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(54) Title: MULTIVALENT BINDING COMPOSITION FOR NUCLEIC ACID ANALYSIS

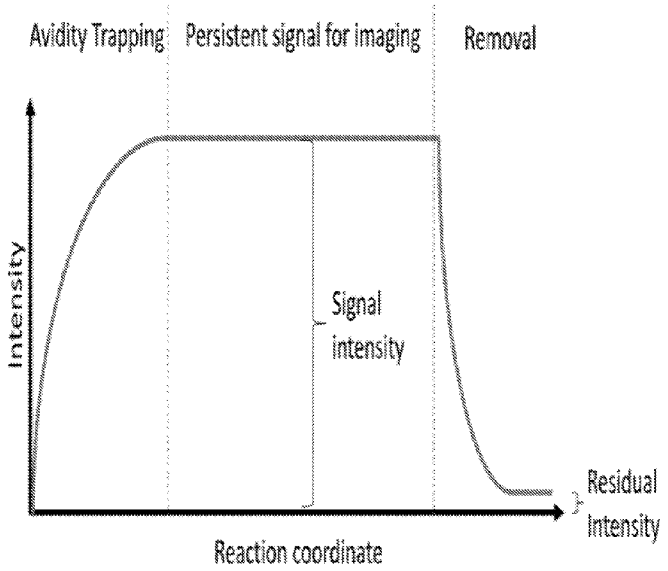

FIG. 6

(57) Abstract: Multivalent binding compositions including a particle-nucleotide conjugate having a plurality of copies of a nucleotide attached to the particle are described. The multivalent binding compositions allow one to localize detectable signals to active regions of biochemical interaction, e.g., sites of protein-protein interaction, protein-nucleic acid interaction, nucleic acid hybridization, or enzymatic reaction, and can be used to identify sites of base incorporation in elongating nucleic acid chains during polymerase reactions and to provide improved base discrimination for sequencing and array based applications.

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MULTIVALENT BINDING COMPOSITION FOR NUCLEIC ACID ANALYSIS

CROSS-REFERENCE

[0001] This application is a continuation-in-part of U.S. Patent Application No. 16/579,794, filed on September 23, 2019, and claims the benefit of U.S. Provisional Application No. 62/897,172 filed on September 6, 2019, and of U.S. Provisional Application No. 62/852,876 filed on May 24, 2019, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present disclosure relates generally to multivalent binding compositions and their use in analyzing nucleic acid molecules. In particular, the inventive concept relates to a multivalent binding composition having multiple copies of a nucleotide attached to a particle or polymer core which effectively increases the local concentration of the nucleotide and enhances the binding signals. The multivalent binding composition can be applied, for example, in the field of sequencing and biosensor microarrays.

BACKGROUND

[0003] Nucleic acid sequencing can be used to obtain information in a wide variety of biomedical contexts, including diagnostics, prognostics, biotechnology, and forensic biology. Various sequencing methods have been developed including Maxam-Gilbert sequencing and chain-termination methods, or de novo sequencing methods including shotgun sequencing and bridge PCR, or next-generation methods including polony sequencing, 454 pyrosequencing, Illumina sequencing, SOLiD sequencing, Ion Torrent semiconductor sequencing, HeliScope single molecule sequencing, SMRT® sequencing, and others. Despite advances in DNA sequencing, many challenges to cost effective, high throughput sequencing remain unaddressed. The present disclosure provides novel solutions and approaches to addressing many of the shortcomings of existing technologies.

SUMMARY

[0004] Disclosed herein are methods of determining an identity of a nucleotide in a target nucleic acid sequence comprising: a. providing a composition comprising: i. two or more copies of said target nucleic acid sequence; ii. two or more primer nucleic acid molecules that are complementary to one or more regions of said target nucleic acid sequence; and iii. two or more polymerase molecules; b. contacting said composition with a polymer nucleotide conjugate under conditions sufficient to allow a multivalent binding complex to be formed between said polymer-nucleotide conjugate and said two or more copies of said target nucleic acid sequence in said composition of (a), wherein the polymer-nucleotide conjugate comprises two or more copies of a nucleotide moiety and

