。抗体通过含有生物素和链霉抗生物素蛋白(例如,生物素-链霉抗生物素蛋白-生物素接头)的接头连接于对接链。

[0138] 图3A显示固定的HeLa细胞内的微管网络的使用抗体-DNA缀合物和Atto655-标记的成像链进行的超分辨率图像(10,000帧,10Hz帧率)[比例尺:5μm]。图3B显示图3A中突出显示的区域的高倍图像[比例尺:1μm]。图3C显示图3B中相同区域的衍射极限图。箭头突出显示其中图像分辨率的增加清楚可见的位置。在图3B中位置<i>上具有约46nm的表观宽度的相邻微管间隔约79nm[比例尺:1μm]。图3D显示使用抗体-DNA缀合物、Cy3b-标记的成像链(对于微管)(线状结构)和Atto655-标记的成像链(对于线粒体)(片状结构)获得的固定的HeLa细胞内的微管和线粒体的双色超分辨率图像(15,000帧,10Hz帧率)[比例尺:5μm]。图3E显示图3D中突出显示的区域的高倍图像[比例尺:1μm]。图3F显示图3E中显示的相同区域的衍射极限图像[比例尺:1μm]。

[0139] 图4A显示本公开内容的使用光谱上无差别的成像链(例如,各自用相同颜色荧光团标记的)的一个实施方案。在步骤[1]中,3个不同种类的对接链(a,b,c)标记表面。这样的标记可使用单独的或连接于蛋白质-结合分子(例如,抗体)或核酸-结合分子来进行,所述结合分子结合目标表面/生物分子。在步骤[2]中,引入多个拷贝的成像链a*(其中a*具有与a互补的序列),并且对利用对接链a标记的点进行成像。在步骤[3]中,将成像链a*的拷贝冲刷走,并且将成像链b*引入以对b标记的点成像。获取图像,并将成像链b*洗掉。在步骤[4]中,以相同的方式对c标记的点成像。在步骤[5]中,将来自[2-4]的图像赋予伪彩色并且将其组合以产生最终图像。虽然伪彩色可用于图像的最终渲染,但实际上用相同颜色染料(例如,荧光团)标记所有成像链。图4B(1)-4B(3)显示减小图像密度同时使可达到的分辨率增加高达系数$2\sqrt{2\ln 2} \approx 2.35$.此处,先前被定义为重建的定位的FWHM的分辨率可被理解为如在利用稀疏点的定位显微术中的定位的标准差。图4B(1)显示以线性几何间隔10nm的7个点(上图)。具有约14nm分辨率的模拟超分辨率数据(中图)。不能解析的点。横截面直方图数据显示宽峰(底图)。图4B(2)和4B(3)显示每隔一个位点进行成像允许个体斑点的定位。随后可将这些定位组合以形成完全模式的最终图像。图4C显示展示相距10nm间隔的对接链的DNA折纸结构的图像。

[0140] 图5A显示本公开内容的使用类似编号0-3(0、1、2和3)的在指定位置上具有不同种类的对接链的DNA折纸结构的一个实施方案。对于每一回合,将各自的成像链序列添加至成像室,进行图像获取,并且洗掉成像链。在每一个成像回合中,对设计的编号成像,从而显示在不同回合之间无串扰的非常序列特异性的相互作用[比例尺:50nm]。注意,用相同颜色染料标记所有成像链,尽管将每个结构(例如,0-3(0、1、2和3))渲染不同的颜色(例如,紫色、黄色、蓝色或红色;未显示颜色渲染)。图5B(i)-(v)显示本公开内容的使用类似编号0-9(0、1、2、3、4、5、6、7、8和9)的在指定位置上具有不同种类的对接链的DNA折纸结构的另一个实施方案。图5B(i)显示交换-PAINT示意图,其显示使用利用相同荧光团标记的成像链的多个靶的连续成像。图5B(ii)显示展示类似数字4的对接链的DNA折纸(70100nm)的示意图。图5B(iii)显示所有10个交换-PAINT循环的组合概括图像,显示了在成像循环之间无串扰的与各自的靶的特异性相互作用。比例尺:250nm。图5B(iv)显示数字0至3的4-"色"图像,所述数字均呈现在相同的DNA折纸(每个10,000帧,5Hz帧率;底部的示意图)上。比例尺:25nm。图5B(v)显示10个不同折纸结构的伪彩色图像,其每一个渲染不同的颜色(例如,橙色、绿色、蓝色、紫色、粉色等;颜色渲染未显示),以高分辨率(条状特征的FWHM<10nm)和特异性在一个样品中展示数字0至9。仅使用一个荧光团(Cy3b)通过10个成像-洗涤循环(成像:7,500帧/循环,5Hz帧率;洗涤:每循环1-2分钟)获得的图像。比例尺:25nm。

[0141] 图6A显示本公开内容的使用固定的HeLa细胞的一个实施方案的实验示意图,其中在一个回合中,将对接链结合于靶,随后添加经标记的成像链,获得图像,并洗掉成像链。利用特异于不同靶的对接链以及不同的经标记的成像链重复每一回合。可单独的或连接于结合于目标靶的蛋白质-结合分子(例如,抗体)或核酸结合分子来使用对接链。图6B显示在固定的HeLa细胞中使用Cy3b-标记的成像链的本公开内容的方法的两个回合。此处,利用对接序列a标记微管(绿色伪彩色;颜色渲染未显示)以及利用正交对接序列b标记线粒体(品红伪彩色;颜色渲染未显示)。图6C显示类似于图6B使用ATTO655-标记的成像链在固定的HeLa细胞中进行的本公开内容的方法的两个回合。[比例尺:5μm]。注意,用相同颜色染料标记所有成像链。

[0142] 图7A显示荧光标记的成像链从溶液短暂地结合于目标结构或分子上的互补对接链。所述短暂结合产生如在具有特征性荧光接通和关断时间(分别为τ_b和τ_d)的结合相对于时间轨迹中显示的表观闪烁。来自溶液的成像链的检测到的结合频率线性地依赖于给定图像区域中可获得的对接链的数目(即,对接链越多,结合频率越高)。结束事件之间的时间,即荧光关断时间(τ_d)与对接链的数目成反比。图7B显示平均荧光关断时间(τ_d)可通过计算关断时间分布的累积分布函数(CDF)来测定。给定已知的缔合常数k_{on}和成像链浓度c,结合位点的数目可通过:结合位点的数目$=(\tau_d \cdot c \cdot k_{on})^{-1}$来计算。图7C显示被设计来作为概念验证平台展示13个结合位点的DNA折纸结构的超分辨率图像。对接位点的掺入效率不是100%,从而导致实际掺入位点的分布(图7D(1))。所述结构用作理想的测试系统,因为展示的对接位点的数目可通过对点的数目进行计数(直接计数)和将其与使用提出的结合动力学分析计算的对应的位点数目相比较来可视地测定。图7D(1)显示通过直接视觉计数获得的377个折纸结构的结合位点分布。图7D(2)显示通过本公开内容的结合动力学分析(动力学)获得的相同结构的结合位点分布。图7D(3)显示直接和动力学计数之间的"偏离":本公开内容的方法的计数"误差"或不确定性小于7%(通过高斯分布的变异系数测定的)。

[0143] 图8A显示在FISH样杂交方案中在固定的大肠杆菌(Escherichia coli)细胞中使用对接链标记的目标mRNA分子。图8B显示用于测定每个成像颜色的结合频率的读出方案。每个单个mRNA位置的强度相对于时间特征谱产生每个颜色特异性短暂结合模式(闪烁)。结合事件的频率取决于结合位点的数目,从而允许使用结合频率来区分结合位点的不同整数数目。图8C显示对于红色、绿色和蓝色成像链(颜色渲染未显示)的每一个分别展示3、9、22和44个结合位点的DNA折纸结构的体外原理验证实验。对于每一种颜色可清楚地区分不同的结合水平,这表明每颜色4种可能的"频率水平",从而产生用于条形编码例如细胞内的mRNA分子的高达124种不同的可能组合。可通过将荧光接通时间用作另外的编码实体来增加条形编码空间。

[0144] 图9显示展示可独立于荧光关断时间(涉及缔合速率k_{on})来调整荧光接通时间(涉及解离速率k_{off})的图。通过添加单个CG碱基对将成像/对接双链体从9个核苷酸延长至10个核苷酸(nt),使动力学解离速率减小几乎一个数量级(8)。

[0145] 图10A显示条形码探针,其长度大致为50个核苷酸并且可使用后接具有针对红色、绿色或蓝色成像链的8、9或10nt长的结合结构域的组合的大约30nt长的"条形码"区的21nt的靶检测结构域t*来标记生物分子。此处,展现了针对3种颜色分别具有每秒10、1和0.1的k_{off}的8、9或10nt长的对接链(颜色渲染未显示)。图10B显示相较于8nt相互作用结构域针对9nt相互作用结构域的荧光接通时间τ_b增加的特征性强度相对于时间轨迹。图10C显示证明可区分每秒10、1和0.1的k_{off}值的随机模拟。

[0146] 图11A显示在其中将单个荧光团稳定地附接于成像表面(参见图11B(1))的传统检测方法中,发射每"开关"事件有限数目的光子(上图),所有光子从"可补充的"成像链的提取(参见图11B(2))导致每开关事件更高的定位精度(中图),DNA元荧光团(metafluorophore)(参见图11B(3)和图11B(4))产生比图11B(2)中的单荧光团每开关事件显著更大数量的光子(下图)。图11B(1)-11B(3)显示目前成像方法和本公开内容的方法的示意图。图11B(1)显示使用稳定地附接于成像表面的荧光团的传统检测法(例如,于STORM中)。图11B(2)显示本公开内容的利用短暂地结合于成像表面的荧光团的检测法的一个实施方案。图11B(3)显示紧密DNA纳米结构中具有8个荧光团的明亮元荧光团。图11B(4)显示利用淬灭剂(暗点)和仅当短暂地结合于表面时才发荧光的荧光团(星号)修饰的条件元荧光团。图11C显示具有以4×3网格(间隔20nm)排列的对接位点的DNA折纸结构。单个位点以约3nm(目前所证实的最高的分辨率)的精确度光学定位[比例尺:50nm]。图11D显示用作超分辨率成像的测试平台的280nm×240nm DNA纳米矩形(单链瓦片(tile)结构(15),比折纸10x更大的面积),其展示2000个具有7或5nm间距的单链对接链(点)[比例尺:100nm]。

[0147] 图12A(i)举例说明显示漂移校正的每个阶段的原理的示意图。在每个图像中,黑色标记和线表示源数据,灰色值和曲线表示计算的漂移校正。图12A(ii)显示用于每个阶段的主要类型的漂移标记(例如,DNA漂移标记)的示意图。图12B(i)举例说明显示每个阶段或校正后的成像质量的示例性结构,以及图12B(ii)显示在每个阶段12B(i)中的对应的绿色矩形的放大图像。图12B(i)和12B(ii)中显示的比例尺对应于50nm。图12C(i)举例说明每个阶段校正后的示例性漂移轨迹,图12C(ii)显示在每个阶段图12C(i)中的对应矩形的放大图像。图12C(i)中的比例尺对应于x:500nm,t:500s,图12C(ii)中的比例尺对应于x:10nm,t:10s。

[0148] 图13举例说明用于按照一些实施方案进行漂移校正的方法。

[0149] 图14举例说明用于进行对应于图13的阶段230的漂移校正的方法。

[0150] 图15举例说明用于进行对应于图13的阶段240的漂移校正的方法。

[0151] 图16举例说明用作用于3D漂移校正的模板的3D四面体。用对接位点标记4个转角。图16A显示清楚地解析四个转角。图16B举例说明具有～85nm的高度的结构的X-Z投影。

[0152] 图17显示可与本文所述的本公开内容的任何实施方案结合使用的计算机系统600的举例说明性实现。

[0153] 图18A-18C举例说明按照本公开内容的一些实施方案的漂移校正方法中的阶段的替代性代表。显示了单链DNA折纸纳米结构上的间隔10nm的规则网格的超分辨率图像。DNA折纸结构被设计为在垂直和水平方向上间隔10nm的5x 8正方形网格。图18A显示由十字星(cross)代表的收集和过滤的定位的散点图。图18B显示上述结构的二值化2-D直方图视图。图18C显示通过将图18A和18B中的矩形中的所有定位投射至x-轴并且用8个高斯分量最小二乘拟合的1-D直方图。拟合的高斯峰全都具有在1.5-2.4nm的范围内的标准差，从而原则上允许3.5-5.6nm的分辨率；和在9.8-11.0nm的范围内的相邻峰之间的间距，与DNA折纸设计一致。少数(在本情况下5个)点由于组装反应中吻合钉(staple)的不完美掺入而在结构中丢失，但在超分辨成像过程中未丢失。

[0154] 图19A和19B显示RNA适体调节GFP样荧光团的荧光。图19A显示HBI(绿色)(在GFP的背景中)和DMHBI的结构。图19B显示13-2RNA适体通过稳定有利于荧光发射的特定分子排列而增强DMHBI的荧光。在利用365nm的光的照明下对含有DMHBI、13-2RNA、DMHBI与13-2RNA，或DMHBI与HeLa细胞总RNA的溶液照相。图像是在相同图像采集条件下获得的蒙太奇。(图像来自Paige等，参考文献19)

[0155] 图20A和20B显示DFHBI结合动力学的单分子荧光表征。图20A显示使用生物素化的DNA捕捉序列(利用红色染料例如Alexa647标记；颜色渲染未显示)将5'-延长的Spinach(绿色)固定在涂覆有BSA/生物素的玻璃基片上。图20B显示在添加适体之前(底部线)和之后(顶部线)Spinach-DFHBI的主要荧光测量显示DFHBI结合活性在添加用于固定至图20A中的玻璃表面所需的延长部分至Spinach后得以良好维持。

[0156] 图21A和21B显示基准测试Spinach-PAINT性能。图21A显示用于以确定的距离放置两个Spinach分子的六螺旋DNA折纸结构。图21B显示使用DNA-PAINT定位DNA结构(P)和使用Spinach-PAINT以3个不同的距离定位Spinach分子的超分辨重构的模拟表示。

[0157] 图22显示基于Spinach的传感器。基于Spinach的传感器(左)的变构变体包含Spinach结构域(黑色)、转换器模块(中度灰色)和识别模块(浅灰色)。在靶分子不存在的情况下，转换器模块主要处于非结构性状态，这阻止DFHBI活化所需的Spinach结构的稳定化。在靶分子结合后，转换器模块形成双链体，从而导致Spinach模块的结构硬化和DFHBI荧光的激活(参考文献24)。

[0158] 图23A和23B显示体外和原位交换-PAINT室的实例。

[0159] 发明描述

[0160] 本公开内容除其它以外提供了用于使用基于核酸的成像探针(例如，基于DNA的成像探针)例如在细胞环境中进行复用成像的方法、组合物和试剂盒。用于复用荧光成像的方法、组合物和试剂盒不受所达到的分辨率程度的限制。因此，本文中提供的方法、组合物和

试剂盒通常可用于成像。

[0161] 在一些方面，本公开内容还提供了，除其它以外，用于使用基于核酸的成像探针(例如，基于DNA的成像探针)例如在细胞环境中进行复用超分辨率成像的方法、组合物和试剂盒。如本文中所用，“超分辨率”成像是指组合相同区域的一组低分辨率图像以获得具有较高分辨率的单个图像的方法。本公开内容的许多方面可用于将目标靶(例如，生物分子)在荧光接通与关断状态之间“开关(switch)”以允许个体靶的连续或在一些情况下同时的定位。荧光“接通”状态是其中发射荧光的状态。荧光“关断”状态是其中不发射荧光的状态。在一些实施方案中，使用利用可检测标记物(例如，荧光分子)标记的扩散分子实现两个状态之间的开关，所述扩散分子使用包含可检测标记物(例如，荧光分子)并且结合于靶的中间部分与所述靶短暂相互作用。在一些方面，本公开内容的方法、组合物和试剂盒用于检测、鉴定和定量目标靶。

[0162] 本公开内容的结合伴侣-核酸缀合物(“BP-NA缀合物”)短暂地结合于互补的经标记的(任选地荧光标记的)成像链。如本文中所用，“结合伴侣-核酸缀合物”或“BP-NA缀合物”是指连接(例如，通过N-羟琥珀酰亚胺(NHS)接头)于单链核酸(例如，DNA)对接链的分子。缀合物的结合伴侣可以是具有针对目标靶诸如生物分子(例如，蛋白质或核酸)的亲和力(例如，结合于)的任何部分(例如，抗体或适体)。在一些实施方案中，所述结合伴侣为蛋白质。包含连接于对接链的蛋白质(或肽)的BP-NA-缀合物在本文中可被称为“蛋白质-核酸缀合物”或“蛋白质-NA缀合物”。用于本公开内容的缀合物的蛋白质的实例包括，但不限于，抗体(例如，单克隆抗体)、抗原结合抗体片段(例如，Fab片段)、受体、肽和肽适体。可按照本公开内容使用其它结合伴侣。例如，本文考虑了通过静电(例如，静电颗粒)、疏水或磁性(例如，磁性颗粒)相互作用结合于靶的结合伴侣。

[0163] 如本文中所用，“抗体”包括全长抗体及其任意抗原结合片段(例如，“抗原结合部分”)或单链。术语“抗体”包括，但不限于，包含通过二硫键相互连接的至少两个重(H)链和两个轻(L)链的糖蛋白或其抗原结合部分。抗体可以是多克隆或单克隆的；异种的、同种异体的或同基因的；或其修饰形式(例如，人源化的、嵌合的)。

[0164] 如本文中所用，抗体的“抗原结合部分”是指保留特异性结合抗原的能力的抗体的一种或多种片段。抗体的抗原结合功能可通过全长抗体的片段来执行。术语抗体的“抗原结合部分”内包含的结合片段的实例包括：(i)Fab片段，由V_H、V_L、C_L和C_{H1}结构域组成的单价片段；(ii)F(ab')2片段，包含通过在铰链区的二硫键连接的两个Fab片段的双价片段；(iii)由V_H和C_{H1}结构域组成的Fd片段；(iv)由抗体的单臂的V_H和V_L结构域组成的Fv片段，(v)dAb片段(Ward等，Nature 341:544546,1989)，其由V_H结构域组成；和(vi)分离的互补决定区(CDR)或(vii)两个或更多个分离的CDR的组合，其可任选地通过合成接头连接。此外，尽管Fv片段的两个结构域V_H和V_L由单独的基因编码，但可使用重组方法，通过使它们能够被产生为其中V_H和V_L区配对形成单价分子的单个蛋白质链(称为单链Fv(scFv)；参见，例如，Bird等，Science 242:423426,1988；和Huston等Proc.Natl.Acad.Sci.USA 85:5879-5883,1988))的合成接头来连接它们。这样的单链抗体也包含在术语抗体的“抗原结合部分”内。使用本领域技术人员已知的常规技术获得这些抗体片段，并且以与对于完整抗体相同的方式就效用筛选片段。

[0165] 如本文中所用，“受体”是指结合配体例如肽或小分子(例如，低分子量(<900道尔

顿)有机或无机化合物)的细胞来源的分子(例如,蛋白质)。

[0166] 如本文中所用,"肽适体"是指具有插入恒定支架蛋白的可变肽序列的分子(参见,例如,Baines IC等,Drug Discov.Today 11:334-341,2006)。

[0167] 在一些实施方案中,BP-NA缀合物的分子为核酸例如核酸适体。如本文中所用,"核酸适体"是指可形成能够特异性结合蛋白质或其它细胞靶的二级和三级结构的小RNA或DNA分子(参见,例如,Ni X等,Curr Med Chem.18(27):4206-4214,2011)。因此,在一些实施方案中,BP-NA缀合物可以是适体-核酸缀合物。

[0168] 如本文中所用,"对接链"是指长度为约5个核苷酸至约50个核苷酸(或长度为5个核苷酸至50个核苷酸)的单链核酸(例如,DNA)。在一些实施方案中,对接链的长度为约4个核苷酸至约60个核苷酸、约6个核苷酸至约40个核苷酸、约7个核苷酸至约30个核苷酸、约8个核苷酸至约20个核苷酸,或约9个核苷酸至约15个核苷酸。在一些实施方案中,对接链的长度为(或为约)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或更多个核苷酸。

[0169] 对接链可具有一个结构域或多于一个结构域(即,多个结构域),每个结构域与各自的成像链互补。如本文中所用,"对接链结构域"是指与成像链的核苷酸序列互补的对接链的核苷酸序列。对接链例如可以含有1、2、3或更多个结构域,每个结构域与成像链互补。每个互补的成像链可含有不同的标记物(例如,红色荧光团、蓝色荧光团或绿色荧光团),或所有互补成像链可含有相同标记物(例如,红色荧光团)。例如,对于3结构域对接链,所述链可含有与利用红色荧光团标记的成像链互补的第一结构域、与利用蓝色荧光团标记的成像链互补的第二结构域和与利用绿色荧光团标记的成像链互补的第三结构域。或者,3个对接结构域的每一个可与利用红色荧光团标记的成像链互补。在一些实施方案中,对接链具有至少2个、至少3个、至少4个、至少5个或更多个结构域,每个结构域各自与成像链互补。在一些实施方案中,对接链具有1至5、1至10、1至15、1至20、1至25、1至50或1至100个结构域,每个结构域各自与成像链互补。

[0170] 如本文中所用,"成像链"是单链核酸(例如,DNA),其长度为约4至约30个核苷酸、约5至约18个核苷酸、约6至约15个核苷酸、约7至约12个核苷酸或约8至10个核苷酸,并且被荧光标记。在一些实施方案中,成像链的长度可以为(或可以为约)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或30个核苷酸。本公开内容的成像链与对接链互补并与其短暂结合。如果两个核酸或核酸结构域通过Watson-Crick相互作用彼此碱基配对或结合以形成双链核酸分子,则它们彼此"互补"。如本文中所用,"结合"是指在生理条件下因例如静电、疏水、离子和/或氢键相互作用而形成的在至少两个分子之间的缔合。如果成像链与对接链的互补区结合并随后例如在室温下在短时间内与对接链解离(不结合),则其被认为与对接链"短暂结合"。在一些实施方案中,成像链保持与对接链结合,持续约0.1至约10秒、或约0.1至约5秒。例如,成像链可保持与对接链结合,持续约0.1秒、约1秒、约5秒或约10秒。

[0171] 本公开内容的成像链可以使用可检测标记物(例如,荧光标记物,并从而被认为是"荧光标记的")来标记。例如,在一些实施方案中,成像链可包含至少一个(即,一个或多个)荧光团。按照本公开内容使用的荧光团的实例包括,但不限于,呫吨衍生物(例如,荧光素、

罗丹明、俄勒冈绿、曙红和德克萨斯红)、花青衍生物(例如,花青、吲哚碳花青、氧杂碳花青、噻碳花青和部花青)、萘衍生物(例如,丹酰和氟硅酸钠衍生物)、香豆素衍生物、噁二唑衍生物(例如,吡啶基噁唑、硝基苯噁二唑和苯并噁二唑)、芘衍生物(例如,级联蓝),噁嗪衍生物(例如,尼罗红、尼罗蓝、甲酚紫和噁嗪170),吖啶衍生物(例如,原黄素、吖啶橙、吖啶黄)、芳基次甲基衍生物(例如,金胺、结晶紫和孔雀石绿),和四吡咯衍生物(例如,卟吩、酞菁和胆红素)。可按照本公开内容使用其它可检测标记物,例如,金纳米颗粒或其它检测颗粒或部分。

[0172] 如本文中所用,本公开内容的"光谱上不同的"分子(例如,缀合物和/或成像链)是指具有不同光谱信号或波长的标记物(例如,荧光团)的分子。例如,用Cy2荧光团标记的成像链以约510nm的光波长发射信号,而用Cy5荧光团标记的成像链以约670nm的光波长发射信号。因此,Cy2-标记的成像链在本文中被认为在与Cy5-标记的成像链光谱上不同。相反地,本公开内容的"光谱上无差别的"分子在本文中是指具有拥有相同的光谱信号或波长的标记物的分子-即,标记物的发射波长不能用于区分两个光谱上无差别的荧光标记的分子(例如,因此波长是相同的或紧密靠近的)。

[0173] 在一些实施方案中,本公开内容的BP-NA缀合物(例如,蛋白质-核酸缀合物)可包含将分子连接(例如,共价地或非共价地)于对接链的中间接头。所述中间接头可包含生物素和/或链霉抗生物素蛋白。例如,在一些实施方案中,抗体和对接链可各自被生物素化(即,连接于至少一个生物素分子)并通过与中间链霉抗生物素蛋白分子结合的生物素彼此连接,如图2中显示的。可根据本公开内容使用其它中间接头。在一些实施方案中,诸如其中分子为核酸的实施方案中,可不需要中间接头。例如,BP-NA缀合物的对接链可以是核酸分子的延伸(例如,5′或3′延伸),例如核酸适体。

[0174] 本文中提供了多个BP-NA缀合物(例如,蛋白质-核酸缀合物)和成像链。所述多个可以是相同种类或不同种类的群体。相同种类的多个BP-NA缀合物可包含全部结合于相同靶(例如,生物分子)(例如,相同表位或区域/结构域)的缀合物。相反地,不同种类的多个BP-NA缀合物可包含缀合物或缀合物的亚组,每种缀合物或缀合物的亚组结合于相同靶上的不同表位或不同靶。相同种类的多个成像链可包含具有相同核苷酸序列和相同荧光标记物(例如,Cy2、Cy3或Cy4)的成像链。相反地,不同种类的多个成像链可包含具有不同核苷酸序列(例如,DNA序列)和不同荧光标记物(例如,Cy2、Cy3或Cy4)或具有不同核苷酸序列和相同荧光(例如,全部为Cy2)的成像链。给定的多个BP-NA缀合物中的不同种类的数目受到结合伴侣(例如,抗体)的数目和具有不同核苷酸序列的对接链(和从而互补的成像链)的数目的限制。在一些实施方案中,多个BP-NA缀合物(例如,蛋白质-核酸缀合物)包含至少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}个BP-NA缀合物。同样地,在一些实施方案中,多个荧光标记的成像链包含至少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}个荧光标记的成像链。在一些实施方案中,所述多个可含有1至约200个或更多个不同种类的BP-NA缀合物和/或成像链。例如,所述多个可含有至少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或更多个不同的种类。在一些实施方案中,所述多个可含有少于约5至约200个不同种类的BP-NA缀合物和/或成像链。例如,所述多个可含有少于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或200个不同种类。

[0175] 本公开内容还考虑了可直接结合于靶的对接链。例如，如图8A中所示的，除了成像链结合结构域(例如，具有相同或不同荧光团的1、2、3或更多个结构域)以外，对接链可包含与靶例如mRNA或其它核酸互补并且与其结合的靶结构域。

[0176] 方法

[0177] 本文中提供的方法部分地基于核酸对接链和成像链的可编程性。即，例如，可设计对接链和成像链，以使得它们在某些条件下彼此结合持续一定的时期。该可编程性允许成像链与对接链的短暂结合，如本文中提供的。一般地，本文中提供的方法涉及鉴定特定样品(例如，生物样品)中的一种或多种靶(例如，生物分子诸如蛋白质或核酸)。在一些情况下，一种或多种靶是否存在于样品中是未知的。因此，本公开内容的方法可用于测定一种或多种靶在怀疑含有所述靶的样品中的存在或不存在。在本文中提供的方面和实施方案的任一个中，样品可含有一种或多种靶或可怀疑含有一种或多种靶。

[0178] 本文中提供的方法还可用于鉴定单个靶(例如，特定的蛋白质)的绝对量，或单个靶相对于一种或多种其它靶的量。

[0179] 此外，本文中提供的方法可用于鉴定靶在样品内的位置或相对于样品中其它靶的位置。

[0180] 在一些实施方案中，本文中提供的方法可包括将样品与如下物质接触：(a)至少一种包含连接于对接链的结合伴侣的BP-NA缀合物(例如，蛋白质-核酸缀合物)和(b)至少一种与所述至少一种BP-NA缀合物的对接链互补并短暂结合的经标记的(任选地荧光标记的)成像链；以及随后测定所述至少一种BP-NA缀合物是否结合于样品中的至少一个靶(诸如生物分子靶)。在一些实施方案中，所述测定步骤包括对所述至少一种经标记的(任选地荧光标记的)成像链与所述至少一种BP-NA缀合物的对接链的短暂结合进行成像(例如，利用延时荧光显微镜技术)。