optionally one or more detectable labels; and c. detecting said multivalent binding complex, thereby determining the identity of said nucleotide in the target nucleic acid sequence. In some embodiments, the target nucleic acid sequence is DNA. In some embodiments, the detection of the multivalent binding complex is performed in the absence of unbound or solution-borne polymer nucleotide conjugates. In some embodiments, the target nucleic acid sequence has been replicated or amplified or has been produced by replication or amplification. In some embodiments, the one or more detectable labels are fluorescent labels. In some embodiments, detecting the multivalent complex comprises a fluorescence measurement. In some embodiments, the contacting comprises use of one type of polymer-nucleotide conjugate. In some embodiments, the contacting comprises use of two or more types of polymer-nucleotide conjugates. In some embodiments, each type of the two or more types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety. In some embodiments, the contacting comprises use of three types of polymer-nucleotide conjugate and wherein each type of the three types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety. In some embodiments, the polymer-nucleotide conjugate comprises a blocked nucleotide moiety. In some embodiments, the blocked nucleotide is a 3 '-0-azidomethyl nucleotide, a 3 '-0-methyl nucleotide, or a 3 '-0-alkyl hydroxylamine nucleotide. In some embodiments, said contacting occurs in the presence of an ion that stabilizes said multivalent binding complex. In some embodiments, the contacting is done in the presence of strontium ions, magnesium ions, calcium ions, or any combination thereof. In some embodiments, the polymerase molecules are catalytically inactive. In some embodiments, the polymerase molecules have been rendered catalytically inactive by mutation or chemical modification. In some embodiments, the polymerase molecules have been rendered catalytically inactive by the absence of a necessary ion or cofactor. In some embodiments, the polymerase molecules are catalytically active. In some embodiments, the polymer-nucleotide conjugate does not comprise a blocked nucleotide moiety. In some embodiments, the multivalent binding complex has a persistence time of greater than 2 seconds. In some embodiments, the method can be carried out at a temperature within a range of 25°C to 62°C. In some embodiments, the polymer-nucleotide conjugate further comprises one or more fluorescent labels and the two or more copies of the target nucleic acid sequence are deposited on, attached to, or hybridized to a surface, wherein a fluorescence image of the multivalent binding complex on the surface has a contrast to noise ratio in the detecting step of greater than 20. In some embodiments, the composition of (a) is deposited on a surface using a buffer that incorporates a polar aprotic solvent. In some embodiments, the contacting is performed under a condition that stabilizes said multivalent binding complex when said nucleotide moiety is complementary to a next base of said target nucleic acid sequence and destabilizes said multivalent binding complex when said nucleotide moiety is not complementary to

said next base of said target nucleic acid sequence. In some embodiments, said polymer-nucleotide conjugate comprises a polymer having a plurality of branches and said two or more nucleotide moieties are attached to said branches. In some embodiments, said polymer has a star, comb, cross-linked, bottle brush, or dendrimer configuration. In some embodiments, said polymer-nucleotide conjugate comprises one or more binding groups selected from the group consisting of an avidin, a biotin, an affinity tag, and combinations thereof. In some embodiments, the method further comprises a dissociation step that destabilizes said multivalent binding complex formed between the composition of (a) and the polymer-nucleotide conjugate, said dissociation step enabling removal of said polymer-nucleotide conjugate. In some embodiments, the method further comprises an extension step to incorporate a nucleotide that is complementary to a next base of the target nucleic acid sequence into said two or more primer nucleic acid molecules. In some embodiments, the extension step occurs concurrently with or after said dissociation step.

[0005] Disclosed herein are methods of determining an identity of a nucleotide in a target nucleic acid sequence comprising: a. providing a composition comprising: i. two or more copies of said target nucleic acid sequence; ii. two or more primer nucleic acid molecules that are complementary to one or more regions of said target nucleic acid sequence; and iii. two or more polymerase molecules; b. contacting said composition with a polymer nucleotide conjugate under conditions sufficient to allow a multivalent complex to be formed between said polymer-nucleotide conjugate and said two or more copies of said target nucleic acid sequence in said composition of (a), wherein the polymer-nucleotide conjugate comprises two or more copies of a reversibly terminated nucleotide moiety and optionally one or more cleavable detectable labels; and c. detecting said multivalent complex, thereby determining the identity of said nucleotide in the target nucleic acid sequence. In some embodiments, the target nucleic acid sequence is DNA. In some embodiments, the method further comprises contacting the composition of (a) with reversibly terminated nucleotides or polymer-nucleotide conjugates comprising two or more copies of a reversibly terminated nucleotide following the detection of said multivalent binding complex. In some embodiments, the target nucleic acid sequence has been replicated or amplified or has been produced by replication or amplification. In some embodiments, the one or more detectable labels are fluorescent labels. In some embodiments, detecting the multivalent complex comprises a fluorescence measurement. In some embodiments, the contacting comprises use of one type of polymer-nucleotide conjugate. In some embodiments, the contacting comprises use of two or more types of polymer-nucleotide conjugates. In some embodiments, each type of the two or more types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety. In some embodiments, the contacting comprises use of three types of polymer-nucleotide conjugate and wherein each type of the three

types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety. In some embodiments, the polymer-nucleotide conjugate comprises a blocked nucleotide moiety. In some embodiments, the blocked nucleotide is a 3 '-0-azidomethyl, 3 '-0-methyl, or 3 '-0-alkyl hydroxylamine. In some embodiments, said contacting occurs in the presence of an ion that stabilizes said multivalent binding complex. In some embodiments, the polymerase molecules are catalytically inactive. In some embodiments, the polymerase molecules have been rendered catalytically inactive by mutation or chemical modification. In some embodiments, the polymerase molecules are catalytically active. In some embodiments, the polymer-nucleotide conjugate does not comprise a blocked nucleotide moiety. In some embodiments, the method can be carried out at a temperature within a range of 25°C to 80°C. In some embodiments, the polymer-nucleotide conjugate further comprises one or more fluorescent labels and the two or more copies of the target nucleic acid sequence are deposited on, attached to, or hybridized to a surface, wherein a fluorescence image of the multivalent binding complex on the surface has a contrast to noise ratio in the detecting step of greater than 20.

[0006] Also disclosed herein are systems comprising: a) one or more computer processors individually or collectively programmed to implement a method comprising: i) contacting a substrate comprising multiple copies of a target nucleic acid sequence tethered to a surface of the substrate with a reagent comprising a polymerase and one or more primer nucleic acid sequences that are complementary to one or more regions of said target nucleic acid sequence to form a primed target nucleic acid sequence; ii) contacting the substrate surface with a reagent comprising a polymer nucleotide conjugate under conditions sufficient to allow a multivalent binding complex to be formed between said polymer-nucleotide conjugate and two or more copies of said primed target nucleic acid sequence, wherein the polymer-nucleotide conjugate comprises two or more copies of a known nucleotide moiety and a detectable label; iii) acquiring and processing an image of the substrate surface to detect said multivalent binding complex, thereby determining the identity of a nucleotide in the target nucleic acid sequence. In some embodiments, the system further comprises a fluidics module configured to deliver a series of reagents to the substrate surface in a specified sequence and for specified time intervals. In some embodiments, the system further comprises an imaging module configured to acquire images of the substrate surface. In some embodiments, (ii) and (iii) are repeated two or more times thereby determining the identity of a series of two or more nucleotides in the target nucleic acid sequence. In some embodiments, the series of steps further comprises a dissociation step that destabilizes said multivalent binding complex, said dissociation step enabling removal of said polymer-nucleotide conjugate. In some embodiments, the series of steps further comprises an extension step to incorporate a nucleotide that is complementary to a next base of the target nucleic

acid sequence into said two or more primer nucleic acid molecules. In some embodiments, the extension step occurs concurrently with or after said dissociation step. In some embodiments, the detectable label comprises a fluorophore and the images comprise fluorescence images. In some embodiments, the fluorescence images of the multivalent binding complex on the substrate surface has a contrast-to-noise ratio of greater than 20 when the fluorophore is cyanine dye 3 (Cy3) and the image is acquired using an inverted fluorescence microscope equipped with a 20X objective, NA = 0.75, dichroic mirror optimized for 532 nm light, a bandpass filter optimized for Cyanine dye-3 emission, and a camera, under non-signal saturating conditions while the surface is immersed in 25 mM ACES, pH 7.4 buffer. In some embodiments, the series of steps is completed in less than 60 minutes. In some embodiments, the series of steps is completed in less than 30 minutes. In some embodiments, the series of steps is completed in less than 10 minutes. In some embodiments, an accuracy of base-calling is characterized by a Q-score of greater than 25 for at least 80% of the nucleotide identities determined. In some embodiments, an accuracy of base-calling is characterized by a Q-score of greater than 30 for at least 80% of the nucleotide identities determined. In some embodiments, an accuracy of base-calling is characterized by a Q-score of greater than 40 for at least 80% of the nucleotide identities determined.