[0181] 在一些实施方案中，本文中提供的其它方法可包括将样品与如下物质接触：(a)至少两种BP-NA缀合物，其每一种包含连接于对接链的结合伴侣，和(b)至少两种经标记的(任选地光谱上不同的、荧光标记的)成像链，其与所述至少两种不同的BP-NA缀合物的各自的对接链互补并与其短暂结合；以及随后测定所述至少两种BP-NA缀合物是否结合于样品中的至少一种或至少两种靶(诸如生物分子靶)。所述BP-NA缀合物与各自的靶的结合可通过对所述至少两种经标记的(任选地光谱上不同的、荧光标记的)成像链之一与所述至少两种BP-NA缀合物之一的对接链的短暂结合进行成像以产生第一图像，和随后对所述至少两种经标记的(任选地光谱上无不同的、荧光标记的)成像链的另一种对所述至少两种BP-NA缀合物的另一种的短暂结合进行成像以产生第二图像来测定。在一些实施方案中，所述方法还包括将所述第一图像与所述第二图像组合以产生信号(例如，荧光信号)的合成图像，其中所述合成图像的信号(例如，荧光信号)代表至少两种靶。如本文中所用，“合成图像”是指通过组合(例如，重叠)相同(或大体上相似的)区域的多个图像产生的单个图像。合成图像还可被称为超分辨率图像，如本文中其它地方描述的。

[0182] 图3显示其中两个不同种类的BP-NA缀合物(例如，抗体-核酸缀合物)用于标记固定的HeLa细胞样品中的生物分子的本公开内容的一个实施方案。一个种类的抗体-核酸缀合物包含识别并结合线粒体上的表位的抗体。线粒体特异性抗体连接于具有与Cy3b-标记

的成像链互补的序列的对接链。另一种类的抗体-核酸缀合物包含识别并结合微管上的表位的抗体。微管特异性的抗体连接于具有与ATTO655-标记的成像链互补的序列的对接链。随后同时引入两种光谱上不同的种类的成像链：一个种类用Cy3b标记并且与连接于线粒体特异性抗体的对接链互补，另一种类用ATTO655标记并且与连接于微管特异性抗体的对接链互补。虽然Cy3b-标记的成像链和ATTO655-标记的成像链同时存在于具有样品的溶液中，但成像在Cy3b和ATTO655通道中相继进行。

[0183] 在一些实施方案中，本文中提供的其它方法可包括将样品与如下物质接触：(a)至少两种BP-NA缀合物，其每一种包含连接于对接链的蛋白质，和(b)至少两种光谱上无差别的(例如，用相同荧光团标记的)荧光标记的成像链，其与所述至少两种BP-NA缀合物的各自的对接链互补并短暂结合；和随后测定所述至少两种BP-NA缀合物是否结合于样品中的至少两种靶(例如，生物分子靶)。在一些实施方案中，所述方法按照以下顺序的步骤包括：将样品与第一BP-NA缀合物和至少一种其它的BP-NA缀合物接触，将样品与与所述第一BP-NA缀合物的对接链互补并与其短暂结合的第一荧光标记的成像链接触，测定所述第一BP-NA缀合物是否结合第一靶，除去所述第一荧光标记的成像链，将样品与至少一种其它的荧光标记的成像链接触，所述其它荧光标记的成像链与所述至少一种其它的BP-NA缀合物的对接链互补并与其短暂结合，和测定所述至少一种其它的BP-NA缀合物是否结合于至少一种其它的靶。

[0184] 或者，在其它实施方案中，方法按照以下顺序的步骤包括：将样品与第一BP-NA缀合物接触，将样品与与所述第一BP-NA缀合物的对接链互补并与其短暂结合的第一荧光标记的成像链接触，测定所述第一BP-NA缀合物是否结合于第一靶(例如，生物分子)，除去所述第一荧光标记的成像链，将所述样品与至少一种其它的BP-NA缀合物接触，将样品与至少一种其它的荧光标记的成像链接触，所述其它荧光标记的成像链与所述至少一种其它的BP-NA缀合物的对接链互补并与其短暂结合，和测定所述至少一种其它的BP-NA缀合物是否结合于至少一种其它的靶。

[0185] 在一些实施方案中，所述第一测定步骤包括对所述第一荧光标记的成像链与所述第一BP-NA缀合物的对接链的短暂结合成像以产生第一图像，并且第二测定步骤包括对所述至少一种其它的荧光标记的成像链与所述至少一种其它的BP-NA缀合物的对接链的短暂结合成像以产生第二图像。在一些实施方案中，所述方法还包括将伪彩色赋予所述第一图像中的荧光信号，和将至少一种其它的伪彩色赋予所述第二图像中的荧光信号。更进一步，在一些实施方案中，所述方法包括组合所述第一图像与所述第二图像以产生伪彩色化信号的合成图像，其中合成图像的伪彩色化信号代表所述至少两种靶(例如，生物分子靶)。如图4A中步骤[1]中举例说明的，3个不同种类的对接链(a、b、c)标记网格(被选择用于举例说明目的)的表面。在步骤[2]中，引入多个拷贝的成像链a*，并对利用对接链a标记的点进行成像。在步骤[3]中，冲洗掉成像链a*的拷贝，并引入成像链b*以对b标记的点进行进行成像。在步骤[4]中，以相同的方式对c标记的点进行成像。在步骤[5]中，给来自步骤[2-4]的图像赋予人工伪彩色(例如，使用软件程序)并组合以生成最终的合成图像。用相同荧光团标记所有成像链-即，成像链是光谱上无差别的。在一些实施方案中，将对接链连接于结合伴侣(例如，蛋白质诸如抗体，或核酸诸如DNA或核酸适体)。

[0186] 本公开内容的方法的有利方面是划分和连续成像可用于仅使用单一优化荧光染

料获得高达数百个不同种类的复用超分辨图像。通过使用这些方法，不同核苷酸序列(例如，DNA序列)的数目，而不是光谱上不同的染料的数量，限制了复用能力。在本公开内容的一些方法中，例如使用具有9个核苷酸的长度的成像链的方法中，存在可用于单个样品的在动力学紧界内的数百个种类，代表在复用上相较于直接的"传统"成像方法的巨大增加。

[0187] 图5A举例说明使用光谱上无差别的成像链的本公开内容的另一种实施方案。单个DNA纳米结构展示4个不同种类的对接链(任选地连接于蛋白质结合伴侣或核酸结合伴侣)，其被设计来分别类似于0至3的数字。使用简单流动室设置连续进行成像，首先在与数字0的对接链互补的荧光标记的成像链中进行冲洗，随后将溶液换为具有与数字1的对接链互补的序列的荧光标记的成像链，依此类推。所得的图像已被伪彩色化来代表各自的成像循环。如本文中所用，"成像循环"或"成像回合"是指在允许成像链与对接链结合(即使这样的结合是短暂的)的条件下引入与对接链互补的荧光标记的成像链，和获得图像(或对区域进行成像)的过程。

[0188] 本公开内容的方面考虑了使用多结构域对接链(例如，具有多于一个结构域的对接链)的复用检测，如上所述的。为了举例说明目的，在对接链与靶的结合(例如，无中间结合伴侣)方面描述下列实施方案。然而，应当理解，可将所述多结构域对接链连接于结合伴侣(例如，BP-NA缀合物的)，如本文中提供的。

[0189] 在一些实施方案中，方法包括将一种或多种靶与一种或多种对接链接触，所述对接链各自含有两个或更多个结合结构域。在其它实施方案中，方法包括将一种或多种靶与两种或更多种对接链接触，所述对接链各自含有一个结合结构域。所述对接链结构域可具有正交序列。在下面的实例中，所有3种靶(蛋白质#1-#3)存在于样品中。

[0190] 基于光谱分辨率的检测。示例性复用靶检测法如下。样品含有或怀疑含有三个靶种类-蛋白质#1、蛋白质#2和蛋白质#3。设计3种对接链，以使得：含有成像结合结构域A的第一对接链结合蛋白质#1；含有成像结合结构域B的第二对接链结合蛋白质#2；和含有成像结合结构域A和B的第三对接链结合蛋白质#3。互补的成像链A'结合对接链的成像结合结构域A并且用蓝色荧光团标记，并且成像链B'结合对接链的成像结合结构域B并且用红色荧光团标记。首先将样品与对接链接触，随后与成像链A'和B'接触。随后对样品成像。首先在检测蓝色荧光团的条件下对含有对接链和成像链的样品进行成像。对蓝色荧光团的成像检测蛋白质#1和蛋白质#3，其各自被用蓝色荧光团标记的成像链结合。对红色荧光团的成像检测蛋白质#2和蛋白质#3，其各自被用红色荧光团标记的成像链结合。红色和蓝色荧光团的重叠图像仅检测蛋白质#3，其是被用红色荧光团标记的成像链和用蓝色荧光团标记的成像链结合的唯一蛋白质。因此，蛋白质#1-#3的鉴定和位置通过分别检测红色和蓝色荧光团的图像的重叠鉴定。

[0191] 基于成像链的交换的检测。另一种示例性复用靶检测法如下：样品含有或怀疑含有3个靶种类-蛋白质#1、蛋白质#2和蛋白质#3。设计3种对接链，以使得：含有成像结合结构域A的第一对接链结合蛋白质#1；含有成像结合结构域B的第二对接链结合蛋白质#2；和含有成像结合结构域A和B的第三对接链结合蛋白质#3。互补的成像链A'结合对接链的成像结合结构域A并且用蓝色荧光团标记，成像链B'结合对接链的成像结合结构域B并且也用蓝色荧光团标记。首先将样品与对接链接触，随后与成像链A'接触。随后在检测蓝色荧光团的条件下对样品进行成像。对蓝色荧光团的成像检测蛋白质#1和蛋白质#3，其各自被用蓝色荧

光团标记的成像链A'结合。随后洗涤样品以除去成像链A'。然后，将样品与成像链B'接触。随后在检测蓝色荧光团的条件下再次对样品成像。对蓝色荧光团成像现检测蛋白质#2和蛋白质#3，其各自被用蓝色荧光团标记的成像链B'结合。蓝色荧光团的重叠图像(例如，导致相对于非重叠荧光团更强的信号)仅检测蛋白质#3，其是被用蓝色荧光团标记的两种成像链结合的唯一蛋白质。因此，蛋白质#1-#3的鉴定和位置通过检测蓝色荧光团的图像的重叠来鉴定，并且其基于信号强度。

[0192] 基于光谱和交换检测的组合的检测。另一个示例性复用靶检测法如下：样品含有或怀疑含有15个靶种类。设计对接链以使得每个靶种类结合于对接链，每个对接链含有单个不同的结构域或结构域A-D的不同组合(例如，A，或A和B(即，A/B)，或A/C，或A/D，或A/B/C，或A/B/D，或A/C/D，或A/B/C/D，或B，或B/C，或B/D，或B/C/D，或C，或C/D，或D)。成像链被分成两组：第一组(组#1)含有被红色荧光团标记的成像链A'和被蓝色荧光团标记的成像链B'；第二组含有被红色荧光团标记的成像链C'和被蓝色荧光团标记的成像链D'。首先将样品与对接链接触，随后与成像链组#1接触。随后在检测蓝色和红色荧光团的条件下对样品进行成像。被成像链A'结合的靶将被检测为红色并且被成像链B'结合的靶将被检测为蓝色。因此，将在第一图像或第一组图像中检测到具有对接结构域A和B的所有靶种类。随后洗涤样品以除去成像链组#1。随后，将样品与成像链组#2接触。随后在检测蓝色和红色荧光团的条件下再次对样品成像。被成像链C'结合的靶将被检测为红色，并且被成像链D'结合的靶将被检测为蓝色。因此，将在第二图像或第二组图像中检测到具有对接结构域C和D的所有靶种类。通过组合所有收集的图像，可仅使用4种成像链和两种荧光团鉴定15个靶种类的每一个。应当理解，取决于例如靶的数目，可使用多于4种成像链以及多于2种荧光团。

[0193] 基于短暂结合的持续时间的检测。在一些实施方案中，本公开内容考虑了将靶种类与不同的对接链结构域序列和不同长度的那些序列接触。对接链成像结合结构域的长度影响与成像链的短暂结合的持续时间。具有较长结合结构域的对接链结合各自的互补的成像链持续相对于较短的结合结构域更长的持续时间。在以下示例性实施方案中，样品含有或怀疑含有4个靶种类。设计4种对接链，以使得：含有长度为10个核苷酸的结合结构域A(A10)的第一对接链结合蛋白质#1；含有成像结合结构域A10和长度为8个核苷酸的成像结合结构域B(B8)的第二对接链结合蛋白质#2；含有长度为8个核苷酸的成像结合结构域A(A8)和长度为10个核苷酸的成像结合结构域B(B10)的第三对接链结合蛋白质#3；和含有成像链结合结构域B10的第四对接链。成像链A'的长度为10个核苷酸，结合A8和A10，并且用蓝色荧光团标记。成像链B'的长度为10个核苷酸，结合B8和B10，并且用红色荧光团标记。首先将样品与对接链接触，随后与成像链A'和B'接触。随后在检测蓝色荧光团的条件下对样品成像。对蓝色荧光团的成像检测具有较长结合时间(即，成像链与对接链之间的结合的时间)的蛋白质#3和蛋白质#4，以及具有较短结合时间的蛋白质#2。对红色荧光团的成像检测具有较长结合时间的蛋白质#1和蛋白质#2，以及具有较短结合时间的蛋白质#3。蓝色和红色荧光团的重叠图像检测所述4种蛋白质靶的每一种。

[0194] 本公开内容还考虑了组合基于光谱分辨率和持续时间、交换和持续时间以及光谱分辨率、交换和持续时间的复用检测。

[0195] “样品”可包含细胞(或一个细胞)、组织或体液诸如血液(血清和/或血浆)、尿液、精液、淋巴液、脑脊髓液或羊水。样品可获自(或来源于)任何来源，包括，但不限于，人、动

物、细菌、病毒、微生物和植物。在一些实施方案中，样品是细胞裂解物或组织裂解物。样品还可含有来自一个来源或不同来源的材料的混合物。样品可以是空间区域或体积(例如，阵列上的网格或板或盘上的孔)。在一些实施方案中，样品包括靶、BP-NA缀合物和成像链。

[0196] "靶"是希望观察或定量以及针对其存在结合伴侣的任何部分。在一些实施方案中，靶可以是非天然存在的。在一些实施方案中，靶可以是生物分子。如本文中所用，"生物分子"是由活生物产生的任何分子，包括大的大分子诸如蛋白质、多糖、脂质和核酸(例如，DNA和RNA诸如mRNA)，以及小分子诸如初级代谢产物、次级代谢产物和天然产物。生物分子的实例包括，但不限于，DNA、RNA、cDNA或经历逆转录的RNA的DNA产物、A23187(卡西霉素、钙离子载体)、阿维菌素、枞酸、乙酸、乙酰胆碱、肌动蛋白、放线菌素D、腺苷、腺苷二磷酸(ADP)、腺苷一磷酸(AMP)、腺苷三磷酸(ATP)、腺苷酸环化酶、阿东糖醇、肾上腺素(Adrenaline)、肾上腺素(epinephrine)、促肾上腺皮质激素(ACTH)、水母发光蛋白、黄曲霉毒素、琼脂、丙甲菌素、丙氨酸、白蛋白、醛固酮、糊粉、α-鹅膏蕈碱、尿囊素、丙烯菊酯、α-Amanatin、氨基酸、淀粉酶、促蛋白同化甾体、茴香脑、血管紧张素原、茴香霉素、抗利尿激素(ADH)、阿拉伯糖、精氨酸、子囊霉素、抗坏血酸(维生素C)、天冬酰胺、天冬氨酸、不对称二甲基精氨酸、心房钠尿肽(ANP)、生长素、抗生物素蛋白、印楝素A-C35H44O16、细菌素、白僵菌素、荷包牡丹碱、胆红素、生物聚合物、生物素(维生素H)、布雷菲德菌素A、油菜素内酯、马钱子碱、尸胺、咖啡因、骨化醇(维生素D)、降钙素、钙调蛋白、钙调蛋白、钙网织蛋白、樟脑-(C10H16O)、大麻酚、辣椒碱、糖分解酶、碳水化合物、肉碱、角叉菜胶、酪蛋白、半胱天冬酶、纤维素酶、纤维素-(C6H10O5)、浅蓝菌素、西曲溴铵(溴棕三甲铵)-C19H42BrN、白屈菜赤碱、色霉素A3、伴侣蛋白(Chaparonin)、甲壳素、α-氯醛糖、叶绿素、胆囊收缩素(CCK)、胆固醇、胆碱、硫酸软骨素、肉桂醛、柠檬醛、柠檬酸、桔霉素、香茅醛、香茅醇、瓜氨酸、钴胺素(维生素B12)、辅酶、辅酶Q、秋水仙素、胶原蛋白、毒芹碱、皮质类固醇、皮质酮、促肾上腺皮质素释放激素(CRH)、皮质醇、肌酸、肌酸激酶、晶状体、α-环糊精、环糊精糖基转移酶、环巴胺、环匹阿尼酸、半胱氨酸、胱氨酸、胞苷、细胞松弛素、细胞松弛素E、细胞色素、细胞色素c、细胞色素c氧化酶、细胞色素c过氧化物酶、细胞因子、胞嘧啶-C4H5N3O、脱氧胆酸、DON(脱氧瓜萎镰菌醇)、脱氧呋喃核糖、脱氧核糖、脱氧核糖核酸(DNA)、葡聚糖、糊精、DNA、多巴胺、酶、麻黄碱、肾上腺素-C9H13NO3、芥酸-CH3(CH2)7CH=CH(CH2)11COOH、赤藓醇、促红细胞生成素(EPO)、雌二醇、丁香酚、脂肪酸、纤维蛋白、纤连蛋白、叶酸(维生素M)、卵泡刺激素(FSH)、甲醛、甲酸、Formnoci、果糖、烟曲霉毒素B1、γ球蛋白、半乳糖、丙种球蛋白、γ-氨基丁酸、γ-丁内酯、γ-羟基丁酸酯(GHB)、胃泌素、明胶、香叶醇、球蛋白、胰高血糖素、氨基葡萄糖、葡萄糖-C6H12O6、葡萄糖氧化酶、麸质、谷氨酸、谷氨酰胺、谷胱甘肽、谷蛋白、甘油(丙三醇)、甘氨酸、糖原、乙醇酸、糖蛋白、促性腺激素释放激素(GnRH)、粒酶、绿色荧光蛋白、生长激素、生长激素释放激素(GHRH)、GTP酶、鸟嘌呤、鸟苷、鸟苷三磷酸(+GTP)、触珠蛋白、苏木精、血红素、蚯蚓血红蛋白、血蓝蛋白、血红蛋白、血红素蛋白、乙酰肝素硫酸盐、高密度脂蛋白、HDL、组胺、组氨酸、组蛋白、组蛋白甲基转移酶、HLA抗原、高半胱氨酸、激素、人绒毛膜促性腺激素(hCG)、人生长激素、透明质酸、透明质酸酶、过氧化氢、5-羟甲基胞嘧啶、羟脯氨酸、5-羟色胺、靛青染料、吲哚、肌苷、肌醇、胰岛素、胰岛素样生长因子、膜内在蛋白、整合酶、整联蛋白、内含肽、干扰素、菊粉、离子霉素、紫罗兰酮、异亮氨酸、铁-硫簇、K252a、K252b、KT5720、KT5823、角蛋白、激酶、乳糖酶、乳酸、乳糖、羊毛脂、月桂酸、瘦素、细霉素B、亮氨酸、

木质素、柠檬烯、芳樟醇、亚油酸、亚麻酸、脂肪酶、脂质、脂质锚定蛋白、硫辛酰胺、脂蛋白、低密度脂蛋白、LDL、促黄体生成激素(LH)、番茄红素、赖氨酸、溶菌酶、苹果酸、麦芽糖、褪黑激素、膜蛋白、金属蛋白、金属硫蛋白、甲硫氨酸、含羞草碱、光辉霉素A、丝裂霉素C、单体、霉酚酸、肌红蛋白、肌球蛋白、天然酚类、核酸、赭曲霉毒素A、雌激素、寡肽、寡霉素、苷黑酚、食欲肽、鸟氨酸、草酸、氧化酶、催产素、p53、PABA、紫杉醇、棕榈酸、泛酸(维生素B5)、甲状旁腺激素(PTH)、副蛋白、豹蝎剂、小白菊内酯、棒曲霉素、Paxilline、青霉酸、青霉素、青霉震颤素A、肽酶、胃蛋白酶、肽、表霉素、外周膜蛋白、Perosamine、苯乙胺、苯丙氨酸、磷酸原、磷酸酶、磷脂、苯丙氨酸、肌醇六磷酸、植物激素、多肽、多酚、多糖、卟啉、朊病毒、孕酮、催乳素(PRL)、脯氨酸、丙酸、鱼精蛋白、蛋白酶、蛋白质、类蛋白、腐胺、除虫菊酯、吡哆醇或吡哆胺(维生素B6)、吡咯赖氨酸、丙酮酸、醌、根赤壳菌素、棉子糖、肾素、视黄醛、视黄醇(维生素A)、视紫红质(视紫质)、核黄素(维生素B2)、呋喃核糖、核糖、核酶、蓖麻毒素、RNA-核糖核酸、RuBisCO、黄樟脑、水杨醛、水杨酸、Salvinorin-A-C23H28O8、皂甙、胰泌素、硒代半胱氨酸、硒代甲硫氨酸、硒蛋白、丝氨酸、丝氨酸激酶、血清素、粪臭素、信号识别颗粒、生长抑素、山梨酸、角鲨烯、星形孢菌素、硬脂酸、杂色曲霉素、固醇、番木鳖碱、蔗糖(糖)、糖(一般地)、超氧化物、T2毒素、单宁酸、鞣质、酒石酸、牛磺酸、河豚毒素、奇异果甜蛋白、拓扑异构酶、酪氨酸激酶、牛磺酸、睾酮、四氢大麻酚(THC)、河豚毒素、毒胡萝卜内酯、索马汀、硫胺素(维生素B1)-C12H17ClN4OS·HCl、苏氨酸、血小板生成素、胸苷、胸腺嘧啶、Triacsin C、促甲状腺激素(TSH)、促甲状腺激素释放激素(TRH)、甲状腺素(T4)、生育酚(维生素E)、拓扑异构酶、三碘甲状腺原氨酸(T3)、跨膜受体、曲古抑菌素A、营养激素、胰蛋白酶、色氨酸、微管蛋白、衣霉素、酪氨酸、泛素、尿嘧啶、尿素、尿素酶、尿酸-C5H4N4O3、尿苷、缬氨酸、缬氨霉素、Vanabins、加压素、震颤真菌毒素、维生素(一般地)、维生素A(视黄醇)、维生素B、维生素B1(硫胺素)、维生素B2(核黄素)、维生素B3(烟酸或烟碱酸)、维生素B4(腺嘌呤)、维生素B5(泛酸)、维生素B6(吡哆醇或吡哆胺)、维生素B12(钴胺素)、维生素C(抗坏血酸)、维生素D(钙化醇)、维生素E(生育酚)、维生素F、维生素H(生物素)、维生素K(萘醌)、维生素M(叶酸)、渥曼青霉素和木糖。

[0197] 在一些实施方案中,靶可以是蛋白质靶诸如,例如,细胞环境的蛋白质(例如,细胞内或膜蛋白质)。蛋白质的实例包括,但不限于,纤维蛋白诸如细胞骨架蛋白(例如,肌动蛋白、arp2/3、冠蛋白、肌营养不良蛋白、FtsZ、角蛋白、肌球蛋白、伴肌动蛋白、血影蛋白、τ蛋白、肌联蛋白,原肌球蛋白、微管蛋白和胶原)和细胞外基质蛋白(例如,胶原、弹性蛋白,f-脊椎蛋白、皮卡丘素和纤连蛋白);球状蛋白质诸如血浆蛋白(例如,血清淀粉样蛋白P成分和血清白蛋白)、凝血因子(例如,补体蛋白、C1抑制剂和C3-转化酶、因子VIII、因子XIII、纤维蛋白、蛋白C、蛋白S、蛋白Z、蛋白Z相关蛋白酶抑制剂、凝血酶、Von Willebrand因子)和急性期蛋白诸如C-反应蛋白;血红素蛋白;细胞粘附蛋白(例如,钙粘蛋白、窒管膜素、整联蛋白、Ncam和选择蛋白);跨膜转运蛋白(例如,CFTR、血型糖蛋白D和爬行酶)诸如离子通道(例如,配体门控离子通道诸如烟碱乙酰胆碱受体和GABAa受体,和电压门控离子通道诸如钾、钙和钠通道),同向/反向转运蛋白(例如,葡萄糖转运蛋白);激素和生长因子(例如,表皮生长因子(EGF)、成纤维细胞生长因子(FGF)、血管内皮生长因子(VEGF)、肽激素诸如胰岛素、胰岛素样生长因子和催产素,以及类固醇激素诸如雄激素、雌激素和孕酮);受体诸如跨膜受体(例如,G蛋白偶联受体、视紫红质)和细胞内受体(例如,雌激素受体);DNA结合蛋白(例

如，组蛋白、鱼精蛋白、CI蛋白）；转录调节剂（例如，c-myc，FOXP2，FOXP3，MyoD和P53）；免疫系统蛋白质（例如，免疫球蛋白、主要组织相容性抗原和T细胞受体）；营养储存/运输蛋白（例如，铁蛋白）；伴侣蛋白；和酶。

[0198] 在一些实施方案中，靶可以是核酸靶诸如，例如，细胞环境的核酸。如本文中关于靶、对接链和成像链所用的，“核酸”是指具有任何长度的核苷酸（诸如脱氧核糖核苷酸或核糖核苷酸或其类似物）的聚合形式。例如，核酸可以是DNA、RNA或经历逆转录的RNA的DNA产物。核酸的非限制性实例包括基因或基因片段的编码或非编码区、从连锁分析定义的基因座、外显子、内含子、信使RNA（mRNA）、转运RNA、核糖体RNA、核酶、cDNA、重组核酸、支链核酸、质粒、载体、任意序列的分离的DNA、任意序列的分离的RNA、核酸探针和引物。核酸的其它实例包括，但不限于，cDNA、适体、肽核酸（“PNA”）、2'-5'DNA（具有缩短的主链的合成材料，所述主链具有匹配DNA的A构象的碱基间距；2'-5'DNA通常不与以B型的DNA杂交，但其将容易地与RNA杂交）、锁核酸（“LNA”）以及具有经修饰的主链的核酸（例如，天然存在的核酸的碱基-或糖-修饰形式）。核酸可包含经修饰的核苷酸，诸如甲基化的核苷酸和核苷酸类似物（嘌呤和嘧啶的“类似”形式在本领域中是公知的）。如果存在，可在聚合物的组装之前或之后给予对核苷酸结构的修饰。核酸可以是单链、双链、部分单链或部分双链的DNA或RNA。

[0199] 在一些实施方案中，核酸（例如，核酸靶）是天然存在的。如本文中所用，“天然存在的”是指存在于在人干预不存在的情况下天然存在的生物体或病毒中的核酸。在一些实施方案中，核酸天然存在于生物体或病毒中。在一些实施方案中，核酸是基因组DNA、信使RNA、核糖体RNA、micro-RNA、pre-micro-RNA、pro-micro-RNA、病毒DNA、病毒RNA或piwi-RNA。在一些实施方案中，核酸靶不是合成的DNA纳米结构体（例如，包含两个或更多个通过Watson-Crick相互作用彼此杂交以形成2-D或3-D纳米结构的二维（2-D）或三维（3-D）DNA纳米结构）。

[0200] 本文中所述的核酸对接链和成像链可以是上述核酸的任一种（例如，DNA、RNA、经修饰的核酸、核酸类似物、天然存在的核酸、合成核酸）。

[0201] 定量成像

[0202] 本公开内容还提供了用于定量不能使用现有技术的成像技术在空间上解析的致密簇中的荧光部分或发射体的方法。在本发明之前，不存在描述荧光信号的光开关的动力学的系统模型。