[0007] Disclosed herein are compositions comprising: a) a polymer core; and b) two or more nucleotide, nucleotide analog, nucleoside, or nucleoside analog moieties attached to the polymer core; wherein the length of the linker is dependent on the nucleotide, nucleotide analog, nucleoside, or nucleoside analog moiety that is attached to the polymer core. Also disclosed herein are compositions comprising: a) a mixture of polymer-nucleotide conjugates, wherein each polymer-nucleotide conjugate comprises: i) a polymer core; and ii) two or more nucleotide, nucleotide analog, nucleoside, or nucleoside analog moieties attached to the polymer core, wherein the length of the linker is dependent on the nucleotide, nucleotide analog, nucleoside, or nucleoside analog moiety that is attached to the polymer core; and wherein the mixture comprises polymer-nucleotide conjugates having at least two different types of attached nucleotide, nucleotide analog, nucleoside, or nucleoside analog moiety. In some embodiments, the polymer core comprises a polymer having a plurality of branches and the two or more nucleotide, nucleotide analog, nucleoside, or nucleoside analog moieties are attached to said branches. In some embodiments, polymer has a star, comb, cross-linked, bottle brush, or dendrimer configuration. In some embodiments, the polymer-nucleotide conjugate comprises one or more binding groups selected from the group consisting of an avidin, a biotin, an affinity tag, and combinations thereof. In some embodiments, the polymer core comprises a branched polyethylene glycol (PEG) molecule. In some embodiments, the polymer-nucleotide conjugate comprises a blocked nucleotide moiety. In some embodiments, the blocked

nucleotide is a 3 '-0-azidomethyl nucleotide, a 3 '-0-methyl nucleotide, or a 3'-0-alkyl hydroxylamine nucleotide. In some embodiments, the polymer-nucleotide conjugate further comprises one or more fluorescent labels.

[0008] In some embodiments the present disclosure provides methods of determining the identity of a nucleotide in a target nucleic acid comprising the steps, without regard to any particular order of operations, 1) providing a composition comprising: a target nucleic acid comprising two or more repeats of an identical sequence; two or more primer nucleic acids complementary to one or more regions of said target nucleic acid; and two or more polymerase molecules; 2) contacting said composition with a multivalent binding or incorporation composition comprising a polymer-nucleotide conjugate under conditions sufficient to allow a binding or incorporated complex to be formed between said polymer-nucleotide conjugate and the composition of step (a), wherein the polymer-nucleotide conjugate comprises two or more copies of a nucleotide and optionally one or more detectable labels; and 3) detecting said binding or incorporated complex, thereby establishing the identity of said nucleotide in the target nucleic acid polymer. In some further embodiments, the present disclosure provides said method, wherein the target nucleic acid is DNA, and/or wherein the target nucleic acid has been replicated, such as by any commonly practiced method of DNA replication or amplification, such as rolling circle amplification, bridge amplification, helicase dependent amplification, isothermal bridge amplification, rolling circle multiple displacement amplification (RCA/MDA) and/or recombinase based methods of replication or amplification. . In some further embodiments, the present disclosure provides said method, wherein the detectable label is a fluorescent label and/or wherein detecting the complex comprises a fluorescence measurement. In some further embodiments, the present disclosure provides said method wherein the multivalent binding composition comprises one type of polymer-nucleotide conjugate, wherein the multivalent binding composition comprises two or more types of polymer-nucleotide conjugates, and/or wherein each type of the two or more types of polymer-nucleotide conjugates comprises a different type of nucleotide. In some embodiments, the present disclosure provides said method wherein the binding complex or incorporated complex further comprises a blocked nucleotide, especially wherein the blocked nucleotide is a 3’-O-azidomethyl nucleotide, a 3’-O-alkyl hydroxylamino nucleotide, or a 3’-O-methyl nucleotide. In some further embodiments, the present disclosure provides said method wherein the contacting is done in the presence of strontium ions, barium, magnesium ions, and/or calcium ions. In some embodiments, the present disclosure provides said method wherein the polymerase molecule is catalytically inactive, such as where the polymerase molecule been rendered catalytically inactive by mutation, by chemical modification, or by the absence of a necessary ion or cofactor. In some embodiments, the present disclosure also provides said method wherein the

polymerase molecule is catalytically active, and/or wherein the binding complex does not comprise a blocked nucleotide. In some embodiments, the present disclosure provides said method wherein the binding complex has a persistence time of greater than 2 seconds and/or wherein the method is or may be carried out at a temperature of at or above 15 °C, at or above 20 °C, at or above 25 °C, at or above 35 °C, at or above 37 °C, at or above 42 °C at or above 55 °C at or above 60 °C, or at or above 72 °C, or within a range defined by any of the foregoing. In some embodiments, the present disclosure provides said method wherein the binding complex is deposited on, attached to, or hybridized to, a surface showing a contrast to noise ratio in the detecting step of greater than 20. In some embodiments, the present disclosure provides said method wherein the composition is deposited under buffer conditions incorporating a polar aprotic solvent. In some embodiments, the present disclosure provides said method wherein the contacting is performed under a condition that stabilizes said binding complex when said nucleotide is complementary to a next base of said target nucleic acid and destabilizes said binding complex when said nucleotide is not complementary to said next base of said target nucleic acid. In some embodiments, the present disclosure provides said method wherein said polymer-nucleotide conjugate comprises a polymer having a plurality of branches and said plurality of copies of said first nucleotide are attached to said branches, especially wherein said first polymer has a star, comb, cross-linked, bottle brush, or dendrimer configuration. In some embodiments, the present disclosure provides said method wherein said polymer-nucleotide conjugate comprises one or more binding groups selected from the group consisting of avidin, biotin, affinity tag, and combinations thereof. In some embodiments, the present disclosure provides said method further comprising a dissociation step that destabilizes said binding complex formed between the composition of (a) and the polymer-nucleotide conjugate to remove said polymer-nucleotide conjugate. In some embodiments, the present disclosure provides said method further comprising an extension step to incorporate into said primer nucleic acid a nucleotide that is complementary to said next base of the target nucleic acid, and optionally wherein the extension step occurs currently as or after said dissociation step.

[0009] In some embodiments, the present disclosure provides a composition comprising a branched polymer having two or more branches and two or more copies of a nucleotide, wherein said nucleotide is attached to a first plurality of said branches or arms, and optionally, wherein one or more interaction moieties are attached to a second plurality of said branches or arms. In some embodiments, said composition may further comprise one or more labels on the polymer. In some embodiments, the present disclosure provides said composition wherein the nucleoside has a surface density of at least 4 nucleotides per polymer. In some embodiments, the present disclosure provides said composition comprising or incorporating a nucleotide or nucleotide analog that is modified so