[0203] 使用短的寡核苷酸（例如，成像链）与它们的靶的短暂结合的随机超分辨率成像为定量计数衍射限制区域中的整数量的经标记的分子提供了独特的可能性。本公开内容的方法中从荧光关断状态至接通状态的“开关”分子通过单分子核酸（例如，DNA）杂交事件来促进，这可通过利用二阶缔合速率k_{on}和一阶解离速率k_{off}的非常容易预测的动力学模型来控制：

[0204] $$S_{imager} + S_{docking} \xrightarrow{k_{on}} S_{imager}:S_{docking}$$

[0205] $$S_{imager}:S_{docking} \xrightarrow{k_{off}} S_{imager} + S_{docking}$$

[0206] 现将动力学参数k_{on}和k_{off}与图7A中描绘的荧光接通和关断时间（分别为τ_b和τ_d）直接联系起来。荧光接通时间τ_b是通过解离速率k_{off}测定的：$\tau_b = 1/k_{off}$，荧光关断时间τ_d通过缔合速率k_{on}、溶液中的成像链的浓度c_{imager}和观察到的结合位点的数目bs来测定：

[0207] $$\tau_d = \frac{1}{k_{on} \cdot c_{imager} \cdot bs}$$

[0208] 在使用具有已知数目的结合位点bs校准$k_{on} \cdot c_{imager}$(这可使用例如DNA纳米结构来容易地进行)后,可按照以下方程式获得未知分子或区域的结合位点的数目:

[0209] $$bs = \frac{1}{k_{on} \cdot c_{imager} \cdot \tau_d}$$

[0210] 因此,可使用结合动力学分析软件来自动地进行荧光图像的定量。简言之,以延时方式记录典型图像(例如,具有10Hz的帧率的15000帧)。对衍射极限图像进行荧光斑点检测和拟合(例如,高斯拟合、质心拟合或Bessel拟合),从而获得超分辨率图像。在下一步骤中,选择校准标记(例如,具有如图7C中的确定数目的斑点的DNA折纸结构)。所述软件通过将关断时间分布拟合至累积分布函数来自动计算荧光暗时间τ_d。通过使用上述方程式,可计算$k_{on} \cdot c_{imager}$的乘积。该乘积用于计算对接位点的数目,和从而成像的区域中的靶的数目。

[0211] 在一些实施方案中,可通过应用第二斑点检测步骤例如以计算簇中的靶的数目来自动地进行解析的(例如,超分辨的)图像中的目标区域的选择。

[0212] 因此,在一些实施方案中,本公开内容的方法包括提供包含与荧光标记的成像链直接或间接地短暂结合的靶的样品,获得样品的延时衍射极限荧光图像,对衍射极限图像进行荧光斑点检测和拟合(例如,高斯拟合、质心拟合或Bessel拟合)以获得样品的高分辨率图像,使用具有未知数目的靶的样品校准$k_{on} \cdot c_{imager}$,其中k_{on}为二阶缔合常数,并且c_{imager}为样品中的荧光标记的成像链(包括未结合的成像链)的浓度,通过将荧光关断时间分布拟合至累积分布函数来测定变量τ_d,和基于下述方程式来测定样品中靶的数目:靶的数目$=(k_{on} \cdot c_{imager} \cdot \tau_d)^{-1}$。

[0213] 本公开内容的一些方面涉及拟合函数。如本文中所用,"拟合函数"是指用于拟合分子的强度特征谱的数学函数。用于如本文中提供的使用的拟合函数的实例包括,但不限于,高斯拟合、质心拟合和Bessel拟合。应理解,虽然本公开内容的许多方面和实施方案涉及高斯拟合,但可使用其它拟合函数来取代或附加于高斯拟合。

[0214] 组合物

[0215] 本文中提供了包含至少一种或至少两种(例如,多种)本公开内容的BP-NA缀合物(例如,蛋白质-核酸缀合物)的组合物。BP-NA缀合物可结合于目标靶(例如,生物分子)和/或短暂地结合于互补的荧光标记的成像链。组合物可包含多个相同的种类或不同种类的BP-NA缀合物。在一些实施方案中,组合物可包含至少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}个BP-NA缀合物。在一些实施方案中,组合物可包含至少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}个互补的荧光标记的成像链。在一些实施方案中,组合物可含有1至约200个或更多个不同种类的BP-NA缀合物和/或成像链。例如,组合物可含有至少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或更多个不同的种类。在一些实施方案中,组合物可含有少于约5至约200个不同种类的BP-NA缀合物和/或成像链。例如,组合物可含有少于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或200个不同的种类。

[0216] 应当理解,组合物中的互补的荧光标记的成像链的数目可少于、等于或多于组合

物中的BP-NA缀合物的数目。

[0217] 试剂盒

[0218] 本公开内容还提供了包含本文中提供的一种或多种组分的试剂盒。试剂盒可包含，例如，BP-NA缀合物和/或荧光标记的成像链。试剂盒还可包含用于产生BP-NA缀合物或用于标记成像链的组分。例如，试剂盒可包含结合伴侣(例如，抗体)、对接链和中间接头诸如生物素和链霉抗生物素蛋白分子、和/或成像链。试剂盒可用于对于本领域技术人员来说是显然的任何目的，包括，上文所述的那些目的。

[0219] 试剂盒还可包括其它试剂，例如，用于进行杂交反应的缓冲剂。试剂盒还可包括用于使用试剂盒的组分和/或制备和/或使用BP-NA缀合物和/或经标记的成像链的说明书。

[0220] 在一些实施方案中，试剂盒包含至少一种对接链和至少一种能够短暂地结合于对接链的经标记的成像链。可以将或可以不将对接链缀合于结合伴侣。在一些实施方案中，将对接链缀合于“一般的”非靶特异性的亲和分子(例如，生物素或链霉抗生物素蛋白)，其可用于将对接链连接于由终端用户选择的结合伴侣。在一些实施方案中，所述亲和分子为第二抗体。因此，在一些实施方案中，试剂包含至少一种对接链、至少一种亲和分子诸如第二抗体和至少一种成像链。

[0221] 在一些实施方案中，试剂盒包含(a)至少一种连接于结合伴侣诸如蛋白质(例如，结合至靶的蛋白质)的对接链和(b)至少一种(例如，至少2种、至少3种、至少4种、至少5种、至少10种、至少100种)能够短暂地结合于(例如，短暂结合)对接链的经标记的成像链。对接链可包含，例如，至少两个结构域或至少3个结构域，其中每个结构域结合于各自的互补的经标记的成像链。经标记的成像链的数目可以，例如，少于、多于或等于对接链的数目。所述结合伴侣可以是蛋白质诸如，例如，抗体(例如，单克隆抗体)、抗原结合抗体片段或肽适体。在一些实施方案中，试剂盒包含至少两种不同的结合配偶(例如，蛋白质)，每一种特异于不同的靶。在一些实施方案中，结合伴侣(例如，蛋白质)通过中间接头诸如包含生物素和链霉抗生物素蛋白的接头(例如，生物素-链霉抗生物素蛋白-生物素接头)连接于对接链。在一些实施方案中，修饰对接链以包含可用于将对接链连结于结合伴侣的亲和分子。在一些实施方案中，所述亲和分子为第二抗体。在一些实施方案中，用至少一种荧光标记物(例如，至少一种荧光团)标记成像链。在一些实施方案中，成像链的长度为4至30个核苷酸，或更长。例如，成像链的长度可以为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或30个核苷酸。在一些实施方案中，成像链的长度为8至10个核苷酸。在一些实施方案中，试剂盒包含至少两种成像链，每一种彼此不同。在一些实施方案中，短暂地结合于其互补的经标记的成像链的对接链的热稳定性在其它短暂地结合于其各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

[0222] 在一些实施方案中，试剂盒包含(a)至少一种连接于单克隆抗体或其抗原结合片段(例如，结合于靶的单克隆抗体或或其抗原结合片段)的对接链和(b)至少一种(例如，至少2种、至少3种、至少4种、至少5种、至少10种、至少100种)能够短暂地结合于(例如，短暂结合)对接链的经标记的成像链。对接链可包含例如至少两个结构域，其中每个结构域结合于各自的互补的经标记的成像链。经标记的成像链的数目可以例如少于、多于或等于对接链的数目。在一些实施方案中，试剂盒包含至少两种不同的单克隆抗体或其抗原结合片段，每一种特异于不同的靶。在一些实施方案中，单克隆抗体或其抗原结合片段通过包含生物素

和链霉抗生物素蛋白的中间接头(例如,生物素-链霉抗生物素蛋白-生物素接头)连接于对接链。在一些实施方案中,用至少一种荧光标记物(例如,至少一种荧光团)标记成像链。在一些实施方案中,成像链的长度为4至30个核苷酸或更长。例如,成像链的长度可以为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或30个核苷酸。在一些实施方案中,成像链的长度为8至10个核苷酸。在一些实施方案中,试剂盒包含至少2种成像链,每一种彼此不同。在一些实施方案中,短暂地结合于互补的经标记的成像链的对接链的热稳定性在其它短暂地结合于它们各自的经标记的成像链的对接链的热稳定性的0.5kcal/mol之内。

[0223] 应用

[0224] 本公开内容的BP-NA缀合物(例如,蛋白质-核酸缀合物或抗体-核酸缀合物)可用于,除其它以外,其中使用现有的靶检测技术的任何测定。

[0225] 通常地,测定包括检测测定,所述检测测定包括诊断测定、预后测定、患者监测测定、筛选测定、生物战测定、法医分析测定、产前基因组诊断测定等。测定可以是体外测定或体内测定。本公开内容提供可使用本公开内容的方法从单个样品一次分析许多不同靶的有利方面,即使这样的靶在空间上不能使用现有技术的成像方法进行解析(并因此是空间上无差别的)。这允许例如用于在一个样品上进行的若干诊断测试。

[0226] BP-NA缀合物还可用于简单地观察区域或部位。

[0227] 本公开内容的方法可用于分析获自或源自患者的样品,以测定患病细胞类型是否存在于样品中和/或对疾病进行分期。例如,可按照本文中描述的任何方法测定血液样品以测定癌细胞类型的标志物在样品中的存在和/或量,从而诊断癌症或对其进行分期。

[0228] 或者,本文所述的方法可用于通过测定分别地细菌或病毒的标志物在样品中的存在和/或量来诊断病原体感染(例如细胞内细菌和病毒引起的感染)。因此,使用本公开内容的方法、组合物和试剂盒检测的靶可以是患者的标志物(诸如癌症标志物)或外来试剂的感染的标志物诸如细菌或病毒标志物。

[0229] 本公开内容的定量成像方法可用于例如定量靶(例如,靶生物分子),所述靶的丰度指示生物状态或疾病状况(例如,由于疾病状态而被上调或下调的血液标志物)。

[0230] 此外,本公开内容的方法、组合物和试剂盒可用于提供帮助测定患者的治疗过程的预后信息。例如,可从患者的甚至少量样品精确地定量肿瘤的特定标志物的量。对于某些疾病如乳腺癌,某些蛋白诸如Her2-neu的过表达指示将需要更具攻击性的治疗过程。

[0231] 本公开内容的方法还可用于测定扰乱(包括化合物、突变、温度的变化、生长激素、生长因子、疾病或培养条件的变化)对各种靶的影响,从而鉴定其存在、不存在或水平指示特定生物状态的靶。在一些实施方案中,本公开内容用于阐明和发现疾病状态的组分和途径。例如,存在于疾病组织中的靶的量与"正常"组织的比较允许阐明参与该疾病的重要靶,从而鉴定用于发现/筛选可用于治疗疾病的新药物候选物的靶。

[0232] 进行分析的样品可以为生物样品,诸如血液、痰、淋巴液、粘液、粪便、尿液等。样品可以为环境样品诸如水样品、空气样品、食物样品等。可利用固定的结合反应的一种或多种组分进行测定。因此,可固定靶或BP-NA缀合物。可用未固定的结合反应的一种或多种组分进行测定。鉴于由本公开内容的BP-NA缀合物和荧光标记的成像链提供的复用潜能,测定可包括基本上同时检测样品中的许多靶。作为实例,测定可用于检测特定细胞类型(例如,基

于特定的细胞表面受体)和该特定细胞类型中的特定遗传突变。以此方式,作为实例,终端用户可以能够测定携带目标突变的特定类型的细胞的数目。

[0233] 装置

[0234] 本文中还提供了用于液体处理的流体室装置,如图23A和23B中显示的。在一些实施方案中,装置是基于聚合物(例如,聚二甲基硅氧烷(PDMS))的装置,其包括第一和第二通道,每一个通道在一端连接至样品室。该构造允许以受控的速率将一种或多种流体连续地施用至样品。例如,可将使用注射器通过装置的第一通道将第一流体施用至样品。随后可使用注射器施用第二流体,其穿过装置的第一通道进入样品室内,从而迫使第一流体流出样品室,穿过第二通道并进入例如连接于第二通道的储存器(图23A)。在一些实施方案中,将装置置于载玻片上,以允许从置于装置之下的显微镜物镜进行观察。

[0235] 超分辨率成像

[0236] 在一些实施方案中,可通过使用图11A中描述的两个策略之一增加每定位事件的光子数目来实现具有增加的空间分辨率的超分辨率成像(在本文中称为"超分辨率"成像)。在第一策略中,提取来自单一的可补充的荧光团的最大数目的光子。与荧光团稳定缓冲剂组合的高激光激发功率(16,17)可用于"漂白"对接位点(或对接链的位点)上的短暂结合的荧光团,从而提取每结合事件和染色的最大数目的光子(图11A)。成像链的反复结合允许每一结合链的"光漂白",从而最大程度地利用发射的光子和导致定位精确性相对于传统成像技术的显著提高。在第二策略中,使用明亮的元荧光团。可通过用许多荧光团修饰紧密的DNA纳米结构来构建荧光DNA纳米结构或"元荧光团"(图11B3)。来自元荧光团的个体染料发射的总和被解释为源自相同点源,从而,使用元荧光团替代标准荧光团(例如,Cy3)进一步提高定位精确性。元荧光团的具有活跃本底抑制的更高级形式描绘于图11B4中。此处,蛤壳状结构用作只有当其结合于对接链时才发荧光的条件荧光团。

[0237] 本公开内容还提供了用于斑点检测、拟和和漂移校正的算法,如下文中描述的。

[0238] 用于漂移校正的软件算法

[0239] 一些实施方案涉及用于校正时间序列中记录的图像中的漂移的方法和装置。下文中更详细论述的用于进行漂移校正的技术的非限制性应用是校正本文中描述的在分子尺度上的基于DNA的成像中的漂移,所述成像牵涉对接链与成像链之间的短暂结合。然而,应当理解,本文所述的技术可备选地用于校正其它成像应用中的漂移,在所述应用中,在图像的时间序列过程中记录一个或多个短暂成像事件,并且与漂移校正相关的实施方案不限于在分子尺度上的基于DNA的成像。

[0240] 在一些实施方案中,DNA纳米结构可用作漂移标记。可使用任何合适的DNA纳米结构(参见,例如,(Rothemund US-2007/0117109A1)、单链瓦片(Yin等,"Programming DNA Tube Circumferences,"Science(2008):321:824-826)、DNA发夹(Yin等US-2009/0011956A1;Yin等,"Programming biomolecular self-assembly pathways,"Nature(2008)451:318-323),并且可使用例如DNA折纸技术来制备其。与高级的分析和后处理技术组合使用基于DNA纳米结构的漂移标记的漂移校正具有高精度校正、与长时间成像相容和执行简单的有利方面。并入基于荧光珠粒的漂移标记的基于核酸的常规成像技术受到在漂白珠粒之前有限的成像时间长度的困扰;而明视野成像需要专门的设备,例如,双视场摄像视图。

[0241] 根据本文所述的一些实施方案的漂移校正技术可包括多个阶段，其中每个阶段使用不同的技术来进行漂移校正。在一些实施方案中，将来自一个阶段的输出提供为随后阶段的输入用于另外的漂移校正处理。在第一阶段，通过比较来自相邻帧的定位进行粗略的漂移校正。在第二阶段，选择单个漂移标记，将其时间轨迹用作不同的粗略校正。在第三阶段，自动地或通过用户输入选择一组漂移标记，随后组合它们的时间轨迹以计算更精确的漂移校正。在第四阶段，将来自以确定的和空间上可解析的几何学(例如，4x3网格点)展示斑点的基于模板的漂移标记的定位合并。在第五阶段，进行漂移校正的平滑以进一步减少噪声和使得最终图像的分辨率能够达到分子尺度的分辨率。

[0242] 可按照本文所述的技术进行这5个阶段的任何数目和/或组合。例如，在一些实施方案中，可使用质量测量表征图像的时间序列中的漂移的量，并且至少部分地基于质量测量，可以消除所述阶段的一个或多个阶段。在其它实施方案中，可进行所有5个阶段，因为实施方案不限于此方面。在其它实施方案中，还可使用与本文所述的阶段的至少一个阶段组合使用的另外的漂移校正阶段。

[0243] 在用于进行漂移校正的技术的下列描述中，术语FWHM(半峰全宽)用作“分辨率”的数学替代。还使用针对高斯分布的FWHM～＝σ*2.35的近似法，其中σ为标准差。本文所述的用于进行漂移校正的技术涉及处理图像的时间序列。记录的图像堆栈在本文中被称为"影片"并且每个个体图像被称为"帧"。对影片的每一帧利用斑点寻找算法进行操作，随后将局部高斯拟合算法用于每个鉴定的斑点；斑点及其拟合的中心位置定位在本文中可互换地称为“定位”。影片的帧捕捉存在于一些帧中但不存在于其它帧中的一个或多个短暂事件。在其中影片的帧涉及如上讨论的基于核酸的成像的技术的举例说明性应用中，成像链与对接链的杂交直至它们的解离在本文中称为结合"事件"；从而事件可具有一系列相邻帧中的几个定位，并且通常将由所述定位组成。虽然结合事件在下文中被更详细地讨论为可使用本文所述的技术来分析用于进行漂移校正的一个举例说明性短暂事件，但应当理解，可替代地使用在时间序列中成像的其它类型的短暂事件。在某一视野区域内，在整个完整影片中的所有定位的集合被总地称为“时间轨迹”，其反映观察到的结构的运动，并且用于以几个不同的方式进行漂移校正，如在下文中更详细描述的。

[0244] 漂移校正技术的概述

[0245] 图12举例说明可按照本文所述的技术进行的漂移校正程序的5个阶段的示意图。所述阶段被举例说明为以从未处理的图像至最终图像的顺序连续地进行，所述最终图像已使用五个阶段的每一个的技术进行了处理。在下文中将更详细地讨论这些阶段的每个阶段。简言之，图12A(i)举例说明显示漂移校正的每个阶段的原理的示意图。在每个图像中，黑色标记和线表示源数据，红色值和曲线表示计算的漂移校正。图12A(ii)显示每个阶段中使用的主要类型的漂移标记(例如，DNA漂移标记)的示意图。图12B(i)举例说明显示每个阶段或校正后的成像质量的示例性结构，以及图12B(ii)显示每个阶段上图12B(i)中的对应的绿色矩形的放大的图像。图12B(i)和12B(ii)中显示的比例尺对应于50nm。图12C(i)举例说明每个阶段校正后的示例性漂移轨迹，图12C(ii)显示在每个阶段图12C(i)中的对应矩形的放大图像。图12C(i)中的比例尺对应于x:500nm，t:500s，图12C(ii)中的比例尺对应于x:10nm，t:10s。图18举例说明按照一些实施方案的漂移校正方法中的阶段的替代性代表。

[0246] 在一些实施方案中，来自第一阶段的图像分辨率输出可为约1μm。

[0247] 在一些实施方案中，来自第二阶段的图像分辨率输出可以为约200nm。

[0248] 在一些实施方案中，来自第三阶段的图像分辨率输出可为约20nm。

[0249] 在一些实施方案中，来自第四阶段的图像分辨率输出可为约5nm。

[0250] 在一些实施方案中，来自第四阶段的图像分辨率输出可以为约小于5nm。

[0251] 成像质量和可达到的分辨率的极限

[0252] 漂移校正的图像的最优可能质量受到个体定位的质量的限制，所述个体定位的质量通过在成像会话(例如，显微成像会话)过程中使用的各种条件来测定。为了在不同成像条件的质量之间进行定量评估和有效比较，将被称为相邻帧定位之间的距离(DNFL)的量定义为源自相同短暂事件(例如，结合事件)的从连续图像帧检测到的定位之间的平均间距。

[0253] 如下概述用于计算图像的DNFL的方法。对于每一对NF(相邻帧，例如帧#1和#2)，将来自两个帧的所有定位合并，并计算来自不同帧的每一对定位(例如，来自帧#1的一个定位相对于来自帧#2的另一个定位)之间的距离，假定帧之间无漂移。将所得的来自所有NF对的距离合并以提供双峰分布。所述双峰分布的第一模式在振幅上是宽、高的，并且横跨视野的宽度。双峰分布的第二模式在振幅上是尖锐、低的，和接近于零，并对应于来自相同结合事件的定位。可测定第二模式的最大值并且可围绕最大值进行局部高斯拟合算法以测定峰的中心。该值可被认为是某一图像的DNFL。在不组合来自连续帧的定位的情况下，DNFL值设置了可从某一图像获得的最优可能分辨率的极限，其中最佳实现的分辨率与DNFL之间的数学关联为：最佳可实现的分辨率＝DNFL/sqrt(2)*2.35。

[0254] 漂移校正质量和支持的分辨率

[0255] 可通过表征单个结合位点的点扩散函数(PSF)来评估最终的漂移校正图像的质量。超过数千个单个对接位点的图像的统计叠加可被产生为PSF分布的参照。可对统计叠加进行2-D高斯拟合以测定PSF的标准差(σ)，其进而测定所产生的图像的最佳支持的分辨率，由与上述相似的公式给出：图像支持的分辨率＝σ*2.35。可借助于辅助DNA纳米结构进行单个分离的对接位点的分离和叠加。该结构具有良好分离的对接位点的已知模式(例如，网格模式)，并且可以是用于基于模板的漂移校正阶段的相同结构，在下文中进行了更详细论述。由于激光强度的变化，光学表面沉积和其它系统因素的不均匀性以及成像过程的可能的随机性质，完整图像的上述测定的图像质量可能不反映成像视野中的每个单个样品物体的真实成像质量。通常地，与成像视野的中心更接近并且更好地固定于光学表面的结构被更好地照明，并且显示比在外周上并且固定得不太好的那些结构更好的分辨率。可在与上述类似的方法中测定单个分子的成像质量和分辨率。可沿最佳分离对接位点的方向(在网格结构的情况下，这将沿着任何网格方向)获取辅助DNA纳米结构(与上述相同)的单分子的投影，并且可对投影的1-D分布进行多高斯拟合。随后可测定拟合的高斯峰的标准差，并将其类似地用于推断单分子图像的分辨率。

[0256] 漂移评估和漂移校正阶段的选择

[0257] 在一些实施方案中，连续地操作下文中更详细讨论的并入用于进行漂移校正的技术的五个阶段，以减小未处理的图像的漂移，其中每个连续阶段进一步减小漂移，并且使漂移低至足以用于下一阶段的成功操作。取决于所捕捉的图像中的漂移的量，可使用少于所有五个阶段。例如，如果测定原始图像中存在低的漂移，则每一帧中的定位可以是可分离的，并且处理可始于第二阶段而无需通过第一阶段的处理。如果测定在原始图像中存在甚

至更低的漂移，则处理可始于第三阶段而无最终图像质量的显著损失。由于漂移的复杂来源(其可包括除其它以外热波动和膨胀、因电动机活动而导致的显微镜载物台移动、来自建筑和光学台复合体的振动)，控制原始图像中的漂移往往非常困难，并且包括所述前两个阶段在产生具有所需分辨率的最终图像中常常是有用的。例如，包括本文所述的所有五个阶段为漂移校正提供了稳健策略，其适用于在大多数生物实验室中采集的图像，而无需专门的硬件或建筑要求。

[0258] 在一些实施方案中，原始未处理的图像的漂移的量和整体图像质量可使用任何合适的技术来测定，并且所测定的图像质量可用于选择所选择的漂移校正阶段来用于进行漂移校正。例如，用于测定图像质量的技术可比较相同图像的不同时间区段。在此举例说明性技术中，原始图像可通过单独地合并分别来自影片的前半部分和后半部分的定位来分成两半。可计算两个图像之间的交互关系，并且可估计最佳偏移以提供整体漂移的指征。可将整体漂移的指征与一个或多个阈值相比较，以测定在进行按照本文所述技术的漂移校正过程中是否可跳过一个或多个漂移校正阶段。

[0259] 图13举例说明用于按照一些实施方案进行漂移校正的方法。在行为210中，通过考虑影片的相邻帧间的定位的差异来进行漂移校正。随后将过程进行至行为220，其中选择单个漂移标记，测定描述漂移标记在影片过程中随时间过去的运动的时间轨迹，并将单个漂移标记的时间轨迹用于进行图像的漂移校正。随后将过程进行至行为230，其中测定在图像中鉴定的多个漂移标记的每一个的时间轨迹。如在下文中更详细地讨论的，时间轨迹之间的差异可用于提供最终图像的进一步漂移校正。随后将过程进行至行为240，其中进行使用几何约束的模板的漂移校正。随后将过程进行至行为250，其中通过使用适当的平滑技术使漂移轨迹平滑来进一步漂移校正图像，如在下文中更详细讨论的。在下文中更详细地描述了用于按照本文所述的技术进行漂移校正的五个阶段的每一个。如应当从前述讨论所理解的，并非所有实施方案需要使用漂移校正处理的所有五个阶段。例如，在一些实施方案中，可仅进行行为230和240。在其它实施方案中，可进行行为230、240和250。在其它实施方案中，可进行行为220、230、240和250而不包括行为210。

[0260] 第一阶段漂移校正

[0261] 通过比较来自影片中相邻帧的定位来操作漂移校正的第一阶段(例如，图13的行为210)。与上述DNFL计算类似的方法(或任何其它适当的技术)可用于鉴定源自相同短暂事件(例如，单个结合事件)的成对定位。可将源自相同短暂事件的所有成对定位合并，以产生双峰分布。在生成来自相邻图像的定位的双峰分布后，可自动地测定截断值以将来自相同事件的那些成对定位(紧密的定位)与来自不同事件的那些成对定位分离。随后，合并相同的相邻帧(NF)的所有成对紧密定位，并且计算每一对之间的偏离。将所有偏离的矢量平均值输出为漂移校正。对于不具有鉴定出的合格紧密定位的NF对，可输出零漂移。漂移校正的第一阶段通常校正具有高振幅(在偏移上超过1μm)的全局漂移，这有效地除去不同漂移标记之间的干扰，并且允许下一阶段的并入。如上所讨论的，在其中不同的漂移标记可容易地彼此分开的一些实施方案中，可省略处理的第一阶段。处理的第一阶段可否可被省略的确定可使用图像质量因素分析(如上文中所讨论的)或使用任何其它合适的技术(例如，手动检查)来进行。

[0262] 第二阶段漂移校正

[0263] 漂移校正的第二阶段(例如,图13的行为220)在单个漂移标志或样品物体上操作。可以任何合适的方式选择用于漂移校正处理的此阶段的单个漂移标记。例如,在一些实施方案中,可从所有鉴定的漂移标记的组随机选择单个漂移标记。在其它实施方案中,与所需质量(例如,在整个影片上漂移的平均量)相关的特定漂移标记可被选择作为单个漂移标记以用于此阶段。在选择单个漂移标记后,自动测定、平滑其时间轨迹,并将其输出为漂移校正。或者,可在其中漂移标记之间的分离难以自动鉴定的情况下手工绘制漂移轨迹。

[0264] 漂移校正的第二阶段进一步减小影片中的全局漂移(通常地<200nm),并且允许在以下阶段中进行漂移标记的自动化批量鉴定。

[0265] 漂移校正的第三阶段

[0266] 漂移校正的第三阶段(例如,图2的阶段230)组合多个漂移标记的时间轨迹来以比漂移校正的第二阶段更好的分辨率计算漂移校正。每个漂移标记具有大量的对接位点,以允许高的时间覆盖;并且将大量的这些漂移标记与样品一起沉积在成像表面上。可适当地选择每个漂移标记上的结合位点的数目和表面上的漂移标记的浓度来确保高质量的漂移校正,因为进行此阶段中的漂移校正改善主要通过每个漂移标记在图像中的散步和每帧漂移标记的有效数目来决定。