as to prevent its incorporation into an extending nucleic acid chain during a polymerase reaction. In some embodiments, said composition may comprise or incorporate a nucleotide or nucleotide analog that is reversibly modified so as to prevent its incorporation into an extending nucleic acid chain during a polymerase reaction. In some embodiments, the present disclosure provides said composition wherein one or more labels comprise a fluorescent label, a FRET donor, and/or a FRET acceptor. In some embodiments, said composition may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or more branches or arms, or 2, 4, 8, 16, 32, 64, or more, branches or arms. In some embodiments, the branches or arms may radiate from a central moiety. In some embodiments, said composition may comprise one or more interaction moieties, which interaction moieties may comprise avidin or streptavidin; a biotin moiety; an affinity tag; an enzyme, antibody, minibody, receptor, or other protein; a non-protein tag; a metal affinity tag, or any combination thereof. In some embodiments, the present disclosure provides said composition wherein the polymer comprises polyethylene glycol, polypropylene glycol, polyvinyl acetate, polylactic acid, or polyglycolic acid. In some embodiments, the present disclosure provides said composition wherein the nucleotide or nucleotide analog is attached to the branch or arm through a linker; and especially wherein the linker comprises PEG, and wherein the PEG linker moiety has an average molecular weight of about 1K Da, about 2K Da, about 3K Da, about 4K Da, about 5K Da, about 10K Da, about 15K Da, about 20K Da, about 50K Da, about 100K Da, about 150K Da, or about 200K Da, or greater than about 200K Da. In some embodiments, the present disclosure provides said composition wherein the linker comprises PEG, and wherein the PEG linker moiety has an average molecular weight of between about 5K Da and about 20K Da. In some embodiments, the present disclosure provides said composition wherein at least one nucleotide or nucleotide analog comprises a deoxyribonucleotide, a ribonucleotide, a deoxyribonucleoside, or a ribonucleoside; and/or wherein the nucleotide or nucleotide analog is conjugated to the linker through the 5' end of the nucleotide or nucleotide analog. In some embodiments, the present disclosure provides said composition wherein one of the nucleotides or nucleotide analogs comprises deoxyadenosine, deoxyguanosine, thymidine, deoxyuridine, deoxycytidine, adenosine, guanosine, 5-methyl-uridine, and/or cytidine; and wherein the length of the linker is between 1 nm and 1,000 nm. In some embodiments, the present disclosure provides said composition wherein at least one nucleotide or nucleotide analog is a nucleotide that has been modified to inhibit elongation during a polymerase reaction or a sequencing reaction, such as wherein the at least one nucleotide or nucleotide analog is a nucleotide that lacks a 3' hydroxyl group; a nucleotide that has been modified to contain a blocking group at the 3' position; and/or a nucleotide that has been modified with a 3'-O-azido group, a 3'-O-azidomethyl group, , a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, or a 3'-O-benzyl group.

In some embodiments, the present disclosure provides said composition wherein at least one nucleotide or nucleotide analog is a nucleotide that has not been modified at the 3' position.

[0010] In some embodiments, the present disclosure provides a method of determining the sequence of a nucleic acid molecule comprising the steps, without regard to any particular order, of 1) providing a nucleic acid molecule comprising a template strand and a complementary strand that is at least partially complementary to the template strand; 2) contacting the nucleic acid molecule with the one or more nucleic acid binding composition according to any of the embodiments disclosed herein; 3) detecting binding of the nucleic acid binding composition to the nucleic acid molecule, and 4) determining an identity of a terminal nucleotide to be incorporated into said complementary strand of said nucleic acid molecule. In some embodiments, the present disclosure provides a method of determining the sequence of a nucleic acid molecule comprising the steps, without regard to any particular order, of 1) providing a nucleic acid molecule comprising a template strand and a complementary strand that is at least partially complementary to the template strand; 2) contacting the nucleic acid molecule with the one or more nucleic acid binding composition according to any of the embodiments disclosed herein; 3) detecting partial or complete incorporation of the nucleic acid binding composition to the nucleic acid molecule, and 4) determining an identity of a terminal nucleotide to be incorporated into said complementary strand of said nucleic acid molecule from the partial or complete incorporation of the embodiments described herein. In some embodiments, the present disclosure provides said method, further comprising incorporating said terminal nucleotide into said complementary strand, and repeating said contacting, detecting, and incorporating steps for one or more additional iterations, thereby determining the sequence of said template strand of said nucleic acid molecule. In some embodiments, the present disclosure provides said method, wherein said nucleic acid molecule is tethered to a solid support; and especially wherein the solid support comprises a glass or polymer substrate, at least one hydrophilic polymer coating layer, and a plurality of oligonucleotide molecules attached to at least one hydrophilic polymer coating layer. In some embodiments, the present disclosure provides said method, further comprising embodiments wherein at least one hydrophilic polymer coating layer comprises PEG; and/or wherein at least one hydrophilic polymer layer comprises a branched hydrophilic polymer having at least 8 branches. In some embodiments, the present disclosure provides said method, wherein the plurality of oligonucleotide molecules is present at a surface density of at least 500 molecules/mm^2, at least 1,000 molecules/mm^2, at least 5,000 molecules/mm^2, at least 10,000 molecules/mm^2, at least 20,000 molecules/mm^2, at least 50,000 molecules/mm^2, at least 100,000 molecules/mm^2, or at least 500,000 molecules/mm^2. In some embodiments, the present disclosure provides said method, wherein said nucleic acid molecule has been clonally-amplified on a solid support. In some embodiments, the

present disclosure provides said method, wherein the clonal amplification comprises the use of a polymerase chain reaction (PCR), multiple displacement amplification (MDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, bridge amplification, isothermal bridge amplification, rolling circle amplification (RCA), circle-to-circle amplification, helicase-dependent amplification, recombinase-dependent amplification, single-stranded binding (SSB) protein-dependent amplification, or any combination thereof. In some embodiments, the present disclosure provides said method, wherein the one or more nucleic acid binding compositions are labeled with fluorophores and the detecting step comprises use of fluorescence imaging; and especially wherein the fluorescence imaging comprises dual wavelength excitation/four wavelength emission fluorescence imaging. In some embodiments, the present disclosure provides said method, wherein four different nucleic acid binding compositions, each comprising a different nucleotide or nucleotide analog, are used to determine the identity of the terminal nucleotide, wherein the four different nucleic acid binding compositions are labeled with separate respective fluorophores, and wherein the detecting step comprises simultaneous excitation at a wavelength sufficient to excite all four fluorophores and imaging of fluorescence emission at wavelengths sufficient to detect each respective fluorophore. In some embodiments, the present disclosure provides said method, wherein four different nucleic acid binding compositions, each comprising a different nucleotide or nucleotide analog, are used to determine the identity of the terminal nucleotide, wherein the four different nucleic acid binding compositions are labeled with cyanine dye 3 (Cy3), cyanine dye 3.5 (Cy3.5), cyanine dye 5 (Cy5), and cyanine dye 5.5. (Cy5.5) respectively, and wherein the detecting step comprises simultaneous excitation at any two of 532 nm, 568 nm and 633 nm, and imaging of fluorescence emission at about 570 nm, 592 nm, 670 nm, and 702 nm respectively; and/or wherein the fluorescence imaging comprises dual wavelength excitation / dual wavelength emission fluorescence imaging. In some embodiments, the present disclosure provides said method, wherein four different nucleic acid binding compositions, each comprising a different nucleotide or nucleotide analog, are used to determine the identity of the terminal nucleotide, wherein one, two, three, or four different nucleic acid binding compositions are respectively labeled, each with a with distinct fluorophore or set of fluorophores, and wherein the detecting step comprises simultaneous excitation at a wavelength sufficient to excite one, two, three, or four fluorophores or sets of fluorophores, and imaging of fluorescence emission at wavelengths sufficient to detect each respective fluorophore. In some embodiments, the present disclosure provides said method, wherein three different nucleic acid binding or incorporation compositions, each comprising a different nucleotide or nucleotide analog, are used to determine the identity of the terminal nucleotide, wherein one, two, or three different

nucleic acid binding or incorporation compositions are respectively labeled, each with a with distinct fluorophore or set of fluorophores, and wherein the detecting step comprises simultaneous excitation at a wavelength sufficient to excite one, two, or three, fluorophores or sets of fluorophores, and imaging of fluorescence emission at wavelengths sufficient to detect each respective fluorophore, and wherein detection of the fourth nucleotide is determined or determinable with reference to the location of "dark" or unlabeled spots or target nucleotides. In some embodiments, the present disclosure provides said method, wherein the multivalent binding or incorporation composition may comprise three types of polymer-nucleotide conjugates and wherein each type of the three types of polymer-nucleotide conjugates comprises a different type of nucleotide. In some embodiments, the present disclosure provides said method, wherein the detection of the binding or incorporation complex is performed in the absence of unbound or solution-borne polymer nucleotide conjugates.