[0267] 图14举例说明用于进行对应于图2的阶段230的漂移校正的方法。在行为310中,鉴定了多个漂移标记的位置。鉴定多个漂移标记的位置可包括合并来自影片的所有帧的定位以计算二维(2D)直方图。随后,可通过适当地调整直方图区间化尺寸和其它选择标准的组合来鉴定漂移标记的位置。例如,可调整直方图的区间化尺寸以反映漂移标记的特征尺寸,例如,它们的整体尺寸的四分之一或三分之一。选择标准的范围包括,但不限于,直方图值的下限阈值和基于几何性质(例如,面积、尺度)的过滤。邻近漂移标记之间的充足分离通常是排除错误定位所必需的,所述错误定位例如产生于双结合事件的伪定位。在一组(例如,数千)漂移标记的鉴定后,将方法进行至行为320,其中测定每个漂移标记的时间轨迹并且计算测定为每个时间轨迹与组合轨迹的中心的偏离的相对时间轨迹。因为图像捕捉短暂事件(例如,核酸-结合事件),所以并非漂移标记的所有时间轨迹可覆盖影片中的所有时间点。在这样的情况下,时间轨迹可被线性地内插在"缺失点"以获得更好和更平滑的结果。

[0268] 在测定多个漂移标记的每一个的时间轨迹后,将方法进行至行为330,其中基于针对每个漂移标记测定的时间轨迹测定图像的漂移校正。时间轨迹的任何适当的组合可用于测定漂移校正,并且实施方案不限于此方面。在一些实施方案中,时间轨迹的加权平均用于测定从此阶段的漂移校正输出。例如,可将一组相对时间轨迹的加权平均计算为漂移校正的结果,其中通过漂移标记轨迹的质量对校正进行加权。可以以任何适当的方式测定漂移标记轨迹的质量,所述方式包括,但不限于,通过评估漂移标记质量或个体定位质量来测定质量。在一些实施方案中,通过获得随时间过去轨迹的标准差(σ)来计算每个漂移标记轨迹的质量。每个轨迹的此测量的倒数(例如,1/σ)可用作加权系数。或者,时间轨迹内的每个个体定位的质量可被计算为通过Thomson,2002中的公式给出的定位不确定性。此计算的标准差的倒数可用作每个时间轨迹的加权系数。在测定漂移校正后,方法进行至行为340,其中使用测定的漂移校正来校正图像。

[0269] 可进行漂移校正的此阶段任何次数。在一些实施方案中,利用不同的用于每次迭代的参数迭代进行漂移校正的此阶段。随着漂移校正进行,剩余的漂移幅度被进一步减小,

漂移标记的空间扩展变得越来越小，并且可越来越严格地进行漂移标记的选择。因此，在最初的迭代中，可将阈值直方图计数设置至较低的值，并且可在更后的迭代中将该值调整至较高的值。或者，在一些实施方案中，可在初始迭代中将漂移标记的有效面积和尺度设置至较大的值，之后在随后的迭代中将其调整至较小的值。此外，在一些实施方案中，漂移标记之间的间隔在更后的迭代中可从较大值改变至较小的值。在一些实施方案中，可测定迭代之间的交互质量检查(手工地或自动地进行)，以促进进一步操作的确定。

[0270] 取决于成像质量，漂移校正的此阶段通常将所获得的图像分辨率带至允许的最佳分辨率的系数2之内(即，如果每个个体定位的精确度支持～5nm的分辨率，则此阶段通常产生～10nm的分辨率)。通常地，在良好成像条件的情况下，可在利用此阶段的处理后获得<10nm的分辨率。

[0271] 漂移校正的第四阶段

[0272] 漂移校正的第四阶段(例如，图13的行为240)使用漂移标记"模板"，从而此阶段被称为"模板化漂移校正"。将一个或多个漂移标记模板(例如，具有处于已知和良好分离的几何排列的对接位点的DNA纳米结构)与上文中结合阶段3讨论的"普通"漂移标记一起沉积至成像表面上。这些对接位点之间的间隔优选被选择为不小于从先前阶段(例如，阶段3)所得的分辨率的2倍，从而允许来自不同对接位点的定位之间的容易分离。这些模板上的对接位点的数目以及表面上的对接位点的浓度优选被选择为实现有效的模板校正，类似于针对第三阶段所描述的。

[0273] 图15举例说明用于进行对应于图2的阶段240的漂移校正的方法。在行为410中，从在影片的所有帧上合并的定位的2-D直方图鉴定多个漂移校正模板。为了区分漂移模板与用于第三阶段的漂移标记，除了如上提及的选择标准的范围以外，还可并入直方图计数中的额外上限阈值。在已鉴定漂移模板后，方法进行至行为420，其中测定每个漂移模板的时间轨迹。因为对接位点被设计来在模板中良好地分离，所以可从完全漂移模板的每个时间轨迹分离来自个体对接位点的几个非重叠时间轨迹。可以以与来自完整图像的漂移标记的鉴定类似的方式进行此鉴定和分离步骤。例如，可使用针对每个漂移模板的个体时间轨迹的标准差的局部直方图阈值化和过滤的组合。

[0274] 在已测定每个漂移模板的时间轨迹后，方法进行至行为430，其中将时间轨迹的组合用于测定此阶段的漂移校正。在一些实施方案中，这通过计算每个时间轨迹的相对时间轨迹来实现，并且至少部分地使用相对时间轨迹来测定漂移校正。可以以任何适当的方式使用相对时间轨迹来测定漂移校正。例如，在一些实施方案中，可将个体对接位点的所有时间轨迹的加权平均进行平均以产生最终的漂移校正。可以以任何适当的方式测定加权系数。例如，加权系数可基于每个个体位点的质量，或时间轨迹中的每个个体定位的质量。在测定漂移校正后，方法进行至行为440，其中使用测定的漂移校正来校正图像。

[0275] 取决于成像质量，漂移校正的此阶段可导致与最佳可能分辨率接近的最终图像的分辨率。即，如果每个个体定位的精确度支持～5nm的分辨率，则按照本文所述的技术进行模板漂移校正可达到接近～6nm的分辨率。在一些实施方案中，对于在第3阶段后达到的<10nm的分辨率，可将具有在间隔20nm的4x3网格中排列的12个对接位点的DNA纳米结构用作漂移校正模板。使用此部分中描述的技术的基于模板的漂移校正可使得能够在此阶段后实现6-7nm的分辨率。

[0276] 第五阶段漂移校正

[0277] 漂移校正的第五阶段(例如,图13中的行为250)进行漂移校正轨迹的平滑,并且可将经平滑的漂移校正轨迹用于进行漂移校正。例如,在测定上文中讨论的第二、第三和第四阶段的一个或多个的漂移校正后,可使用任何适当的技术对所得的漂移校正进行平滑,以产生最终的漂移校正结果。平滑通过考虑相邻帧中的定位有效地增加每一帧中漂移标记或漂移模板的数目。可使用任何适当的窗口期,以任何适当的方式进行平滑。例如,在一些实施方案中,利用在通过特征性漂移时间尺度测定的窗口期上操作的稳健局部回归法进行平滑。平滑窗口期的非限定性实例可以为10-30s。

[0278] 至3D成像的扩展

[0279] 上述所有阶段也可直接用于校正3D图像(例如,超分辨率图像)。在3D超分辨率成像中,例如,可将散光透镜引入成像路径,并且可将所得的高斯发射特征谱的椭圆率用于测定分子的z-位置。可以以一对一的方式使用上述折纸漂移标记结构(例如,几何约束的模板)以进行漂移校正的阶段。对于基于模板的漂移校正阶段,可使用具有确定的3D形状(例如,四面体)的DNA折纸结构。

[0280] 图16举例说明用作用于3D漂移校正的模板的3D四面体。用对接位点标记4个转角。图16A显示清楚地解析了四个转角。图16B举例说明具有~85nm的高度的结构的X-Z投影。

[0281] 至多色成像的扩展

[0282] 关于对在图像中使用单色鉴定的短暂事件进行成像而描述了用于校正多个时间序列图像中的漂移的上述技术。然而,应当理解,这些技术可扩展至多色成像,其中使用可在相同图像中鉴定的不同颜色标记不同短暂事件(例如,不同核酸漂移标记与对接位点的结合)。当使用多色成像时,上述用于基于模板的漂移校正的几何模板可包括描述对应于不同颜色的不同短暂事件的特定已知几何学的信息。例如,与在3x4网格中的单个对接位点上发生的仅单个结合事件不同,可呈现在相同几何模板中使用不同颜色进行颜色编码的多个结合事件,并且可使用来自多个结合事件的信息进行漂移校正。还考虑了用于将本文所述的技术扩展至多色成像的其它方法,并且实施方案不限定于此方面。

[0283] 示例性计算机系统

[0284] 可结合本文所述的本公开内容的任何实施方案使用的计算机系统600的举例说明性实现示于图17中。计算机系统600可包括一个或多个处理器610和一个或多个计算机可读的非暂时性存储介质(例如,内存620和一个或多个非易失性存储介质630)。处理器610可以以任何适当的方式控制数据从内存620和非易失性存储设备630的读写,本文所述的本公开内容的各方面不限于此方面。为了进行本文所述的任何功能性,处理器610可执行存储在一个或多个计算机可读存储介质(例如,内存620)中的一条或多条指令,所述计算机可读存储介质可用作存储用于通过处理器610执行的指令的非短暂性计算机可读存储介质。

[0285] 可以以许多方式的任何方式实现本公开内容的上述实施方案。例如,可使用硬件、软件或其组合实现所述实施方案。当在软件中实现时,可在任何适当的处理器或处理器的集合上执行软件代码(无论是单个计算机中提供的还是分配在多个计算机之间的)。应当理解,进行上述功能的任何组件或组件的集合可以通常被视为控制上述功能的一个或多个控制器。可以以许多方式,诸如利用专用的硬件,或利用使用微代码或软件编程以进行上述功能的通用硬件(例如,一个或多个处理器)实现所述一个或多个控制器。

[0286] 在这方面，应当理解本公开内容的实施方案的一个实现包括至少一个利用计算机程序(即，多条指令)编码的非暂时性计算机可读存储介质(例如，计算机内存、软盘、光盘、磁带等)，所述计算机程序当在处理上执行时进行本公开内容的实施方案的上述功能。计算机可读存储介质可以是便携式的，以使得存储在其上的程序可被加载至任何计算机来源以实现本文所讨论的本公开内容的各方面。另外，应当理解，对当执行时进行上述功能的的计算机程序的提及不限于在主机上运行的应用程序。相反地，术语计算机程序在本文中以一般意义用于指可用于对处理器编程以实现本公开内容的上术方面的任何类型的计算机代码(例如，软件或微代码)。

[0287] 等同物

[0288] 虽然已在本文中描述和说明了几个本发明的实施方案，但本领域普通技术人员将容易地设想用于进行所述功能和/或获得本文所述的结果和/或一个或多个有利方面的各种其它方法和/或结构，并且这样的变化和/或修改的每一种被认为在本文所述的本发明的实施方案的范围内。更通常地，本领域技术人员将容易地理解，本文所述的所有参数、尺度、材料和构型意欲为示例性的并且实际参数、尺度、材料和/或构型将取决于对其使用本发明的教导的具体应用。本领域技术人员将认可或能够确定本文所述的特定的本发明的实施方案的许多等同物(通过使用不超过常规实验)。因此，应理解，前述实施方案仅通过举例的方式提出，并且在所附权利要求和其等同物的范围内，本发明的实施方案可按与明确描述和要求保护的方式不同的方式来实施。本公开内容的本发明的实施方案涉及本文所述的各种个体特性、系统、物品、材料、试剂盒和/或方法。另外，如果这样的特性、系统、物品、材料、试剂盒和/或方法不是相互矛盾的，则两个或更多个这样的特性、系统、物品、材料、试剂盒和/或方法的任意组合包括在本公开内容的发明范围内。

[0289] 如本文中所定义和使用的，所有定义应当被理解为优先于词典定义、通过引用并入的文献中的定义和/或所定义的术语的一般含义。

[0290] 本文中公开的所有参考文献、专利和专利申请在关于引用其每一个的主题方面通过引用并入本文，这在一些情况下可包括文献的完整内容。

[0291] 除非明确地指示与之相反，否则不定冠词“一种/一个(a)”和“一种/一个(an)”，如本文中在说明书和权利要求中所用，应当被理解为意指“至少一种/一个”。

[0292] 短语“和/或”，如在本文中在说明书和权利要求中所用，应当被理解为意指所连接的元素的“任一个或两者”，即元素在一些情况下结合地存在以及在其它情况下分离地存在。利用“和/或”列出的多个元素应当以相同的方式来解释，即所连接的元素的“一个或多个”。除通过“和/或”从句明确确定的元素外，还可任选地存在其它元素，无论与明确确定的那些元素相关还是无关。因此，作为非限定性实例，对“A和/或B”的提及，当与开放性措辞诸如“包含/包括”结合使用时，在一个实施方案中可仅指A(任选地包括除B外的元素)；在另一个实施方案中可仅指B(任选地包括除A外的元素)；在另一个实施方案中，可指A和B(任选地包括其它元素)；依此类推。

[0293] 如本文中在说明书和权利要求中所用，“或”应当被理解为具有与如上定义的“和/或”相同的含义。例如，当在列表中分开各项时，“或”或“和/或”应当被理解为是包含性的，即包含至少一个，但也包括许多个元素或一列元素中的多于一个，以及任选地，包括另外未列出的项。只有明确地指明相反的术语，例如“……中的仅一个”或“……中的恰好一个”或

当用于权利要求中时的“由……组成”将指包含多个元素或一列元素中的正好一个元素。一般地，如本文中所用的术语“或”应当仅在冠有排他性的术语诸如“任一”、“……之一”、“……中的仅一个”或“……中的恰好一个”时被解释为表示排他性选择(即“一个或另一个但非两个”)。“基本上由……组成”，当用于权利要求中时，应当具有其在专利法领域中使用的普通含义。

[0294] 如本文中在说明书和权利要求中所用，关于一列一个或多个元素的短语“至少一个”应当被理解为意指选自一列元素中的任何一个或多个元素的至少一个元素，但不一定包括元素列表内明确列出的每一个元素的至少一个并且不排除元素列表中的元素的任何组合。该定义还允许可任选地存在除短语“至少一个”所指的元素列表内明确确定的元素外的元素，无论与那些明确确定的元素相关还是不相关。因此，作为非限定性实例，“A和B的至少一个”(或，等同地，“A或B的至少一个”或，等同地“A和/或B的至少一个”)可在一个实施方案中指至少一个(任选地包括不止一个)A而无B存在(且任选地包括除B外的元素)；在另一个实施方案中指至少一个(任选地包括不止一个)B而无A存在(且任选地包括除A外的元素)；在另一个实施方案中指至少一个(任选地包括不止一个)A和至少一个(任选地包括不止一个)B(且任选地包括其它元素)；依此类推。

[0295] 还应当理解，除非明确地指明与之相反，否则在包括不止一个步骤或行为的本文请求保护的任何方法中，所述方法的步骤或行为的顺序不必须地限定于其中叙述所述方法的步骤或行为的顺序。

[0296] 在权利要求中，以及在上述说明书中，所有过渡短语诸如“包含”、“包括”、“携带”、“具有”、“含有”、“拥有”、“牵涉”、“持有”、“由……组成”等被理解为开放性的，即意指包括但不限于。只有过渡短语“由……组成”和“基本上由……组成”应当分别是封闭性的或半封闭性的过渡短语，如美国专利局专利审查程序手册第2111.03节中所示的。

实施例

[0297] 实施例1：细胞成像

[0298] 通过将对接链连接于抗体来实现固定的细胞中的细胞内组分的复用超分辨率成像(图2)。通过首先将生物素化的对接链与链霉抗生物素蛋白反应，和随后与针对目标蛋白质的生物素化抗体温育来形成这些抗体-DNA缀合物。随后使用针对β-微管蛋白的预装配的抗体-DNA缀合物来对固定的HeLa细胞进行免疫染色。在成像之前，将ATTO655-标记的成像链引入杂交缓冲液(1×PBS，补充有500mM NaCl)中的样品，使用斜射照明进行单分子成像(9)。所得的超分辨率图像显示与衍射极限图相反的空间分辨率的清楚增加(图3a-3c)。在图3B中位置<i>上获取的横断面特征谱在两个相邻微管之间产生≈79nm的距离，其中每个微管具有≈47和≈44nm的表观宽度，这与针对免疫染色的微管的早期报导一致(13)。本公开内容的抗体-DNA缀合法产生高标记密度，并且几乎没有至没有成像链与非标记的细胞组分的非特异性结合发生。

[0299] 为了证实本公开内容的标记方案的多色扩展，其中将正交成像链序列偶联于光谱上不同的染料，用具有针对Cy3b-标记的链的对接序列的预装配的抗体-DNA缀合物标记固定的HeLa细胞中的微管网络，并且使用连接于针对ATTO655成像序列的正交序列的第二抗体对线粒体进行染色。虽然Cy3b-和ATTO655-标记的成像链同时存在于溶液中，但在Cy3b和

ATTO655通道中连续进行成像，所得的超分辨率图像显示空间分辨率相较于衍射极限图的清楚增加(图3d-3f)。如在单色情况下，观察到几乎没有至没有成像链与细胞环境中的非标记组分的非特异性结合。此外，与体外情况类似，未观察到两种颜色之间的串扰，这表明成像链的序列特异性相互作用。

[0300] 实施例2:复用

[0301] 假定固定的定位精确度，可一般地获得成像分辨率的直接2倍的增加。这可以通过使具有相同对接链序列的斑点(例如，图4中的对接位点a)比实际的当前分辨率极限间隔得更开，从而将这些位点鉴定为超分辨图像中的单个斑点来实现。因为现可将所有获得的定位赋予至特定位点，因此可获得的成像分辨率不再是重构斑点的半峰全宽(FWHM)，而是标准差(约为FWHM的1/2.35)。这描述于图4B1中，其中以≈14nm的分辨率对具有10nm间距的一组7个点进行成像，从而使个体点不可解析。横断面直方图数据显示宽峰(底图)。在图4B2和4B3中，每隔一个位点进行成像允许个体斑点的定位。随后组合这些定位以形成具有增加的分辨率的最终合成图像。

[0302] 图5A显示单个DNA纳米结构，其展示四个不同组的DNA序列，被设计来分别类似0至3的数字。使用简单的流动室设置连续进行成像，首先在与数字0的对接链互补的成像链中进行冲洗，随后将溶液替换为具有与数字1的对接链互补的序列的成像链，依此类推。对所得的图像进行伪彩色化以代表各自的成像回合。使用DNA折纸结构的证明显示高成像效率以及在连续成像回合之间无串扰。

[0303] 为了证实使用交换-PAINT的DNA结构的十“色”超分辨率成像，设计10种独特的矩形DNA折纸结构，每一种结构显示类似0至9的数字的不同模式的正交对接链(关于模式“4”参见图5B(ii))。在所有十个结构的表面固定后，利用便于液体操作的定制流动室(图23A)进行连续成像。将全部用Cy3b标记的10个正交成像链(P1*至P10*)用于进行交换-PAINT。来自所有10个成像回合的所得数字示于图5B(v)中。以高空间分辨率解析每个靶。沿数字条的横断面直方图显示分布的10nm以下的FWHM(数据未显示)。注意，对于所有数字维持高分辨率，因为每一循环中使用相同的优化染料(Cy3b)和成像条件。

[0304] 图5B(iii)显示了所有10个回合的组合图像，证实成像链与各自的靶的特异性相互作用不具有可观察到的循环之间的串扰。数字8和9不存在于所选择的区域中。观察到明显的“绿色”数字5而非2(图5B(iii)中的<i>)。这可能不是来自串扰的虚假成像的数字5，而是“镜像”数字2。镜像图像可因颠倒固定的折纸而产生，其中对接链被捕获在底部，但对于成像链仍然是可及的。

[0305] 流体设置被设计来通过将流体储存器和注射器与实际流动室“解偶联”(通过柔性管道)来最大限度减少样品运动。为了避免样品失真，需要特别小心，以确保在洗涤步骤中轻柔的流体流动。为了验证样品确实表现出极少运动和极少至没有失真，进行了10轮交换-PAINT实验。在第一回合中针对数字4对DNA折纸进行成像，并在10轮缓冲液交换后对其再次进行成像。总样品运动(流动室相对于物镜的物理运动)小于2μm，这可以很容易地使用基准标记来校正。用于选择结构的标准化互相关分析产生相关系数0.92，这表明几乎没有样品失真(也参见在细胞成像部分中的讨论)。

[0306] 最后，通过使用交换-PAINT，在相同的DNA折纸结构上成功地对4个不同的数字模式进行成像(图5B(iv))。因此，交换-PAINT不限于空间上分离的种类，并且可解析相同结构

上的亚衍射模式而无可观察的串扰或样品失真。通过使用基于DNA折纸的漂移标记直接对齐来自不同交换-PAINT回合的图像。另外，因为使用相同染料进行成像，所以无需校正成像回合之间的色差。

[0307] 通过靶向固定的HeLa细胞中的微管和线粒体(类似于图3)，但只使用单色荧光团或光谱上无差别的成像链，显示了本公开内容的方法在细胞环境中的适用性。使用用相同染料标记的成像链连续地进行成像(两个回合，图6)。

[0308] 实施例3:定量成像

[0309] 为了证明本公开内容的定量方法的可行性，使用具有以网格状排列的13个结合位点的DNA折纸纳米结构(图7C)。对接位点的掺入效率非100%，从而导致实际掺入位点的分布(图7C和7D1)。然而，所述结构是理想的测试系统，因为可用的位点的数目可通过计数斑点的数量("直接")和将其与使用提出的结合动力学分析计算的对应的位点数目相比较来可视地测定。图7D1显示通过直接计数获得的377个折纸结构的结合位点分布。针对通过结合动力学分析获得的相同结构的结合位点分布示于图7D2中。最后，作为基准测试，计算每个结构的直接计数与动力学计数之间的"偏离"。所述方法的计数"误差"或不确定性小于7%(通过高斯分布的变异系数测定的)，成像时间为~25分钟。

[0310] 实施例4:动力学条形编码

[0311] 通过结合频率分析而非几何或频谱编码来获得高度复用超分辨率条形编码。BP-NA缀合物或对接链与目标分子的结合频率线性地取决于该分子上的结合位点的数目。给定某一浓度的荧光标记的成像链和缔合速率，结合频率与结合位点的数目呈线性比例，从而可用于鉴定。例如，使用3种颜色和每颜色4个水平的结合频率产生124种独特的动态"闪烁标志"(图8)。相较于几何编码，此方法表征紧凑得多的非结构化探针。相较频谱编码(14)，本公开内容的方法更具成本效益、可扩展和更容易实现。在此频率编码方法中只需要3种荧光标记的成像链，从而使得其对于高通量筛选实验非常具有成本效益。对DNA折纸测试结构的体外试验示于图8C中。

[0312] 除了使用事件间寿命τ_d或结合频率来测定可用结合位点的数目和条形编码分子以外，还可使用荧光接通时间或τ_b和从而解离常数k_{off}来编码信息。k_{off}可通过对接链和成像链的双链体的碱基组成和/或长试来精确调整。此方法的可行性在图9中进行了举例说明:将$1/\tau_d$和$1/\tau_b$相对于对接/成像双链体的长度作图。$1/\tau_d$和从而k_{on}不依赖于双链体的稳定性。然而，通过添加单个CG碱基对将成像/对接双链体从9个核苷酸延长至10个核苷酸(nt)，使动力学解离速率减小几乎一个数量级。

[0313] 最后，"微条形码"(使用我们检测成像/对接双链体的热力学稳定性的差异的能力的最小条形码)被产生来使用短的、经济的和非结构化的探针鉴定大量分子。所述条形码只包含长度大约50nt的单个DNA分子，其用于使用后接具有针对红色、绿色或蓝色成像链的8、9或10nt长的结合结构域的组合的大约30nt长的"条形码"区的21nt的靶检测结构域t*来标记生物分子(图10A)。尽管长度只有30nt，但其可用于利用仅3种光谱上不同的颜色和3种热动力学不同的序列长度来呈现$3^3=27$个不同的条形码。图10举例说明了针对3种颜色分别具有每秒10、1和0.1的k_{off}的8、9或10nt长的对接链。图10A显示由针对红色成像链的8nt长的结合结构域、分别针对绿色和蓝色成像链的两个9nt长的结合结构域组成的示例性条形码。这产生相较于8nt相互作用结构域针对9nt相互作用结构域的荧光接通时间τ_b增加的特

征性强度相对于时间轨迹(图10B)。随机模拟显示区分分别为每秒10、1和0.1的k_{off}值是显然可能的(图10C)。

[0314] 实施例5:遗传编码的活细胞超级分辨率成像

[0315] 荧光成像的显著有利方面在于其使活细胞中的生物分子过程可视化的潜能。然而在证实活细胞超分辨率成像中存在挑战(参考文献22-22)。一个这样的挑战在于以生物相容性方式将足够浓度的合成成像探针递送至活细胞,同时还允许恰当的成像条件。另一个挑战是未结合探针在活细胞环境的背景中的本底荧光的程度,在所述活细胞环境中,相较于固定的细胞环境大分子拥挤可以是更加显著的因素,并且"活的"解旋酶可影响结合动力学。

[0316] 本公接通过利用遗传编码的RNA探针解决了这些和其它挑战,所述RNA探针可特异性结合于活细胞中的靶分子,并且只在此时才开始发荧光和闪烁以使得能够对特定的靶进行超分辨率成像。

[0317] 此条件性"闪烁"探针是具有靶结合结构域(TBD)和条件闪烁结构域(CBD)的小的单链RNA(<100nt)。所述CBD在TBD的机械控制之下并且当TBD未结合于靶T时是暗的。TBD与靶"T"的结合导致CBD的重新配置,并且使得其能够以适合于T的超分辨率成像的强度和频率闪烁。

[0318] 闪烁RNA探针基于Spinach适体系统(参考文献19),其是GFP的荧光RNA模拟物(图19)。Spinach可打开最初暗的小分子DFHBI(与DMHBI类似)的荧光,所述小分子DFHBI是无毒的和细胞可透过性的。通过用Spinach标记靶RNA,可对靶RNA在细菌和哺乳动物细胞中的表达成像(参考文献19)。

[0319] 随着小分子DFHBI结合和不结合Spinach,其将在荧光接通和关断状态之间转换。所得的闪烁行为被用于进行超分辨率荧光显微术,这在本文中被称为"Spinach-PAINT"(图20)。与大多数活细胞超分辨率成像技术不同,Spinach-PAINT不需要特殊的成像条件诸如特殊缓冲系统或外部光开关或激活。更重要的是,DFHBI是小的细胞透过性分子并且已被显示为活细胞提供无毒成像。对超分辨率成像是必要的"闪烁"行为可通过改变荧光接通和关断时间(通过调节溶液中的DFHBI浓度和将Spinach修饰/突变来以与调整成像链与其伴侣的DNA-DNA结合相互作用相同的精神增强/减弱DFHBI对其的结合)来获得。有可能基于单分子测量表征和优化DFHBI与表面固定的Spinach的结合动力学(图20)。就与基于短暂结合的超分辨率显微术的相容性检查结合动力学。

[0320] 生成具有用于超分辨率成像的优化闪烁性质的spinach变体:基于DNA-PAINT,起始点是为了获得显示以≈2Hz的频率发生的至少50ms的接通时间的Spinach变体。已发现,Spinach表现出$0.02s^{-1}$的k_{off},导致≈50s的停留时间。这显著长于超分辨率成像所需的时间。为了鉴定具有$1-20s^{-1}$的k_{off},将使用dNTP混合物制备Spinach变体的"掺杂"文库,所述dNTP混合物以2:1:1:1的比率含有在用于每个位置的Spinach中发现的dNTP和其它三种dNTP。此方法通常用于优化适体序列(SELEX)(参考文献23)。随后将就具有较短的DFHBI停留时间的变体筛选Spinach变体。在5-10轮的SELEX后,将分别地表征克隆。第一步骤是确认所有Spinach变体在结合DFHBI后仍然显示出具有可比较的量子产率和消光系数的Spinach样荧光。减弱适体与小分子之间的结合相互作用通常比增强其容易。

[0321] 第二步骤包括通过单分子成像测量这些Spinach变体的结合和解结合速率常数。

表现出提供足够用于获得精确的超分辨率成像的光子计数的结合持续时间的Spinach变体将被选择。

[0322] 为了优化闪烁频率，我们将用DFHBI进行滴定，因为RNA-荧光团复合物形成的速率通过k_{on}和DFHBI浓度来决定，并且将鉴定产生5-10Hz的闪烁率的浓度。最后，我们将鉴定出一组表现出超分辨率成像所需的优化闪烁的Spinach变体。

[0323] 作为测试平台，我们将通过基于DNA的纳米结构(例如，通过DNA折纸产生的纳米结构)的体外"成像"优化Spinach-PAINT的超分辨率能力。DNA折纸基板具有提供用于以确定的距离和几何学放置多个Spinach分子的可编程环境的优势。此纳米级标尺系统将使我们能够精确地定量该超分辨率成像技术的可获得的分辨率(图21)。

[0324] 在结合微管蛋白后展现闪烁用于细胞骨架蛋白的超分辨率成像的RNA：已产生由代谢产物(参考文献24)和蛋白质(参考文献25)激活的基于Spinach的传感器。本公开内容的方法将用于通过使用本文所述的微管蛋白-结合适体和闪烁Spinach变体产生通过结合微管蛋白激活的基于Spinach的传感器。

[0325] Spinach的变构形式受到融合于"控制"或"感应"模块(由结合目标靶的适体组成)的经修饰的Spinach适体结构域的损害(参考文献24)。靶的结合导致经修饰的Spinach适体至"活性"构象的变构折叠，从而实现DFHBI结合和发荧光(图22)。已描述了几种微管蛋白结合DNA适体(参考文献26)。本公开内容使用之前建立的方案提供了能够感测微管蛋白的Spinach的控制模块的进化。简言之，候选适体将融合于Spinach以获得微管蛋白依赖性Spinach荧光，并且只有在微管蛋白(其将用Cy5标记并且在体外聚合)存在的情况下才检验到Spinach活化。

[0326] 通过使用上述产生的和基于针对体外成像测试的超分辨率条件的条件Spinach探针，可将Spinach-PAINT用于例如活哺乳动物细胞中的微管的超分辨率成像。

[0327] 实施例6：流动室

[0328] 图23A显示出了用于体外DNA折纸实验的实验设置的实例，如本文中提供的。将样品固定在PDMS通道中的玻璃盖玻片上。将成像和洗涤缓冲液添加至储存器中，并通过注射器拉动通过通道。通过柔性管道将储存器和注射器连接至PDMS通道，从而将它们机械地解偶联。图23B显示用于原位细胞成像的实验性设置。在Lab-Tek II室中对细胞成像。一个注射器提供新的缓冲液，第二个除去之前的缓冲液。

[0329] 例如如图23A和23B中描述的使用流动室的交换-PAINT成像的示例性方案如下：

[0330] PDMS流动室容积：40μl

[0331] • 利用100μl 1M KOH漂洗流动室

[0332] • 利用100μl缓冲液A漂洗流动室2次

[0333] • 温育5分钟

[0334] • 利用100μl缓冲液A漂洗流动室

[0335] • 利用50μl 1mg/ml BSA-生物素(于缓冲液A中)漂洗流动室

[0336] • 温育2分钟

[0337] • 利用50μl 1mg/ml BSA-生物素(于缓冲液A中)漂洗流动室

[0338] • 温育2分钟

[0339] • 利用100μl缓冲液A漂洗流动室二次

[0340] • 利用50μl 0.5mg/ml链霉抗生物素蛋白(于缓冲液A中)漂洗流动室

[0341] • 温育2分钟

[0342] • 利用50μl 0.5mg/ml链霉抗生物素蛋白(于缓冲液A中)漂洗流动室

[0343] • 温育2分钟

[0344] • 利用100μl缓冲液A漂洗流动室二次

[0345] • 利用100μl缓冲液B漂洗流动室二次

[0346] • 温育30分钟

[0347] • 利用100μl缓冲液B漂洗流动室二次

[0348] • 利用50μl 1nM折纸(于缓冲液B中)漂洗流动室

[0349] • 温育10分钟

[0350] • 利用100μl缓冲液B漂洗流动室二次

[0351] • 连接管道

[0352] • 在缓冲液B中操作。

[0353] 另外的材料和方法

[0354] 材料。未修饰的DNA寡核苷酸购自Integrated DNA Technologies。荧光修饰的DNA寡核苷酸购自Biosynthesis。抗β-微管蛋白生物素化单克隆抗体(9F3;目录号:6181)和COX IV(3E11;目录号:6014)购自Cell Signaling。抗-PMP70(目录号:ab28499)购自Abcam。抗-TGN46(目录号:NBP1-49643B)购自VWR。链霉抗生物素蛋白购自Invitrogen(目录号:S-888)。牛血清白蛋白(BSA)和BSA-生物素获自Sigma Aldrich(目录号:A8549)。载玻片和盖玻片购自VWR。Lab-Tek II腔式盖玻片购自Thermo Fisher Scientific。M13mp18支架获自New England Biolabs。如19所描述的制备用于微管样DNA折纸结构的p8064支架。"Freeze N Squeeze"柱购自Bio-Rad。TetraSpeck珠粒购自Life Technologies。多聚甲醛、戊二醛和TEM网格(FORMVAR 400目铜网)获自Electron Microscopy Sciences。3种缓冲液用于样品制备和成像:缓冲液A(10mM Tris-HCl,100mM NaCl,0.05%Tween-20,pH 7.5)、缓冲液B(5mM Tris-HCl,10mM MgCl2,1mM EDTA,0.05%Tween-20,pH 8)和缓冲液C(1×PBS,500mM NaCl,pH 8)。

[0355] 光学设置。利用油浸物镜(CFI Apo TIRF 100×,NA 1.49,Oil),使用Nikon TIRF照明器,应用物镜类型的TIRF配置在具有Perfect Focus系统的倒置Nikon Eclipse Ti显微镜(Nikon Instruments)上进行荧光成像。对于2D成像,将另外的1.5倍放大率用于获得≈150-倍的最终放大,对应于107nm的象素尺寸。将3个激光用于激发:488nm(标称200mW,Coherent Sapphire)、561nm(标称200mW,Coherent Sapphire)和647nm(标称300mW,MBP Communications)。将激光束通过清除滤波器(ZT488/10、ZET561/10和ZET640/20,Chroma Technology)并使用多波段分束器(ZT488rdc/ZT561rdc/ZT640rdc,Chroma Technology)将其偶联至显微镜物镜中。用发射滤波器(ET525/50m,ET600/50m和ET700/75m,Chroma Technology)对荧光进行滤光,并在EMCCD照相机(iXon X3DU-897,Andor Technologies)上对其成像。

[0356] DNA折纸自组装。在具有40μl总体积(在折叠缓冲液(含20mM $MgCl_2$的1×TAE缓冲液)中含有10nM支架链(p8064),500nM折叠吻合钉和生物素手柄(handle),750nM生物素抗-手柄和1.1μM DNA-PAINT对接链)的一锅反应中形成微管样DNA折纸结构。在15小时的过程

中使用从80℃至14℃的热坡13对溶液进行退火。在自组装后，通过以4.5V/cm进行琼脂糖凝胶电泳(1.5%琼脂糖，0.5×TBE，10mM $MgCl_2$，1×SybrSafe)1.5小时来纯化单体结构。切取凝胶条带，将其破碎并填充至'Freeze'N Squeeze'柱中，在4℃以1000×g旋转5分钟。在30℃下利用5倍过量的聚合吻合钉(于折叠缓冲液中)进行聚合，持续48小时。将聚合的结构用于成像而无需进一步纯化。在一锅反应(40μl的总体积，20nM M13mp18支架、100nM生物素化的吻合钉、530nM具有DNA-PAINT对接位点的吻合钉、含12.5mM MgCl2的1×TAE)中自组装DNA折纸漂移标记。如先前所述纯化自组装的结构。在一锅反应(40μl的总体积，30nM M13mp18支架、470nM生物素化的吻合钉、400nM具有用于数字成像的对接位点的吻合钉、370nM核心结构吻合钉、含12.5mM MgCl2的1×TAE)中自组装用于四"色"体外交换-PAINT演示的DNA折纸结构。如先前所述纯化自组装结构。在一锅反应(40μl的总体积，30nM M13mp18支架、36nM生物素化的吻合钉、750nM具有用于数字成像的对接位点的吻合钉、300nM核心结构吻合钉、含12.5mM MgCl2的1×TAE)中自组装用于十"色"体外交换-PAINT演示的DNA折纸结构。不纯化结构。在将结构固定于表面上后从样品洗掉过量吻合钉。用于微管样DNA折纸结构的DNA链序列可见于表1中。用于DNA折纸漂移标记的DNA链序列可见于表2中。用于DNA折纸结构(用于十"色"体外交换-PAINT演示)的DNA链序列可见于表3和4(分别针对奇数和偶数数字)中。用于DNA折纸结构(用于体外交换-PAINT演示)的DNA链序列(数字0至3)可见于表5中。用于p8064和M13mp18的支架序列分别对应于SEQ ID NO:882和883。DNA-PAINT成像和对接序列以及用于通过生物素的表面附着的序列列于表6中。

[0357] 抗体-DNA缀合物。在两个步骤中预组装用于用DNA-PAINT对接位点特异性标记目标蛋白质的抗体-DNA缀合物：首先，将3.2μl的1mg/ml链霉抗生物素蛋白(溶解于缓冲液A中)与0.5μl的100μM的生物素化的DNA-PAINT对接链和另外的5.3μl缓冲液A在室温(RT)下反应30分钟，同时轻轻摇动。随后将溶液在第二步骤中与1μl的1mg/ml的针对目标蛋白质的生物素化的单克隆抗体在RT下温育30分钟。将过滤柱(Amicon 100kDa，Millipore)用于从未反应的链霉抗生物素蛋白-寡聚物缀合物纯化预组装的缀合物。

[0358] 细胞免疫染色。用Eagle最低基础培养基(利用10%FBS以及青霉素和链霉素强化的)培养HeLa和DLD1细胞，将其在37℃、5%CO2下温育。在固定之前24小时将每孔约30%汇合细胞接种至Lab-Tek II腔式盖玻片。使用以下程序对微管、线粒体、高尔基体复合物和过氧化物酶体进行免疫染色：于PBS中洗涤；于PBS中的3%多聚甲醛和0.1%戊二醛的混合物中固定10分钟；用PBS洗涤3次；利用≈1mg/ml NaBH4还原7分钟；用PBS洗涤3次；用PBS中的0.25%(v/v)Triton X-100透化10分钟；用PBS洗涤3次；用3%(w/v)牛血清白蛋白封闭30分钟，并用预组装的针对β-微管蛋白、COX IV、PMP70或TGN46的抗体-DNA缀合物(将缀合物在5%BSA中稀释至10μg/ml)染色过夜；用PBS洗涤3次；在3%多聚甲醛和0.1%戊二醛的混合物(于PBS中)中后固定10分钟；以及用PBS洗涤3次。

[0359] 微管样DNA折纸结构的超分辨率DNA-PAINT成像。对于样品制备，利用两条双面胶带将一片盖玻片(编号1.5，18×18mm^2，≈0.17mm厚)和载玻片(3×1英寸^2，1mm厚)夹心在一起，以形成具有≈20μl的内部容积的流动室。首先，将20μl的生物素标记的牛白蛋白(1mg/ml，溶解于缓冲液A中)流入小室中，并温育2分钟。随后使用40μl的缓冲液A洗涤小室。随后将20μl链霉抗生物素蛋白(0.5mg/ml，溶解于缓冲液A中)流过小室，并使其结合2分钟。在用40μl缓冲液A和随后40μl缓冲液B洗涤后，最后将缓冲液B中的20μl生物素标记的微管样DNA

结构(≈300pM单体浓度)和DNA折纸漂移标记(≈100pM)流入小室,并温育5分钟。使用40μl缓冲液B洗涤小室。最终的成像缓冲液含有缓冲液B中的1.5nM的Cy3b-标记的成像链。在进行随后的成像之前用环氧树脂密封小室。将CCD读出带宽设置至16bit的1MHz和5.1的前置放大增益。不使用EM增益。利用561nm下的294W/cm2的激发强度,使用TIR照明进行成像。

[0360] DNA纳米结构的超分辨率交换-PAINT成像。对于流体交换,构建定制流动室。详细制备方案可见于实施例6。在用BSA-生物素官能化成像通道之前,利用1M KOH洗涤其以进行清洁。如先前所述进行折纸结构与流动室的表面的结合。在简短的约1-2分钟的洗涤步骤(由至少3次每次使用200μl缓冲液B的洗涤组成)后进行每个图像获取步骤。随后引入下一个成像链溶液。在整个洗涤过程中监测表面以确保成像溶液的完全交换。重复获取和洗涤步骤直至所有10个靶均被成像。将CCD读出带宽设置至14bit的3MHz和5.1的前置放大增益。不使用EM增益。利用561nm下的166W/cm2(十"色"交换-PAINT利用缓冲液B中的3nM Cy3b-标记的成像链,图5B(iii)和5B(v))和647nm下的600W/cm2(四"色"交换-PAINT利用缓冲液B中的3nM ATTO655-标记的成像链,图5B(iv))的激发强度,使用TIR照明进行成像。

[0361] 细胞的超分辨率DNA-PAINT成像。所有数据利用14bit下5MHz、5.1前置放大增益和255电子倍增增益的EMCCD读出带宽获得。使用HILO照明11进行成像。激光功率密度为283W/cm2(647nm)(图3A中)、142W/cm2(647nm)和19W/cm2(561nm)(图3D中)。成像条件:图3A:缓冲液C中的700pM ATTO655-标记的成像链。图3D:缓冲液C中的600pM Cy3b-标记的成像链和1.5nM ATTO655-标记的成像链。

[0362] 细胞的超分辨率交换-PAINT成像。将Lab-Tek II室改造以适合于流体交换。利用14bit下5MHz、5.1前置放大增益和255EM增益的EMCCD读出带宽获取2D图像(图6B和6C)。利用154bit下3MHz、5.1前置放大增益和无EM增益的CCD读出带宽来获得3D图像(图6)。在两种情况下均使用HILO照明进行成像。如针对2D折纸纳米结构所述(但使用缓冲液C进行洗涤步骤)进行连续成像。

[0363] 3D DNA-PAINT成像。在检测路径(Nikon)中利用圆柱透镜来获取3D图像。将用于NIS组件(Nikon)的N-STORM分析包用于数据处理。在于检测路径中不使用另外的放大倍数进行成像,产生160nm象素尺寸。按照制造商的说明书进行3D校准。

[0364] 成像链浓度测定。根据标记密度凭经验测定最佳成像浓度。通常地,必须确保足够高的荧光关断/接通比率以保证每衍射极限区域仅单个成像链的结合。另外,充足的成像链浓度(和从而足够低的荧光关断时间)是确保足够的结合事件和从而图像获取过程中每个对接链的稳健检测所必需的。

[0365] 超分辨率数据处理。使用LabVIEW10中编程的斑点发现和2D-高斯拟合算法重构超分辨率DNA-PAINT图像。可在DNA-Paint网站(org suffix)上下载获得该软件的简化版本。

[0366] 标准化互相关分析。通过首先标准化各自的重构灰度超分辨率图像和随后在MATLAB R2013b(MathWorks,Natick,MA,USA)中进行互相关分析来获得标准化互相关系数。

[0367] 漂移校正和通道比对。将DNA折纸结构用于漂移校正和用作体外DNA-PAINT和交换-PAINT成像中的比对标记。通过在每个影片的整个持续时间中跟踪每个折纸漂移标记的位置来进行漂移校正。随后对所有检测的漂移标记的轨迹进行平均,并将其用于全局校正最终的超分辨率重构中的漂移。对于交换-PAINT中的不同成像循环之间的通道比对,这些结构通过匹配它们在每个交换-PAINT图像中的位置来用作比对点。对于细胞成像,将100nm

金纳米颗粒(Sigma Aldrich;10nM(于缓冲液C中),在成像前添加)用作漂移和比对标记。金纳米颗粒非特异性地吸附至成像室的玻璃底部。以与针对折纸漂移标记相似的方式进行漂移校正和比对。再次地,在整个影片中跟踪视野中所有金纳米颗粒的表观运动。随后将所获得的轨迹进行平均并将其用于最终超分辨率图像的全局漂移校正。对于图3D-3F中的线粒体和微管的双色成像,在两种颜色通道中均可见金颗粒。相同的金颗粒还在原位交换-PAINT实验中用于不同成像回合的再比对和漂移校正(图6)。

[0368] 透射电子显微术成像。对于成像,将3.5μl未稀释的微管样DNA结构吸附至辉光放电的碳涂布TEM网格上,进行2分钟。随后使用含有25mM NaOH的2%水性超滤(0.2μm滤器)甲酸双氧铀溶液对网格染色10秒。使用在80kV下运行的JEOL JEM-1400进行成像。

[0369] 原子力显微术成像。在具有E-扫描仪的Multimode VIII原子力显微镜(AFM)(Bruker)上使用敲击模式进行成像。使用狭窄的100μm,0.38N/m力常数悬臂的7-9kHz之间的共振频率利用DNP-S氧化物磨尖的氮化硅悬臂和SNL尖锐的氮化物杠杆(Bruker Probes)在TAE/Mg2+缓冲液中进行成像。在折纸结构自组装后将≈20μl的TAE/Mg2+缓冲液沉积在粘于金属定标器(Ted Pella)的新切开的云母表面(Ted Pella)上。30秒后,使用N2的温和气流干燥云母表面,将5μl折纸溶液沉积在云母表面上。再过30秒后,将30μl另外的缓冲液添加至样品。就最佳图像质量优化成像参数,同时维持最高的可能选点以使对样品的破坏降至最低。通过从每条扫描线扣除1阶多项式来对图像进行后处理。驱动振幅为约0.11V,积分增益≈2,以及比例增益≈4。

[0370] 在以下的表1-5中,示出了所述结构中的每个核苷酸位置。第一栏中的每个寡核苷酸位置(例如,n[n]n[n])通过逗号分隔,并且各自对应于相同行内的第二栏中的序列标识号。每个序列标识号鉴定所附序列表(其通过引用并入本文)中的对应的寡核苷酸序列。例如,在表1中,位置0[39]21[39]对应于由SEQ ID NO:1所示的寡核苷酸;位置0[79]1[79]对应于由SEQ ID NO:2所示的寡核苷酸,依此类推。

[0371] 表1.用于微管类似DNA结构的吻合钉序列

[0372]

位置*	序列标识符	描述
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],	SEQ ID NO: 1-SEQ ID NO: 178	结构链

[0373]

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],		

[0374]

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]		
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-PA INT, 对接位点
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	连接链
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'- 生物素，对接位点

[0375]

[0376] 表2.漂移标记的吻合钉序列

[0377]

位置*	序列标识符	描述
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],	SEQ ID NO: 235-SEQ ID NO: 402	DNA-PAINT,对接位点

[0378]

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],		

[0379]

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]		
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'-生物素修饰
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	结构链

[0380] 表3.用于 I·"色"体外交换-PAINT演示的DNA折纸结构的吻合钉序列(奇数数字)

[0381]

位置*	序列标识符	描述(数字)
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], 21[160]22[144], 22[271]20[272], 4[271]2[272], 6[271]4[272], 8[271]6[272], 9[160]10[144]	SEQ ID NO: 428-SEQ ID NO: 445	3,5,9
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],	SEQ ID NO:	1,3,7,9

[0382]

23[32]22[48], 23[64]22[80], 23[96]22[112]	457- SEQ ID NO: 462	
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],	SEQ ID NO: 474- SEQ ID NO: 594	结构吻合钉

[0383]

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]		
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'-生物素

[0384] 表4.用于十"色"体外交换-PAINT演示的DNA折纸结构的吻合钉序列(偶数数字)

[0385]

位置*	序列标识符	描述(数字)
1[160]2[144], 11[160]12[144], 13[160]14[144], 15[160]16[144], 17[160]18[144], 19[160]20[144],	SEQ ID NO: 603- SEQ	2,4,6,8

[0386]

21[160]22[144], 3[160]4[144], 5[160]6[144], 7[160]8[144], 9[160]10[144]	ID NO: 613	
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],	SEQ ID NO: 664- SEQ ID NO: 778	结构吻合钉

[0387]

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],		

[0388]

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]		
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'-生物素

[0389] 表5.用于体外交换-PAINT演示的DNA折纸结构的吻合钉序列(数字0至3)

[0390]

位置*	序列标识符	描述（数字）
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],	SEQ ID NO: 833- SEQ	2

[0391]

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]	ID NO: 857	
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], 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]	SEQ ID NO: 858- SEQ ID NO: 881	3

[0392] 表6.DNA-PAINT对接和成像序列以及生物素对接序列

[0393]

描述	SEQ ID NO:	序列
成像 P1*	884	5'–CTAGATGTAT-染料
成像 P2*	885	5'–TATGTAGATC-染料
成像 P3*	886	5'–GTAATGAAGA-染料
成像 P4*	887	5'–GTAGATTCAT-染料
成像 P5*	888	5'–CTTTACCTAA-染料
成像 P6*	889	5'–GTACTCAATT-染料
成像 P7*	890	5'–CATCCTAATT-染料
成像 P8*	891	5'–GATCCATTAT-染料
成像 P9*	892	5'–CACCTTATTA-染料

[0394]

成像 P10*	893	5'-CCTTCTCTAT-染料
成像 P11*	894	5'-GTATCATCAA-染料
成像 P12*	895	5'-GAATCACTAT-染料
9nt P1 对接位点	896	链-TTATACATCTA-3'
9nt P2 对接位点	897	链-TTATCTACATA-3'
10nt P2 对接位点	898	链-TTGATCTACATA-3'
9nt P3 对接位点	899	链-TTTCTTCATTA-3'
9nt P4 对接位点	900	链-TTATGAATCTA-3'
9nt P5 对接位点	901	链-TTTTAGGTAAA-3'
9nt P6 对接位点	902	链-TTAATTGAGTA-3'
9nt P7 对接位点	903	链-TTAATTAGGAT-3'
9nt P8 对接位点	904	链-TTATAATGGAT-3'
9nt P9 对接位点	905	链-TTTAATAAGGT-3'
9nt P10 对接位点	906	链-TTATAGAGAAG-3'
9nt P11 对接位点	907	链-TTTTGATGATA-3'
9nt P12 对接位点	908	链-TTATAGTGATT-3'
用于抗体偶联的生物素化的 P1 对接位点	909	生物素-TTATACATCTA-3'
用于抗体偶联的生物素化的 P2 对接位点	910	生物素-TTATCTACATA-3'
用于抗体偶联的生物素化的 P3 对接位点	911	生物素-TTTCTTCATTA-3'
用于抗体偶联的生物素化的 P4 对接位点	912	生物素-TTATGAATCTA-3'
用于微管样结构的生物素化的对接位点	913	生物素-GAATCGGTCACAGTACAACCG-3'

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[0043]

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<210> 494
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<210> 495
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<210> 501
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[0068]

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cataaatata gcgtccaata ctgcagacac ca 32

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[0070]

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<210> 610
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<400> 674
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<400> 675
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gaagtttcag caaactccaa cagtgaccat 30

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<400> 677
ggcaaagcat aaagctaaat cgctattttt 30

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<400> 681
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<400> 682
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<400> 691
ataagtccga caaaaggtaa agtaataagg cg 32

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[0083] <400> 695
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gaacggtaat gccggagagg gtaggttgta cc 32

<210> 697
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<400> 697
ctgagagtct acaaaggcta tcaggttcag ct 32

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<400> 698
ccagacgaac gcgcctgttt atcattctaa 30

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<210> 700
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<400> 700
aggcatttta cgagcatgta gaaatcatcg ta 32

<210> 701
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<400> 701
caaaaacaga aaggccggag acagggataa aa 32

<210> 702
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<400> 702
atatgtacat gatattcaac cgtttttgcg 30

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<400> 703
caacattccg tgggaacaaa cgattacgcc agctggcggg taac 44

[0084]

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<211> 48
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<400> 704
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<400> 705
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<210> 706
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<400> 706
tatacaaatt gggttatata actaaagacg ctgagaagag tcaattac 48

<210> 707
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<400> 707
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<210> 708
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tcgcgtctgg gataggtcac gttgtgggaa gg 32

<210> 709
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<400> 709
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<210> 710
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<400> 710
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<210> 713
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<400> 713
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<210> 714
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gaggggacaa tttttgttaa atcaaaaagc cc 32

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<400> 716
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<210> 717
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<400> 717
gcgatcggtt gtaaaacgac ggccgggtgc ct 32

<210> 718
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<400> 718
tcacgacgtg cgggcctctt cgctgcggat tg 32

<210> 719
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<400> 719
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<210> 720
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<400> 720
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[0086]

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<400> 721
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<210> 722
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<400> 722
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<210> 723
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aatgagtggg agaggcggtt tgcgaatccc tt 32

<210> 727
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<400> 727
cggaacgaac cctcagcagc gaaaccggaa ta 32

<210> 728
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<400> 728
acgcgcggag ctaactcaca ttaatttccc ag 32

<210> 729
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[0087]

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<213> 人工的
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<400> 730
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<213> 人工的

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[0104]

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[0106]

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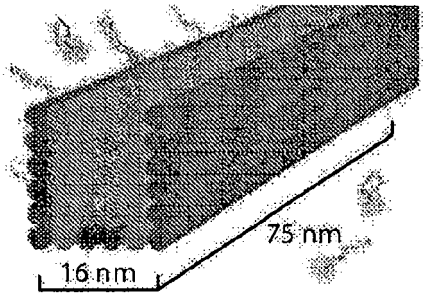

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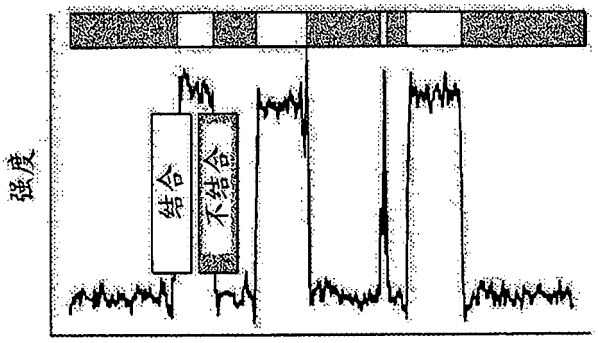

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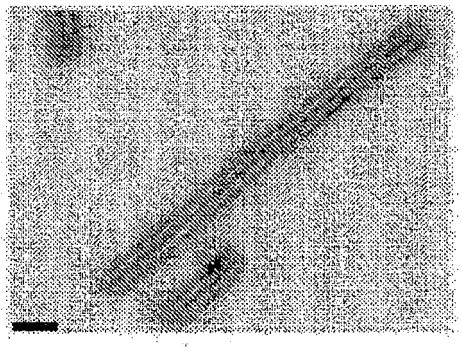

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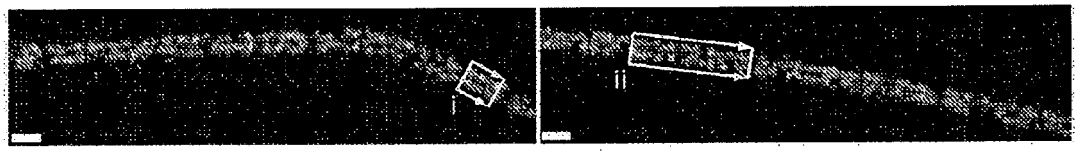

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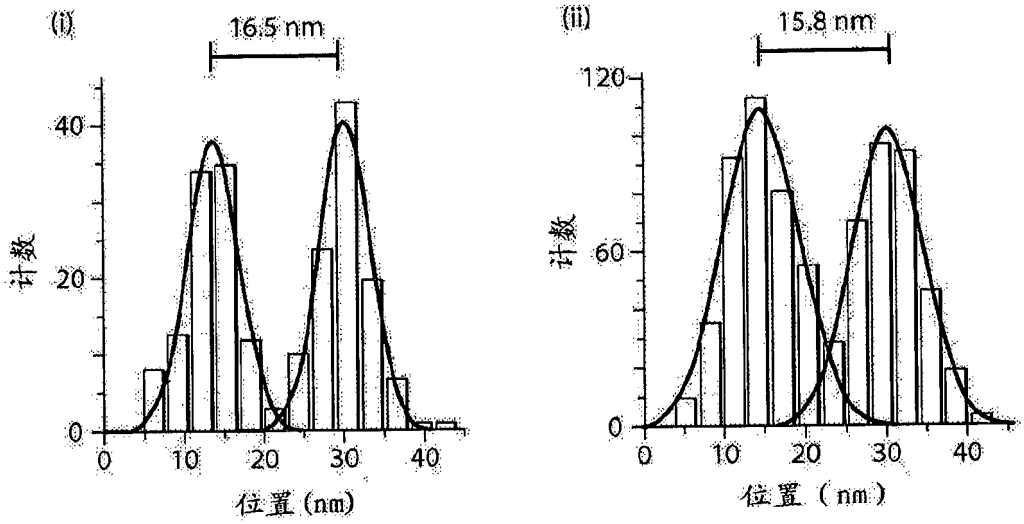

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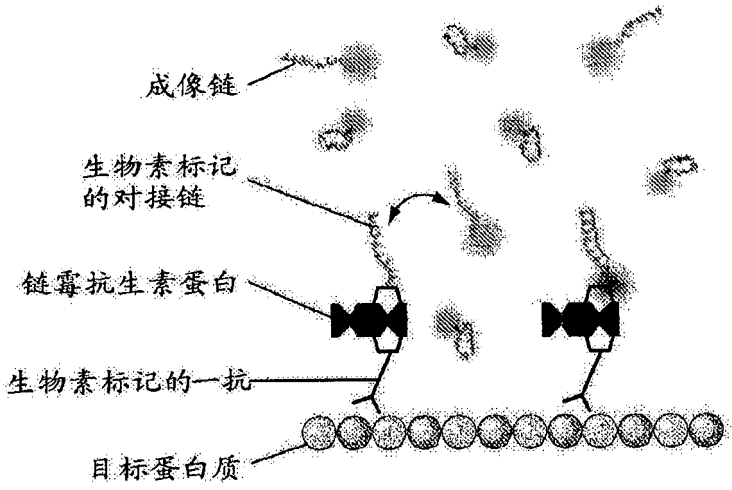

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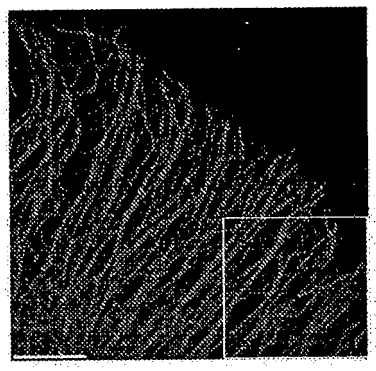


图3A

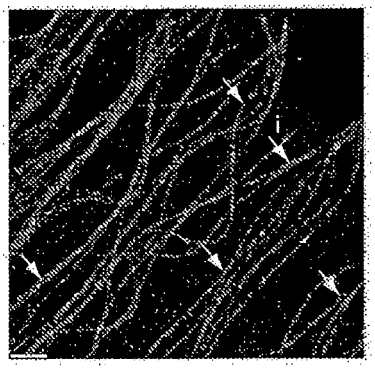


图3B

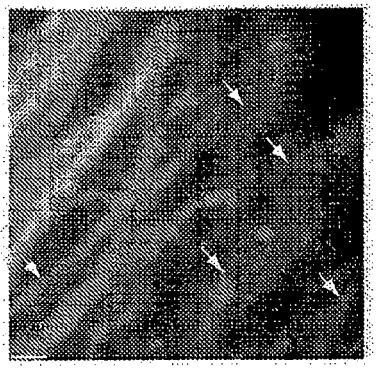


图3C

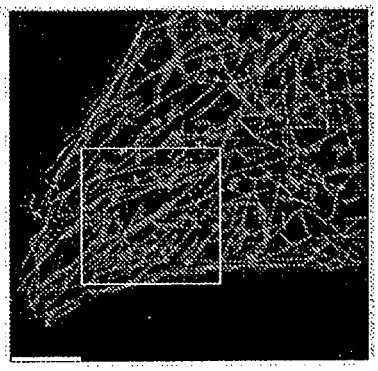


图3D

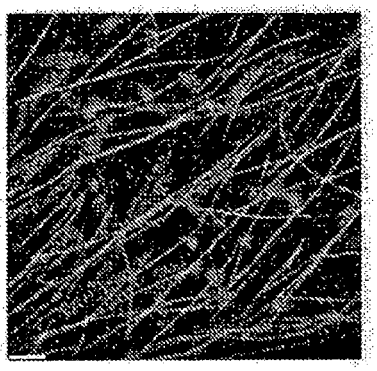


图3E

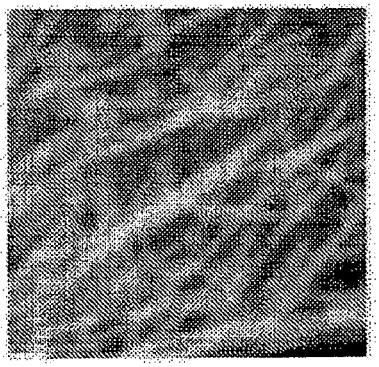


图3F

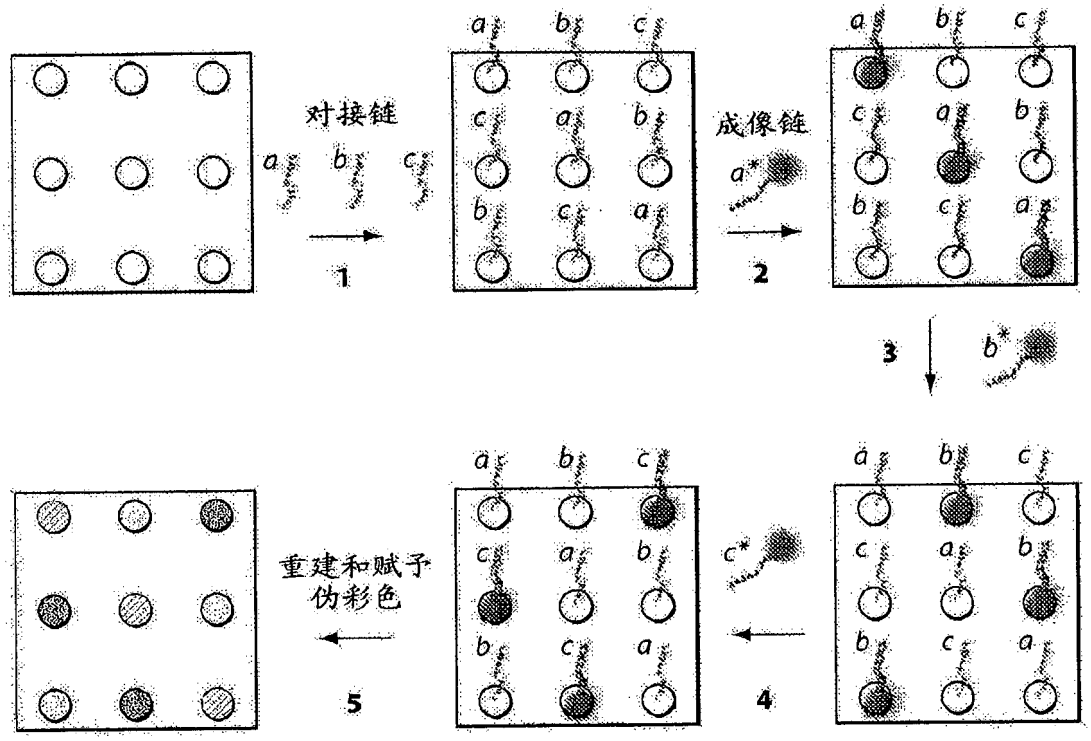


图4A

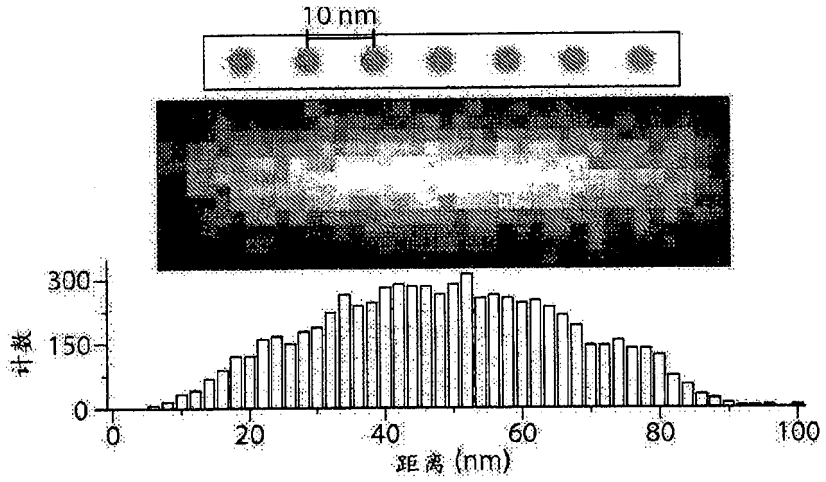


图4B1

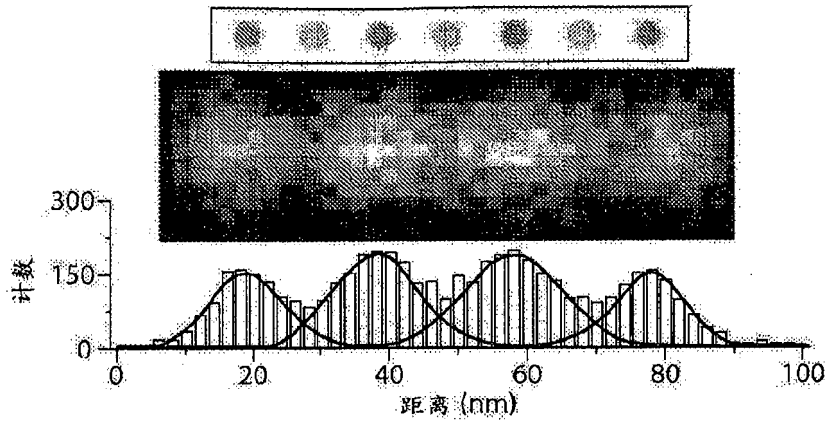

300
150
0
计数
0
20
40
60
80
100
距离 (nm)

图4B2

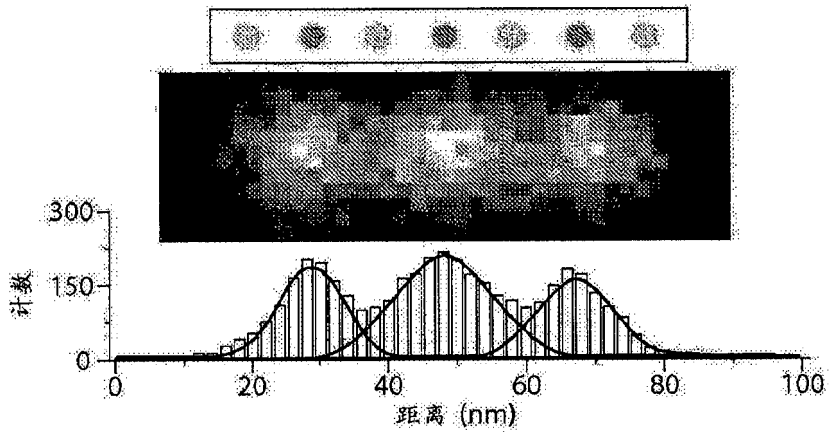
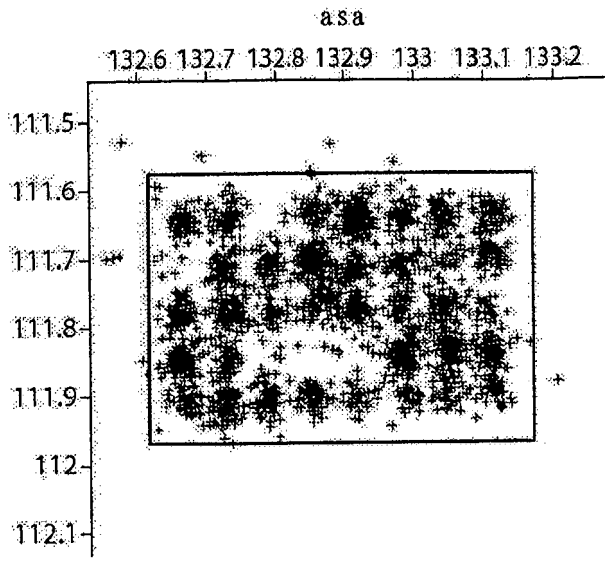
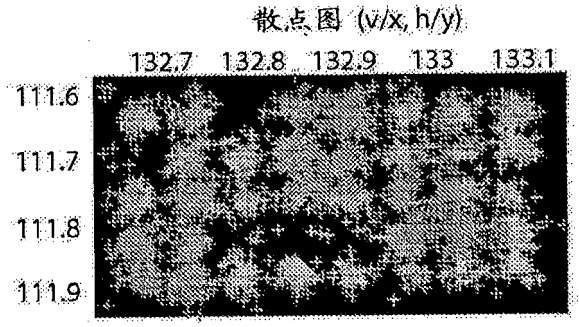
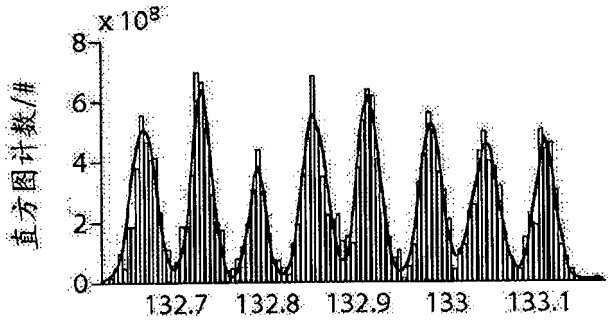

300
150
0
计数
0
20
40
60
80
100
距离 (nm)

图4B3







拟合结果:

间距：	10.4661	9.94118	10.1858	9.83788	10.978	9.80175	10.4411 nm
标准差：	2.1757	1.7805	1.5213	2.163	1.9209	1.9914	2.3486 1.8586 nm

标准化的残差＝3.3e+006, 经修改的卡方＝0.024

图4C

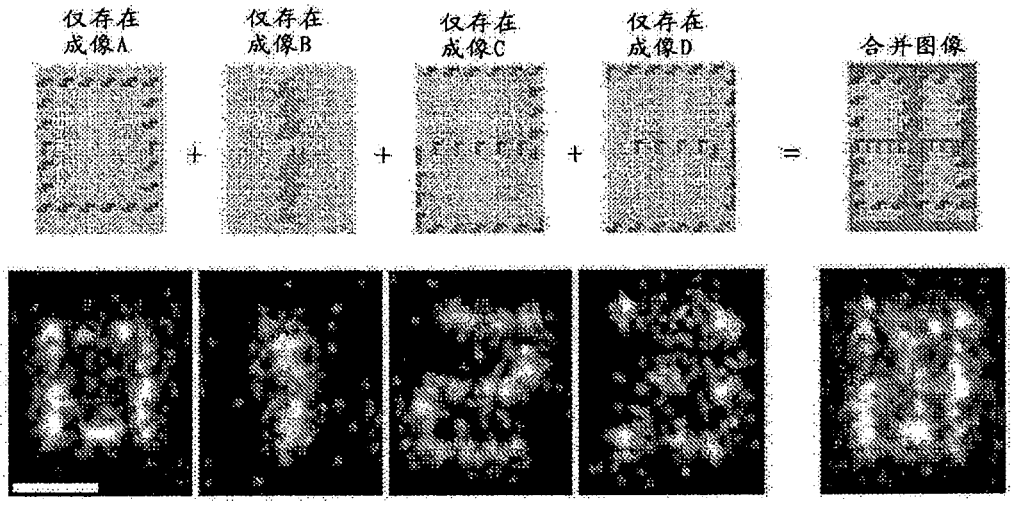


图5A

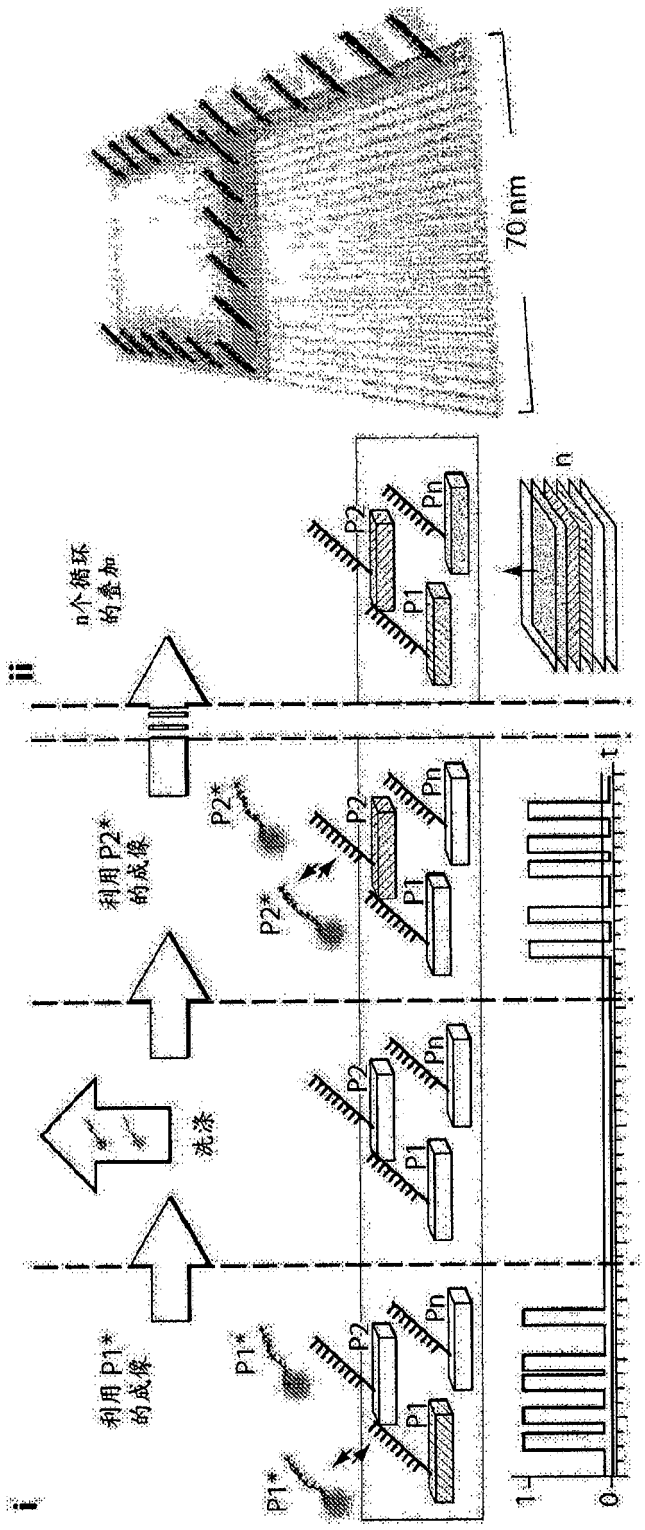


图5B

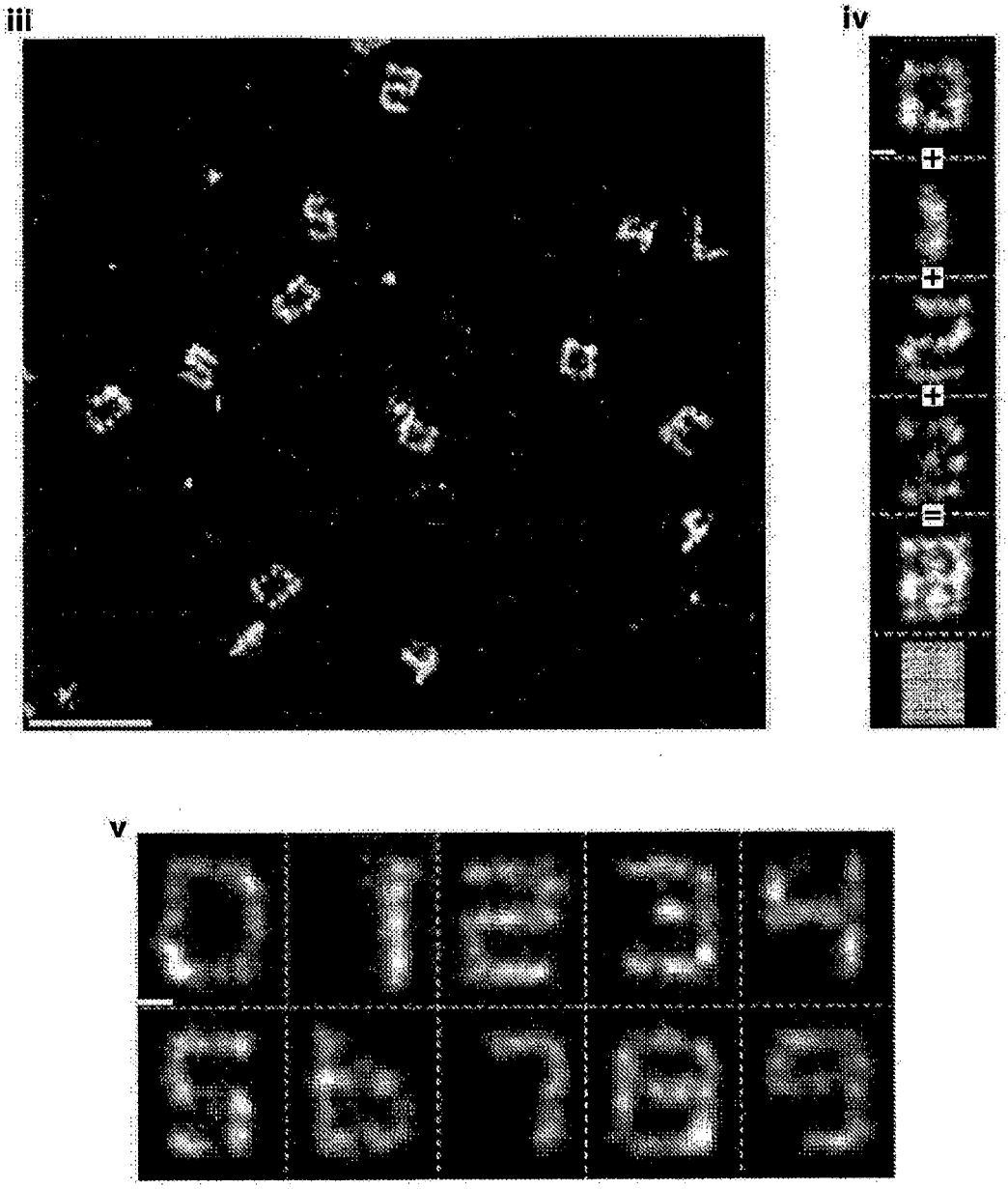


图5B(续)

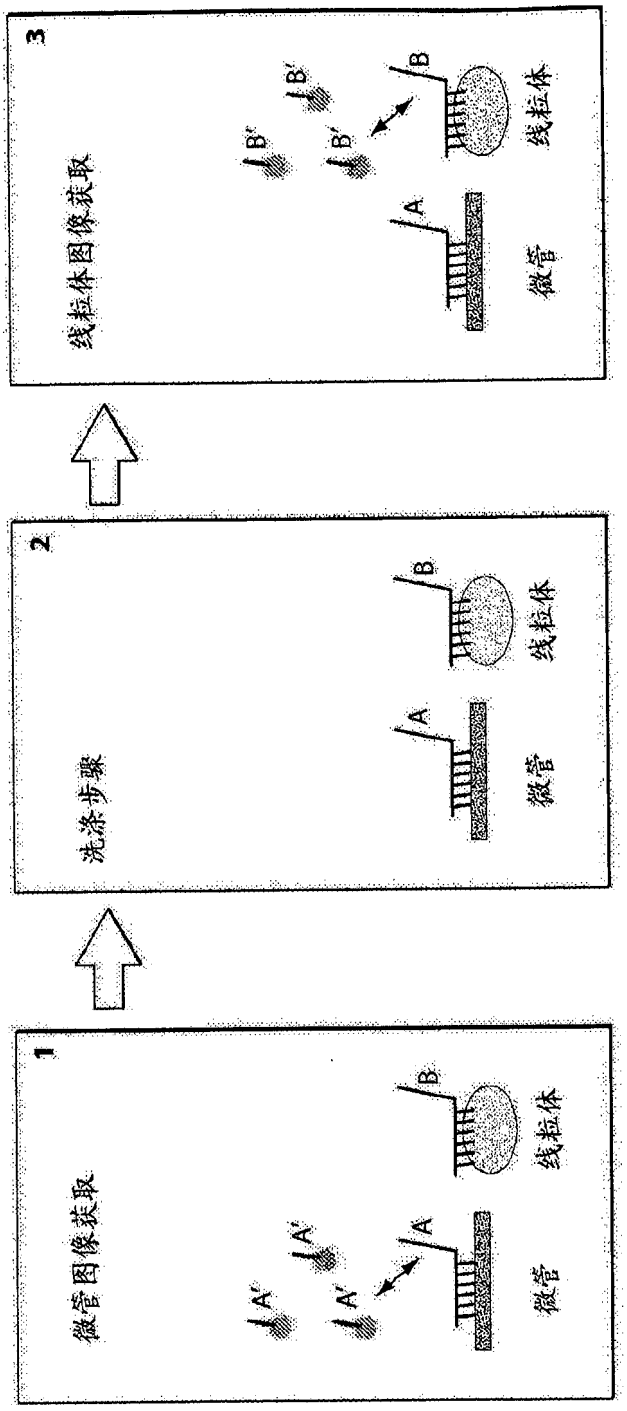


图6A

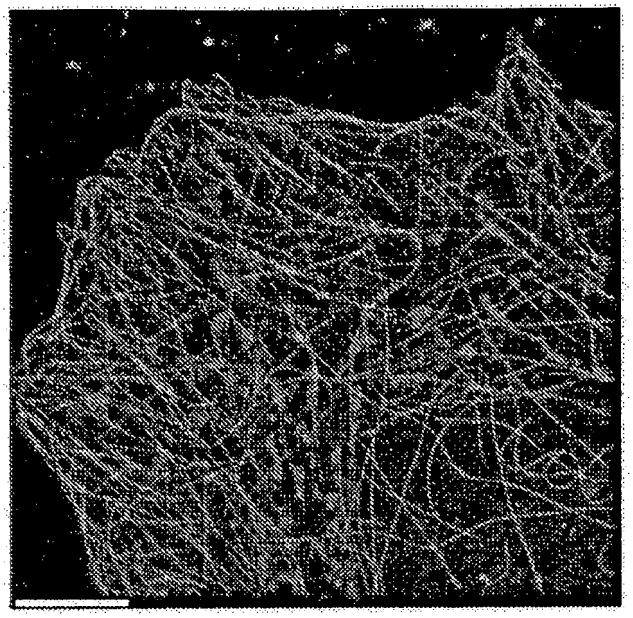


图6B

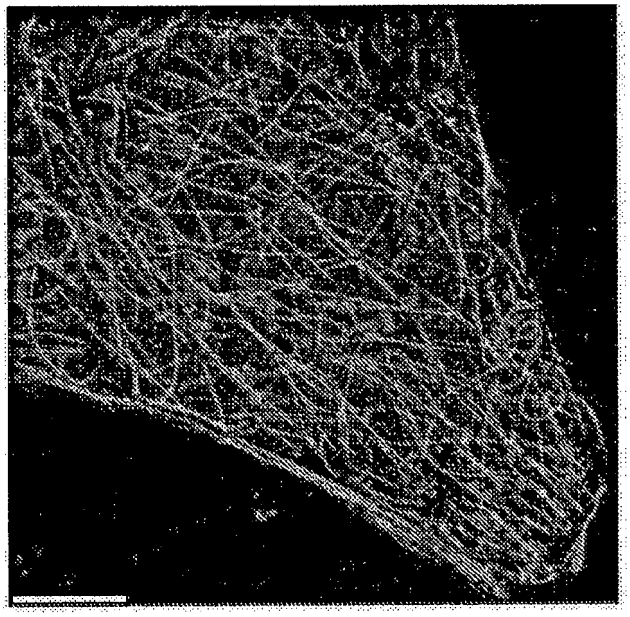


图6C

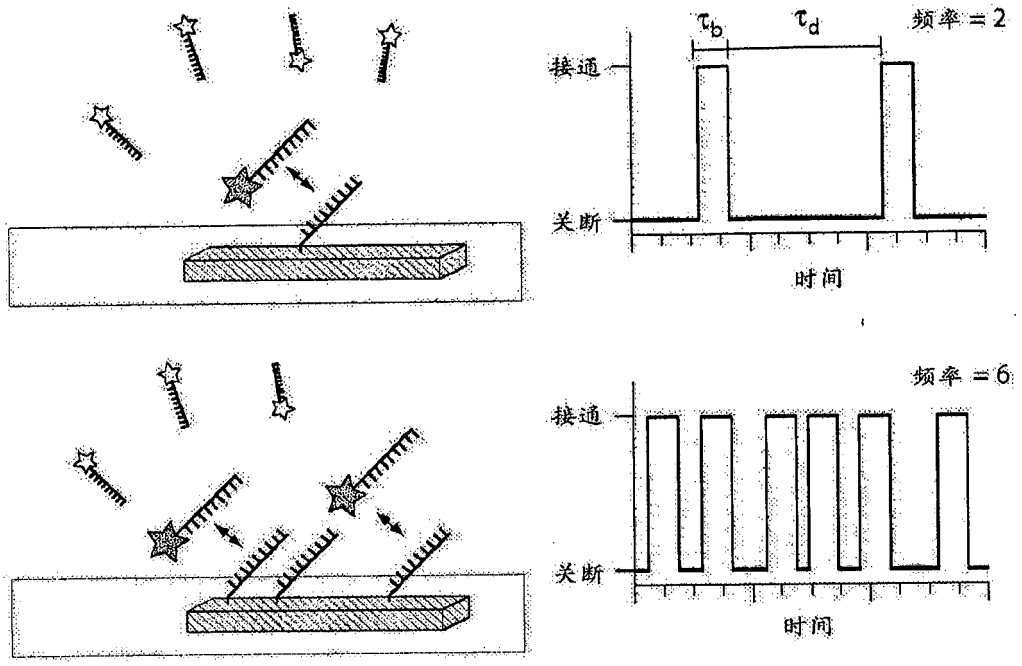


图7A

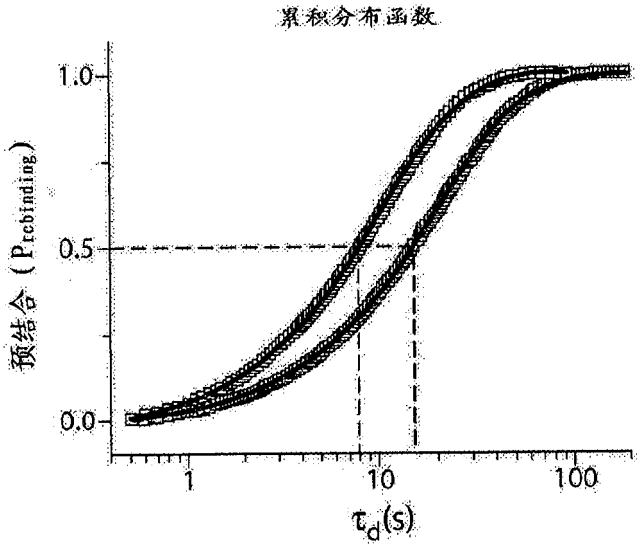


图7B

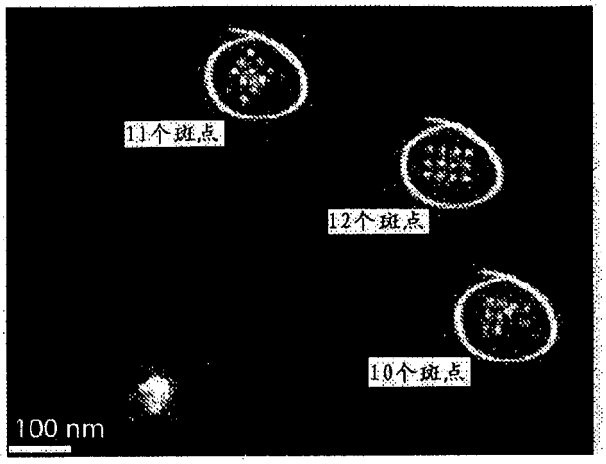


图7C

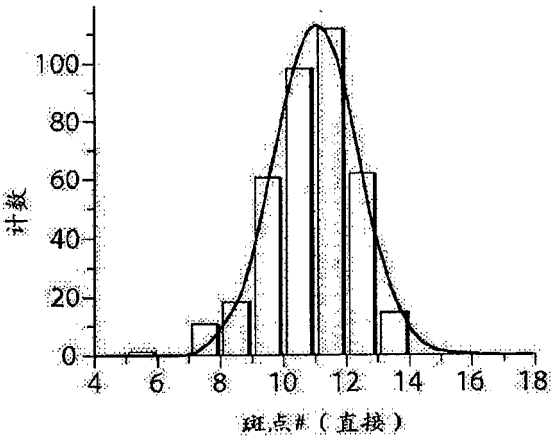


图7D1

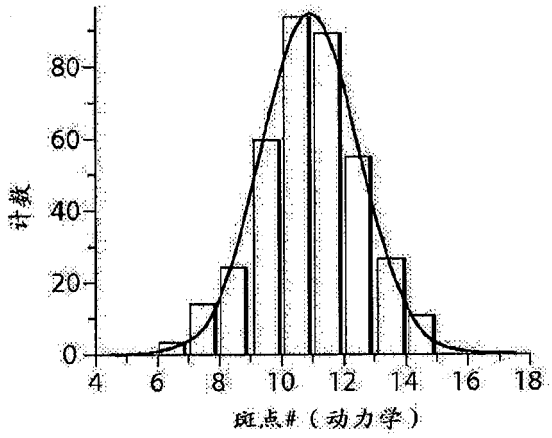


图7D2

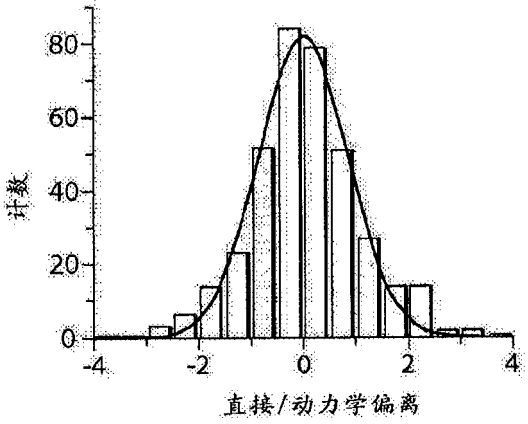


图7D3

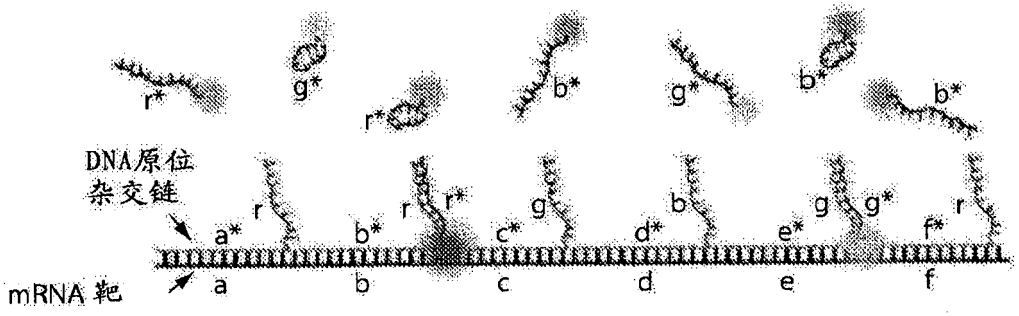


图8A

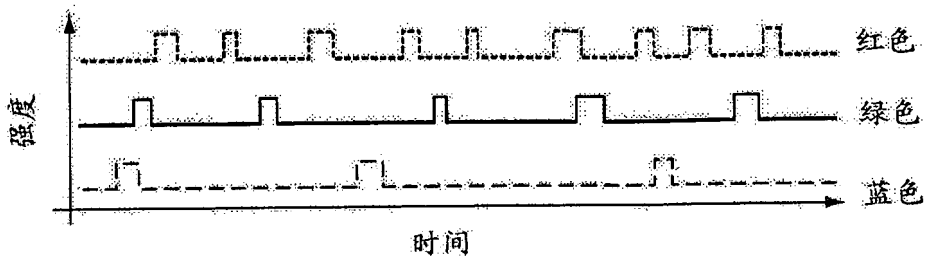


图8B

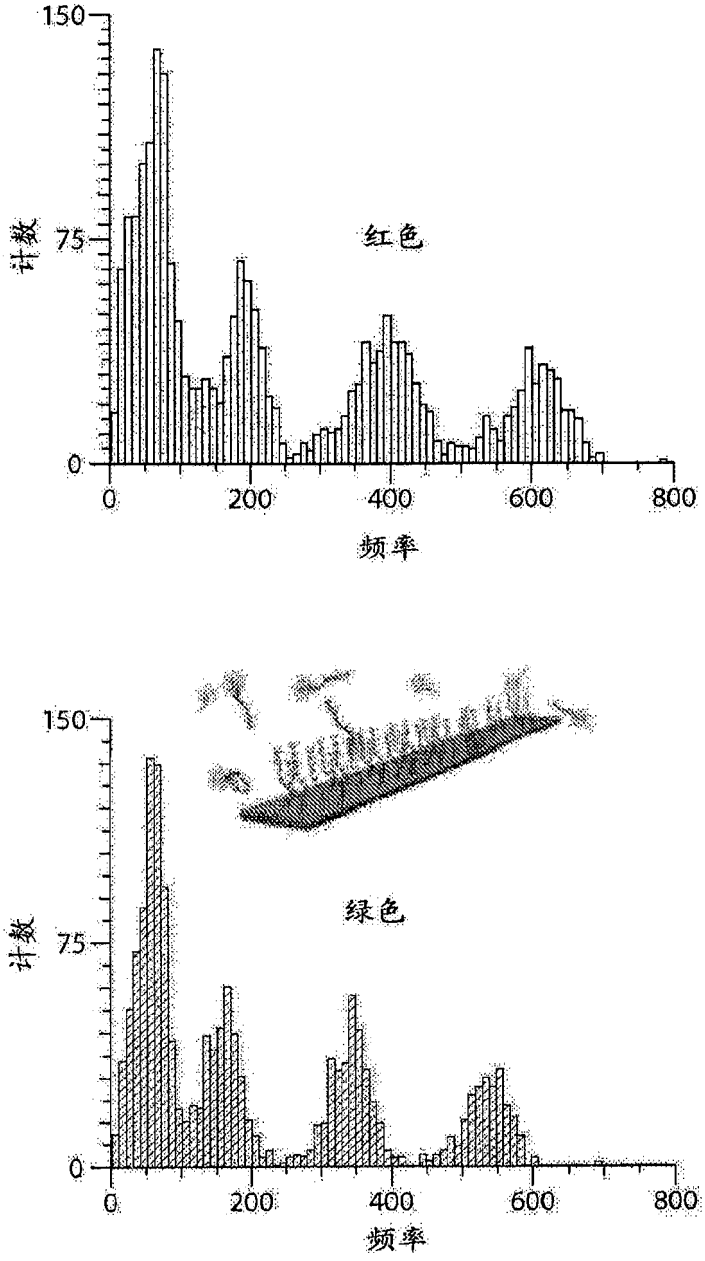

150
75
0
计数
红色
0
200
400
600
800
频率
150
75
0
计数
绿色
0
200
400
600
800
频率

图8C

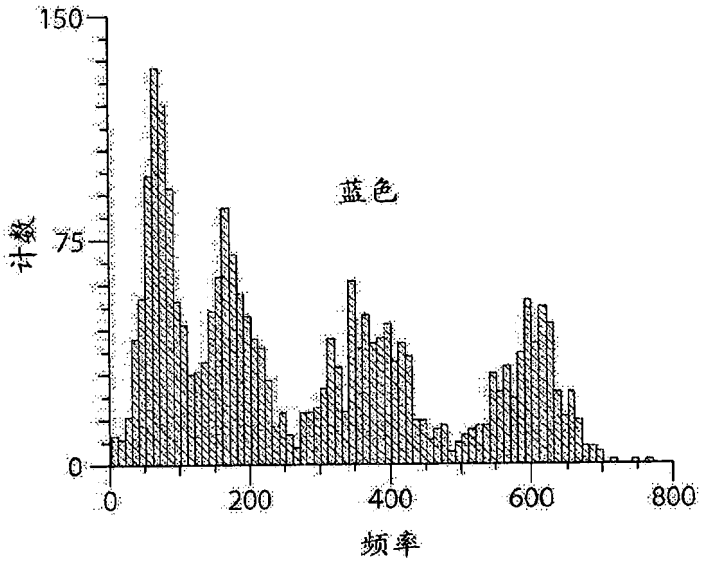

150
75
0
计数
蓝色
0
200
400
600
800
频率

图8C(续)

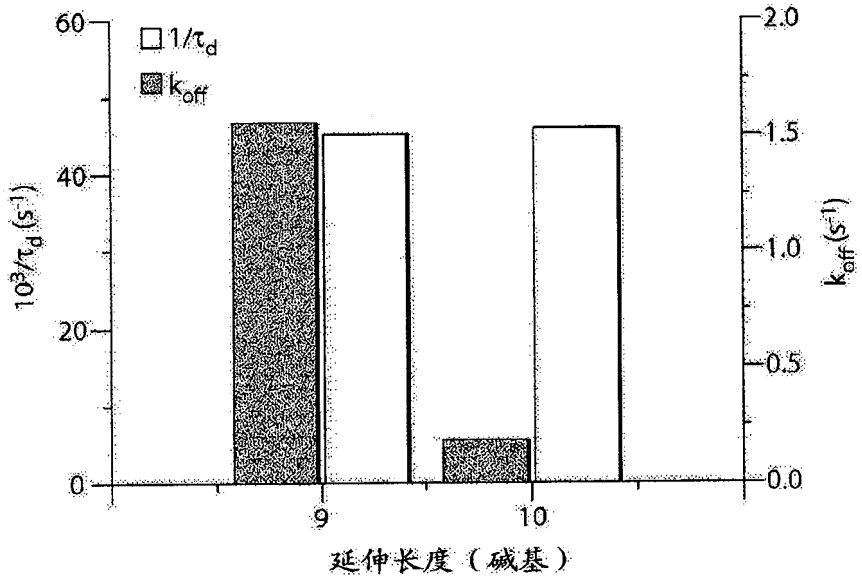

60
40
20
0
$10^3/\tau_d$ (s^{-1})
□ $1/\tau_d$
▨ k_{off}
2.0
1.5
1.0
0.5
0.0
k_{off} (s^{-1})
9
10
延伸长度(碱基)

图9

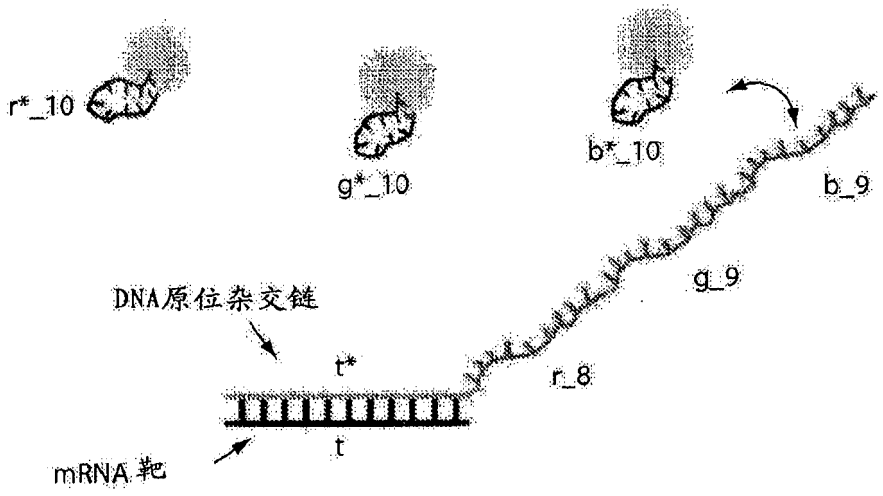


图10A

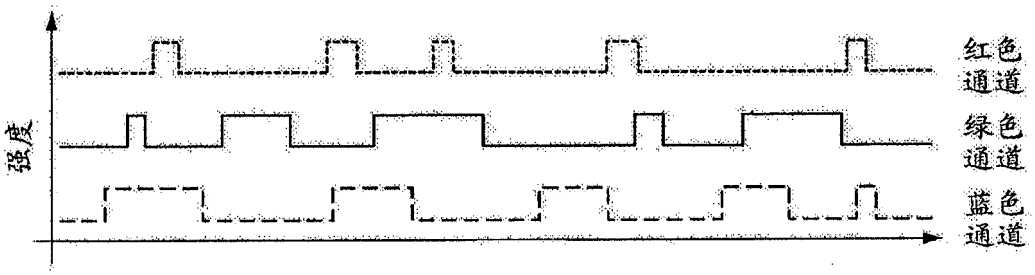


图10B

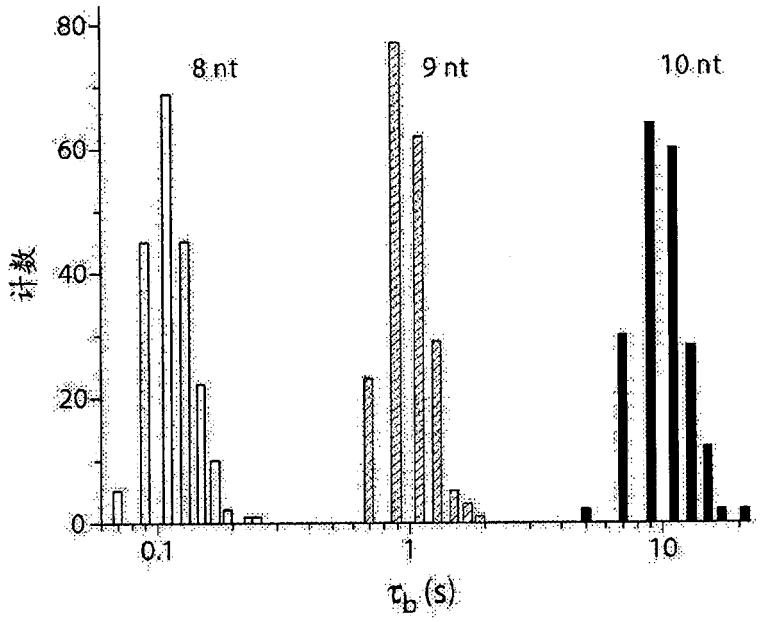


图10C

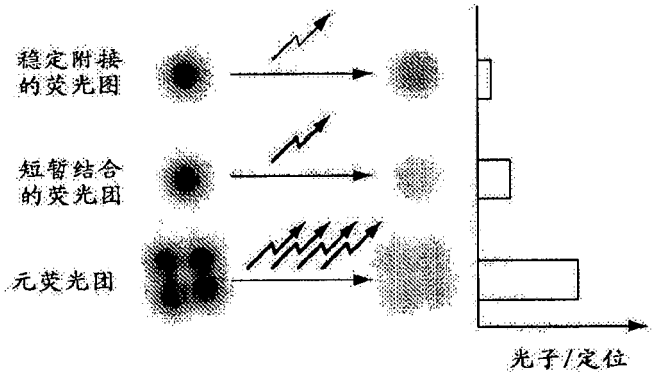


图11A

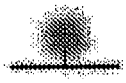


图11B1

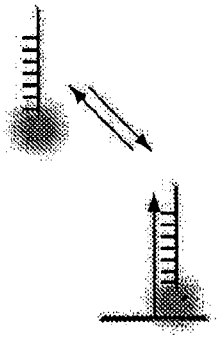


图11B2

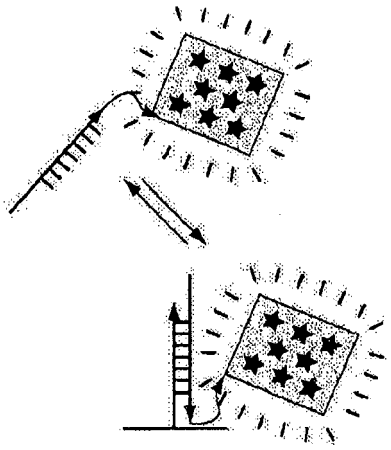


图11B3

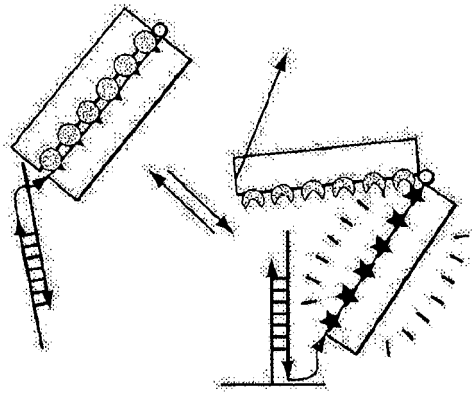
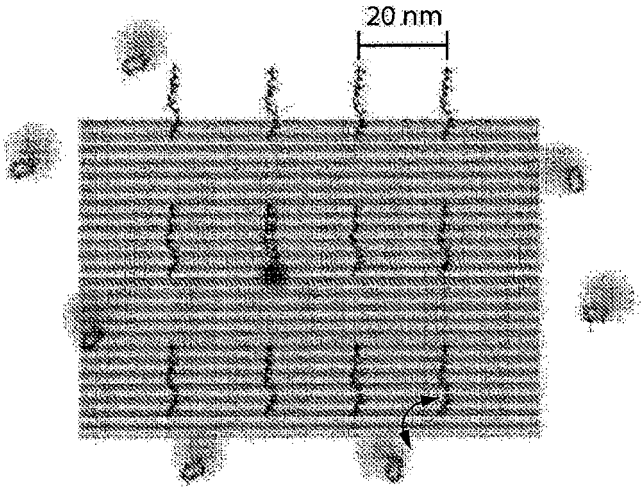


图11B4



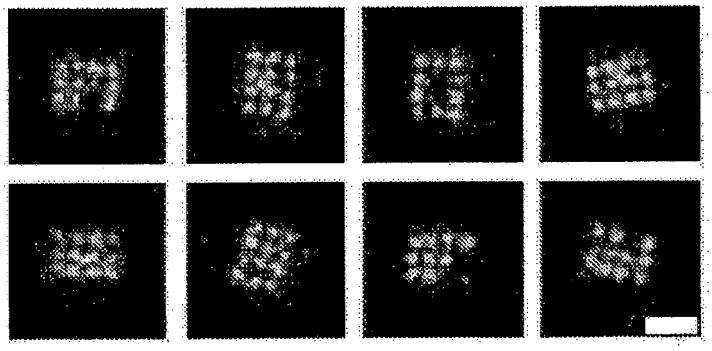


图11C

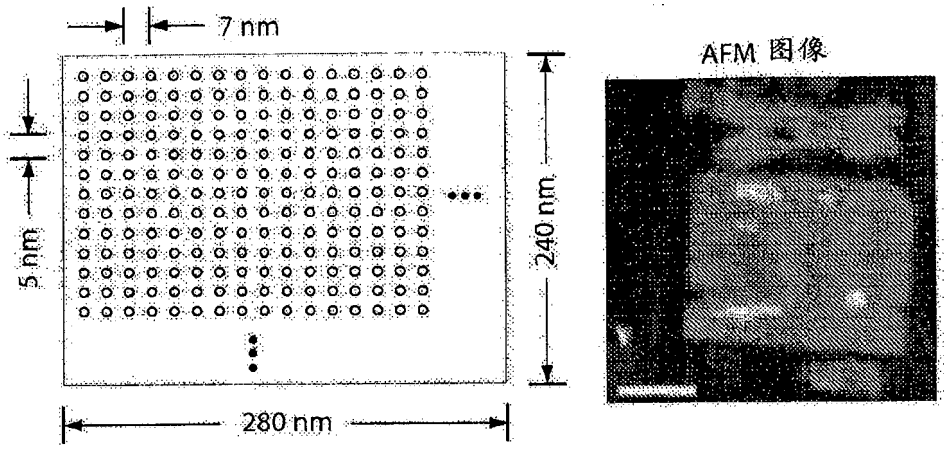


图11D

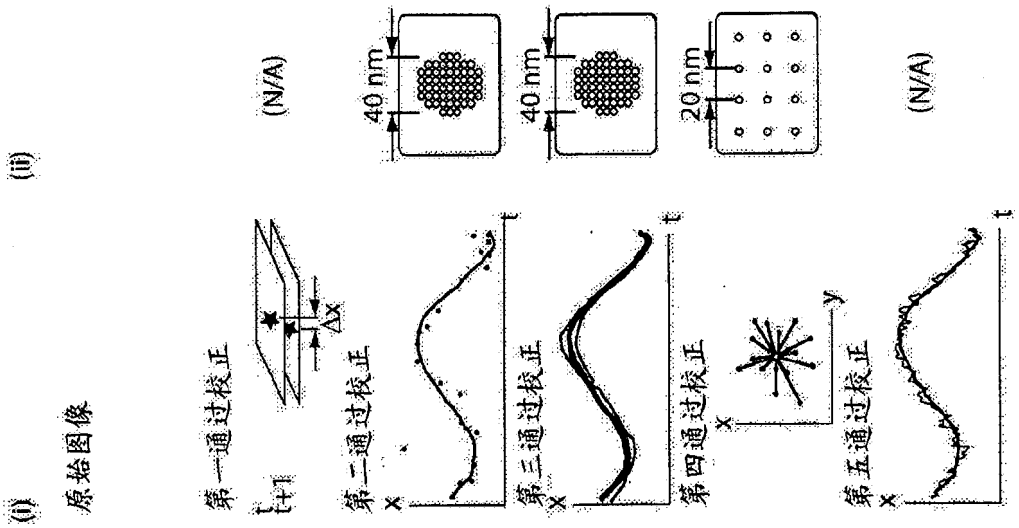


图12A

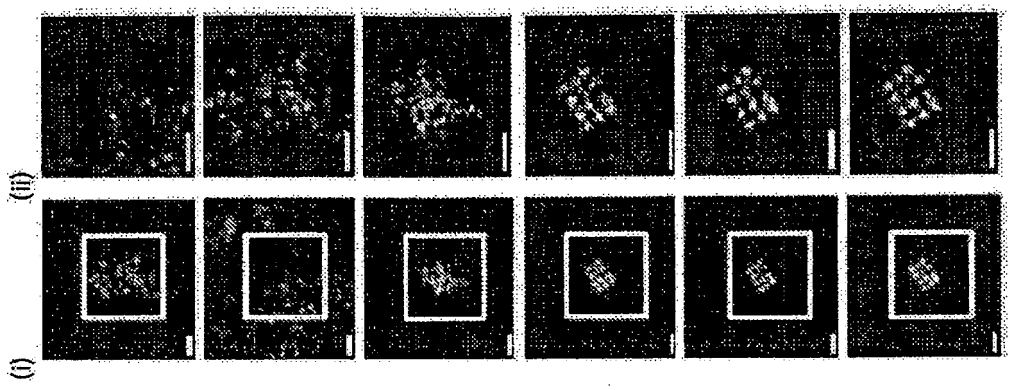


图12B

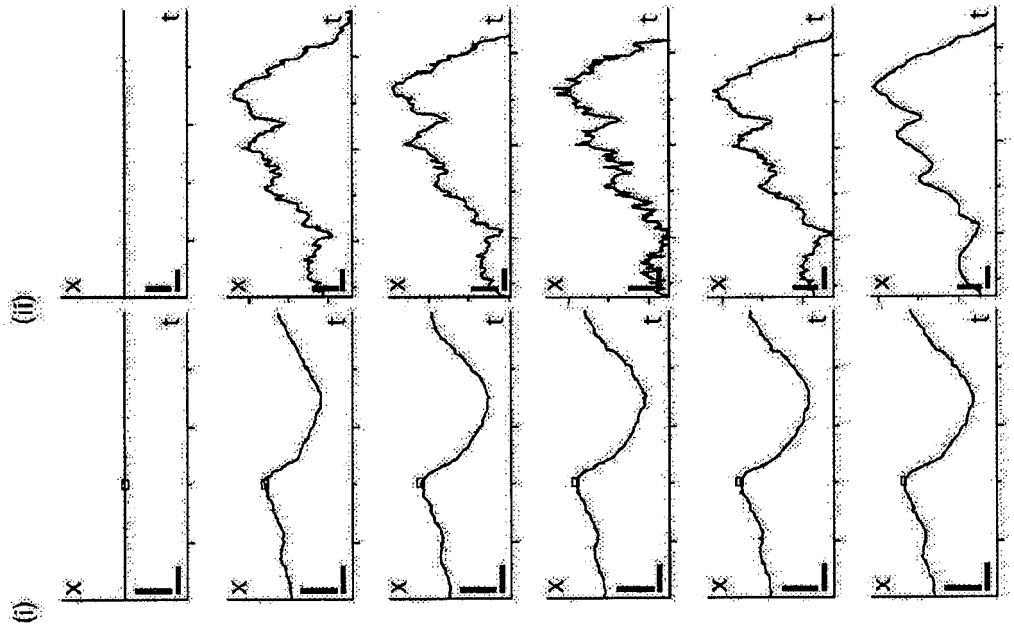


图12C

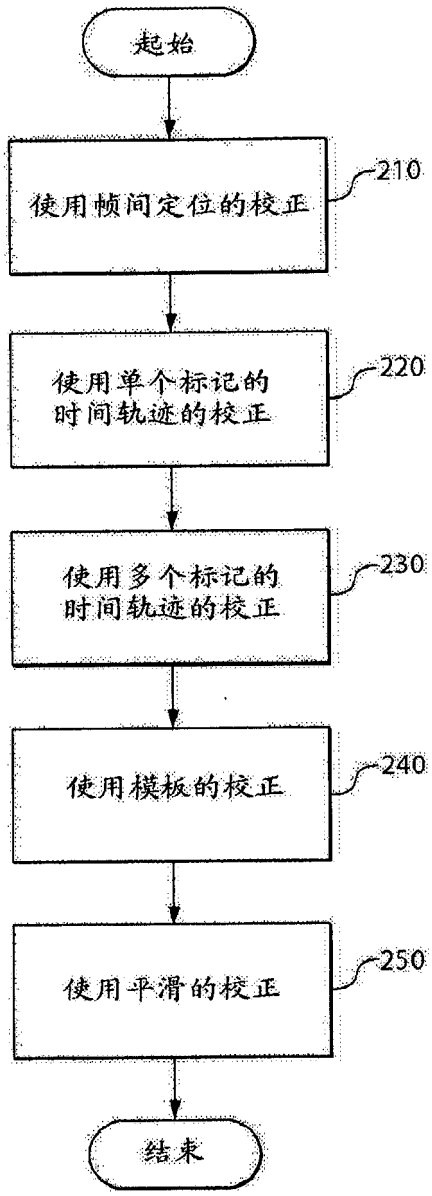

起始
使用帧间定位的校正
210
使用单个标记的时间轨迹的校正
220
使用多个标记的时间轨迹的校正
230
使用模板的校正
240
使用平滑的校正
250
结束

图13

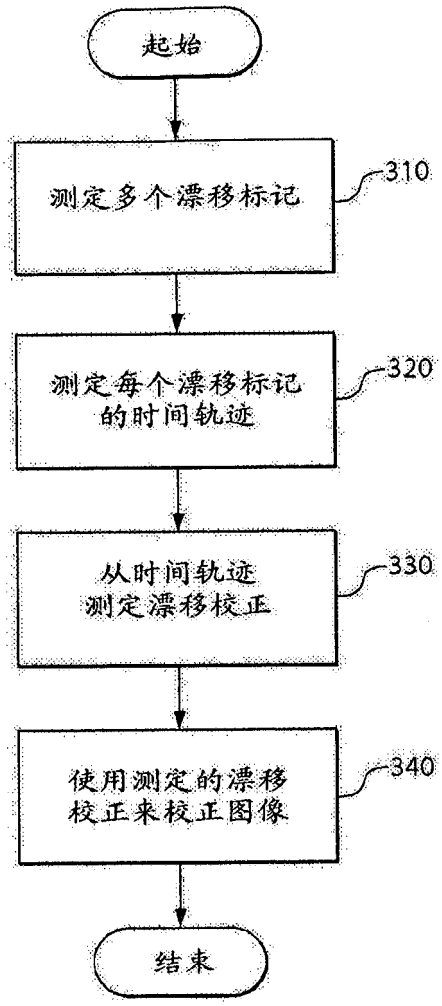

起始
测定多个漂移标记
310
测定每个漂移标记的时间轨迹
320
从时间轨迹测定漂移校正
330
使用测定的漂移校正来校正图像
340
结束

图14

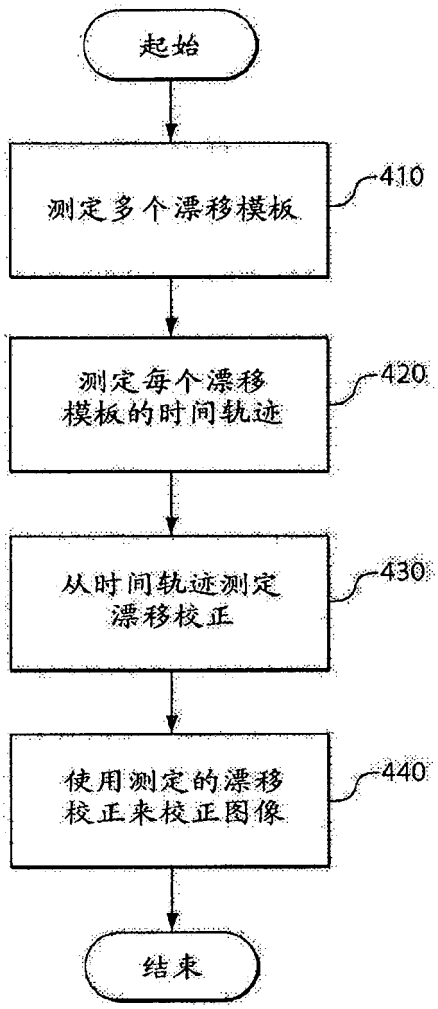

起始
测定多个漂移模板
410
测定每个漂移模板的时间轨迹
420
从时间轨迹测定漂移校正
430
使用测定的漂移校正来校正图像
440
结束

图15

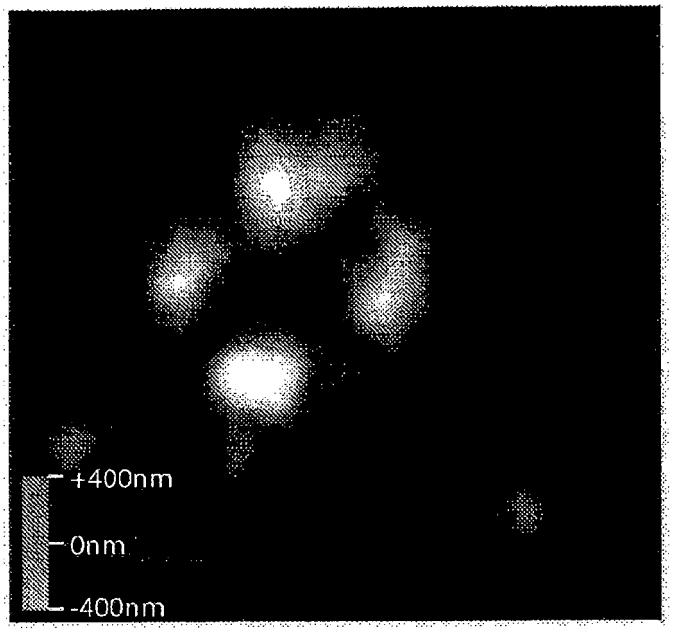

+400nm
0nm
-400nm

图16A

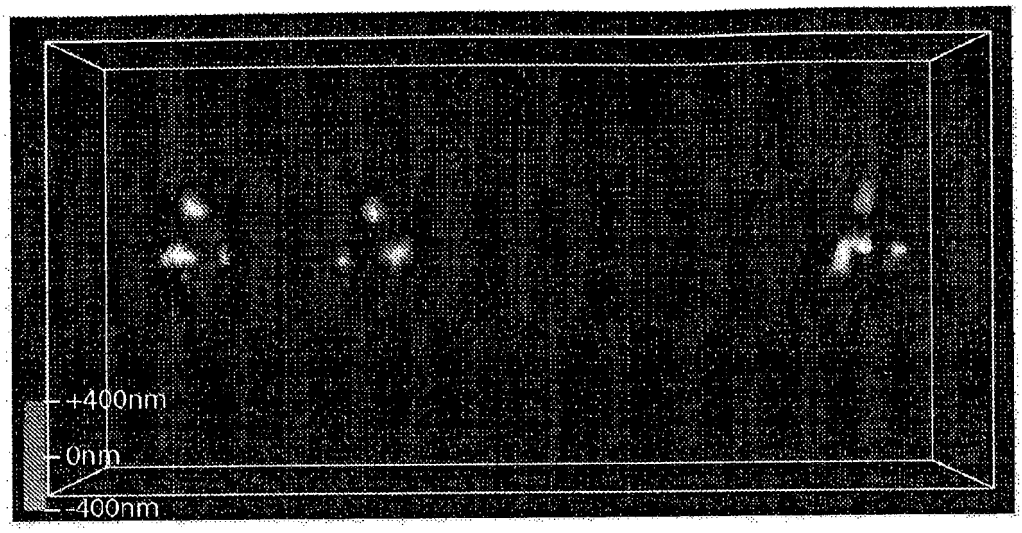


图16B

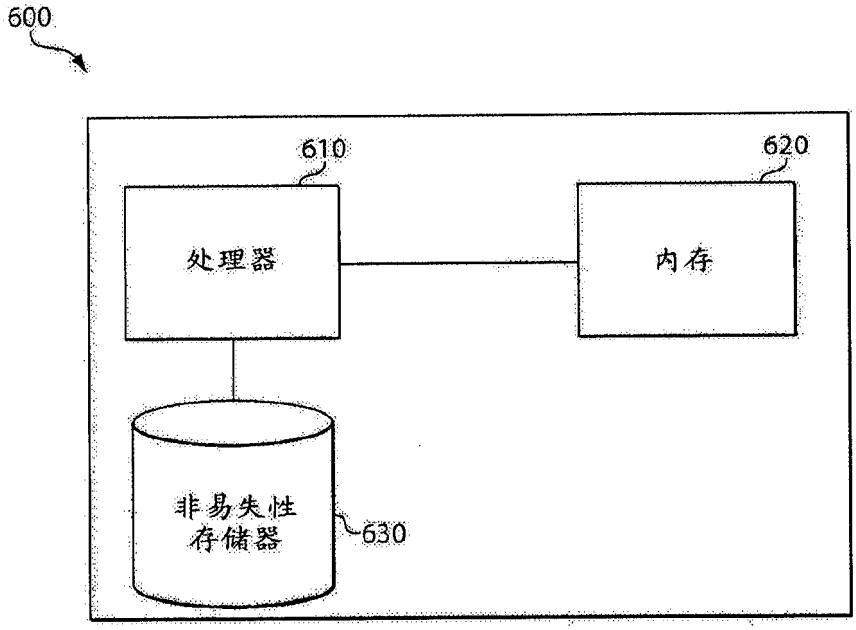


图17

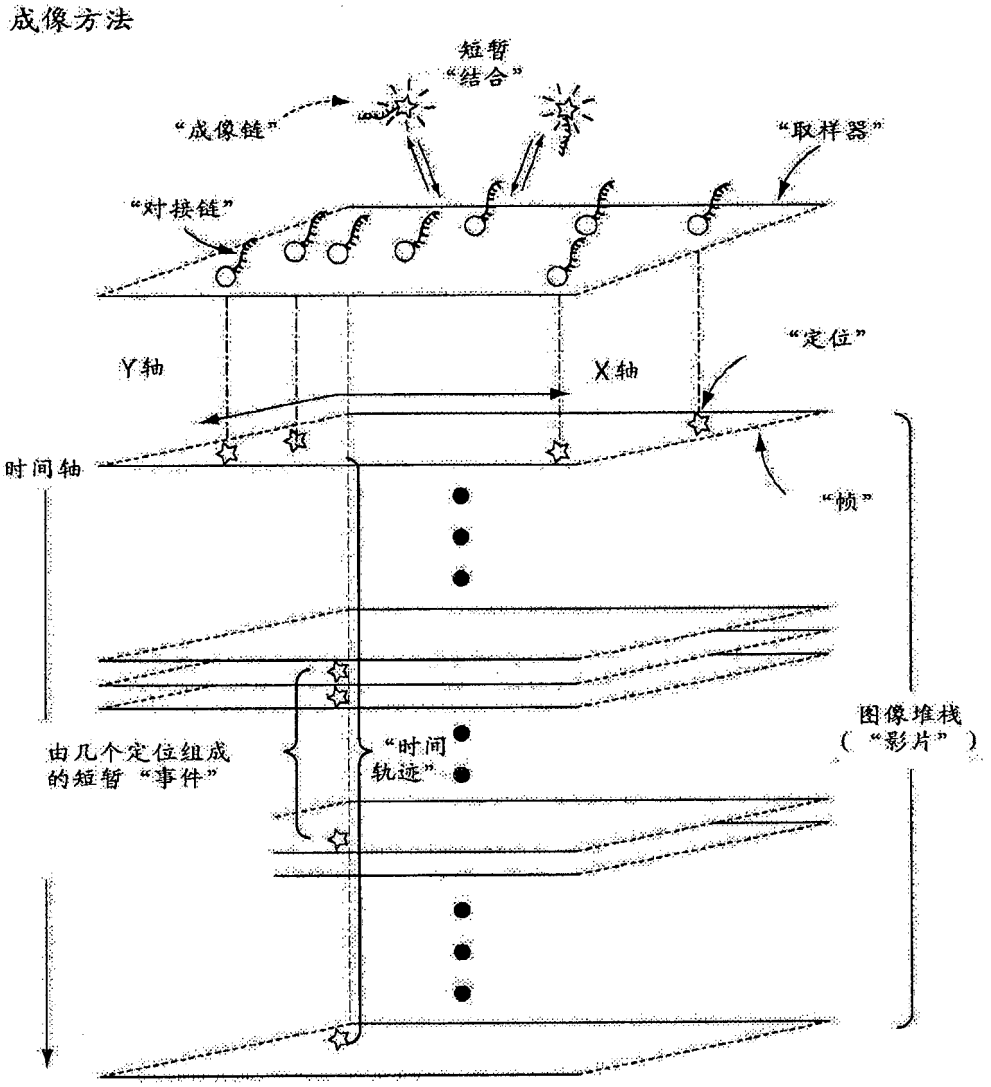


图18A

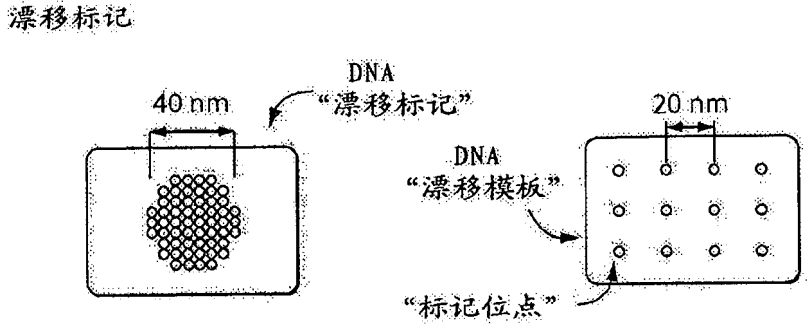


图18B

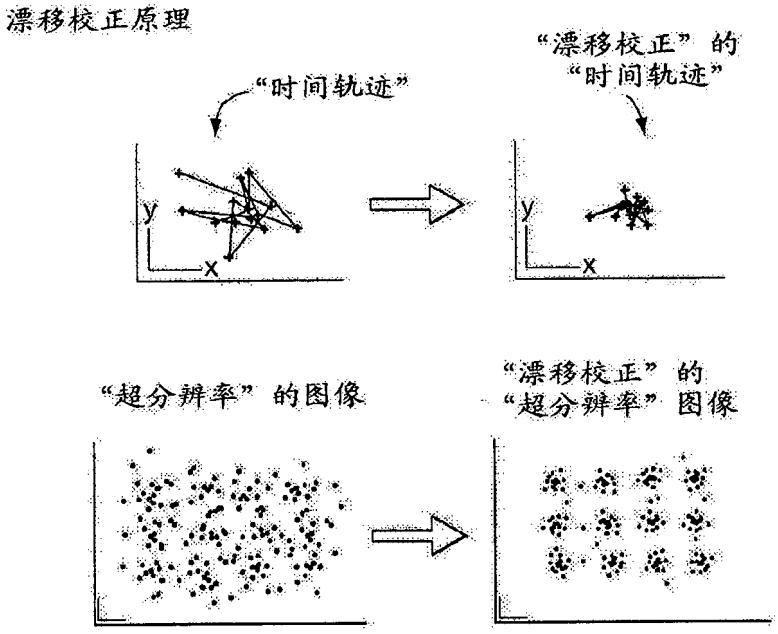


图18C

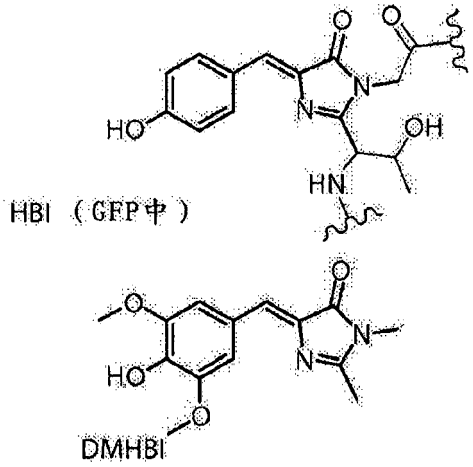


图19A

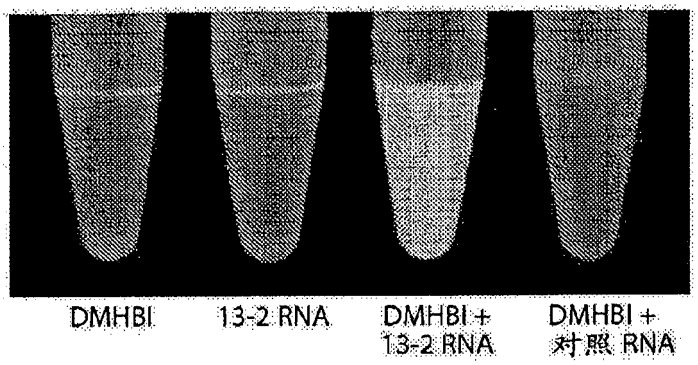


图19B

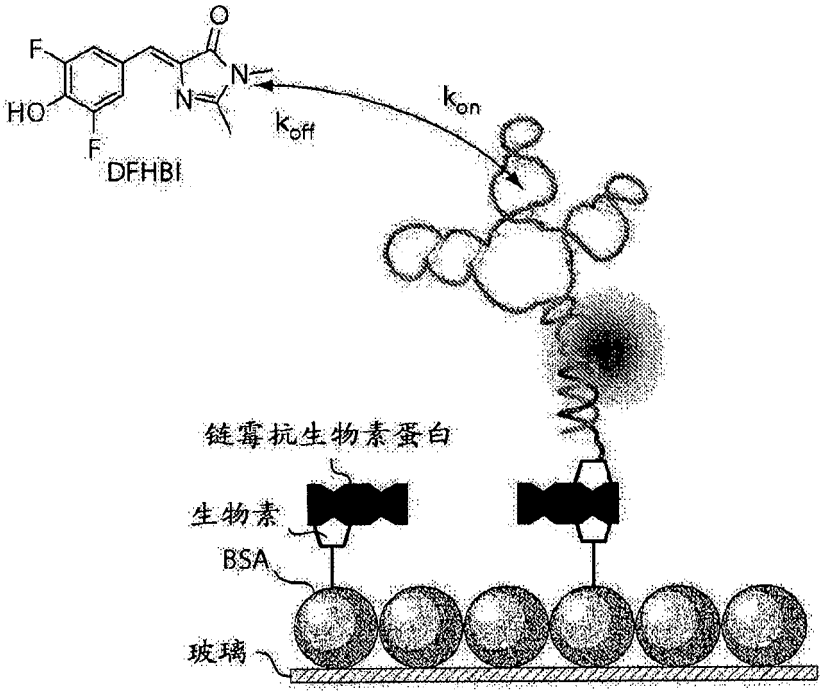


图20A

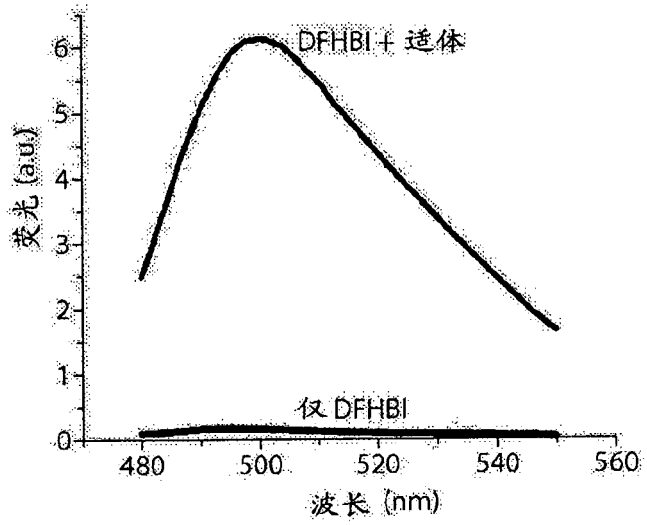


图20B

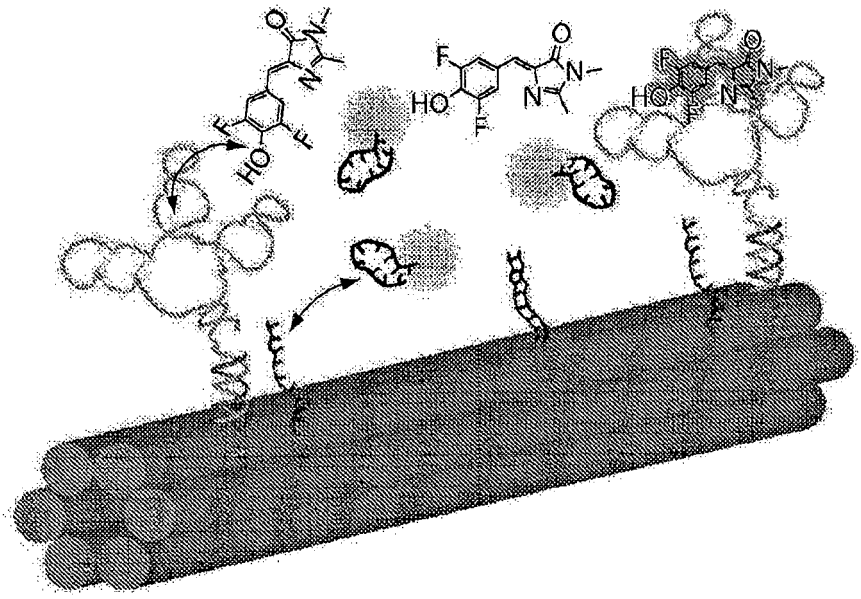


图21A

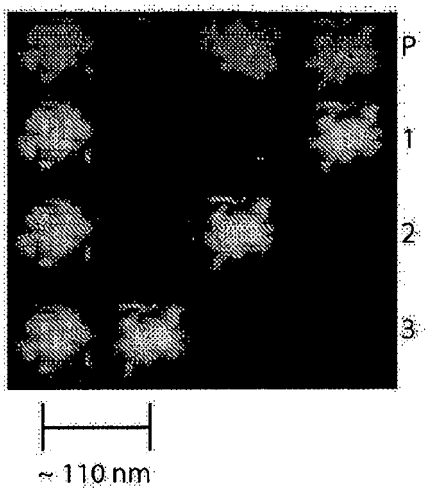


图21B

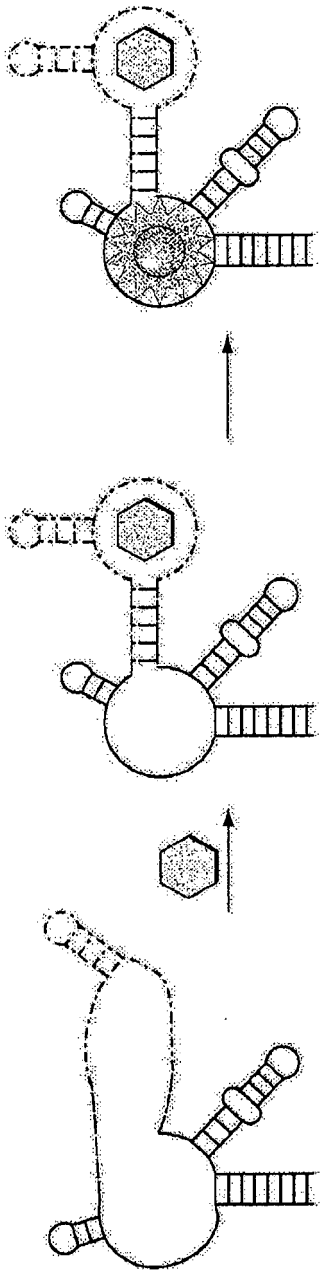


图22

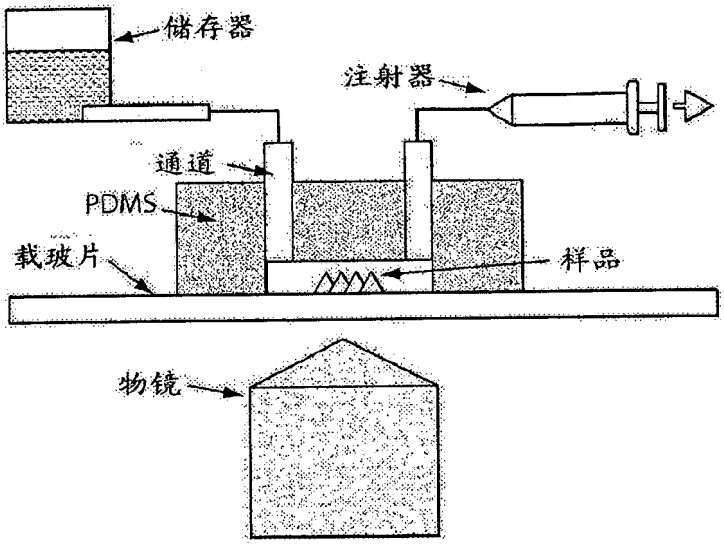


图23A

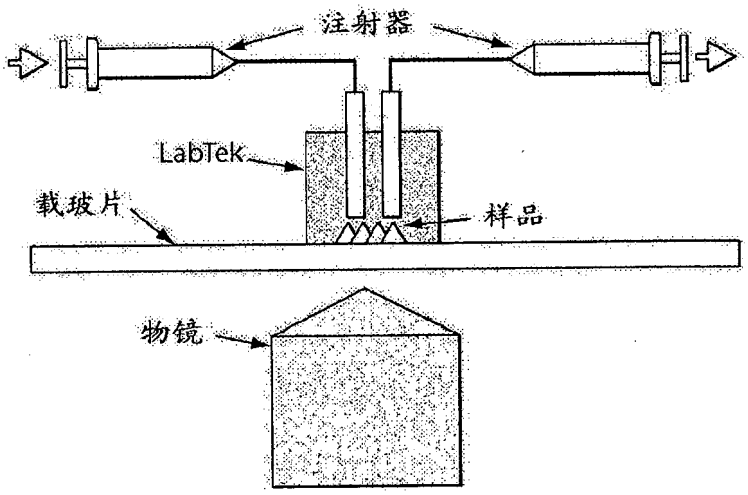


图23B