[0011] In some embodiments, the present disclosure provides said method, wherein four different nucleic acid binding compositions, or three different nucleic acid binding or incorporation compositions, each comprising a different nucleotide or nucleotide analog, are used to determine the identity of the terminal nucleotide, wherein one of the four or three different nucleic acid binding or incorporation compositions is labeled with a first fluorophore, one is labeled with a second fluorophore, one is labeled with both the first and second fluorophore, and one is not labeled or is absent, and wherein the detecting step comprises simultaneous excitation at a first excitation wavelength and a second excitation wavelength and images are acquired at a first fluorescence emission wavelength and a second fluorescence emission wavelength. In some embodiments, the present disclosure provides said method, wherein the first fluorophore is Cy3, the second fluorophore is Cy5, the first excitation wavelength is 532 nm or 568 nm, the second excitation wavelength is 633 nm, the first fluorescence emission wavelength is about 570 nm, and the second fluorescence emission wavelength is about 670 nm. In some embodiments, the present disclosure provides said method, wherein the detection label can comprise one or more portions of a fluorescence resonance energy transfer (FRET) pair, such that multiple classifications can be performed under a single excitation and imaging step. In some embodiments, the present disclosure provides said method, wherein a sequencing reaction cycle comprising the contacting, detecting, and incorporating/extending steps is performed in less than 30 minutes in less than 20 minutes, or in less than 10 minutes. In some embodiments, the present disclosure provides said method, wherein an average Q-score for base calling accuracy over a sequencing run is greater than or equal to 30, and/or greater than or equal to 40. In some embodiments, the present disclosure provides said method, wherein at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the terminal nucleotides identified have a Q-score of greater than 30 and/or greater than or equal to 40. In some

embodiments, the present disclosure provides said method, herein at least 95% of the terminal nucleotides identified have a Q-score of greater than 30.

[0012] In some embodiments, the present disclosure provides a reagent comprising one or more nucleic acid binding compositions as disclosed herein and a buffer. For example, in some embodiments, the present disclosure provides a reagent, wherein said reagent comprises 1, 2, 3, 4, or more nucleic acid binding or incorporation compositions, wherein each nucleic acid binding or incorporation composition comprises a single type of nucleotide. In some embodiments, a reagent of the present disclosure comprises 1, 2, 3, 4, or more nucleic acid binding or incorporation compositions, wherein each nucleic acid binding or incorporation composition comprises a single type of nucleotide or nucleotide analog, and wherein said nucleotide or nucleotide analog may respectively correspond to one or more from the group consisting of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), deoxyadenosine triphosphate (dATP), deoxyadenosine diphosphate (dADP), and deoxyadenosine monophosphate (dAMP); one or more from the group consisting of thymidine triphosphate (TTP), thymidine diphosphate (TDP), thymidine monophosphate (TMP), deoxythymidine triphosphate (dTTP), deoxythymidine diphosphate (dTDP), deoxythymidine monophosphate (dTMP), uridine triphosphate (UTP), uridine diphosphate (UDP), uridine monophosphate (UMP), deoxyuridine triphosphate (dUTP), deoxyuridine diphosphate (dUDP), and deoxyuridine monophosphate (dUMP); one or more from the group consisting of cytidine triphosphate (CTP), cytidine diphosphate (CDP), cytidine monophosphate (CMP), deoxycytidine triphosphate (dCTP), deoxycytidine diphosphate (dCDP), and deoxycytidine monophosphate (dCMP); and one or more from the group consisting of guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), deoxyguanosine triphosphate (dGTP), deoxyguanosine diphosphate (dGDP), and deoxyguanosine monophosphate (dGMP). In some other examples or some further examples, the present disclosure provides a reagent comprising or further comprising 1, 2, 3, 4, or more nucleic acid binding or incorporation compositions, wherein each nucleic acid binding or incorporation composition comprises a single type of nucleotide or nucleotide analog, and wherein said nucleotide or nucleotide analog may respectively correspond to one or more from the group consisting of ATP, ADP, AMP, dATP, dADP, dAMP, TTP, TDP, TMP, dTTP, dTDP, dTMP, UTP, UDP, UMP, dUTP, dUDP, dUMP, CTP, CDP, CMP, dCTP, dCDP, dCMP, GTP, GDP, GMP, dGTP, dGDP, and dGMP.

[0013] Disclosed herein are kits comprising the nucleic acid binding or incorporation composition of any of the embodiments disclosed herein and/or a reagent of any of the embodiments disclosed herein, and/or one or more buffers; and instructions for the use thereof.

[0014] Disclosed herein are systems for performing the method of any embodiment disclosed herein, comprising a nucleic acid binding or incorporation composition of any of the embodiments disclosed herein, and/or a reagent of any of the embodiments disclosed herein. In some embodiments, a system is configured to iteratively perform the sequential contacting of tethered, primed nucleic acid molecules with said nucleic acid binding or incorporation compositions and/or said reagents; and for the detection of binding or incorporation of the disclosed nucleic acid binding or incorporation compositions to the one or more primed nucleic acid molecules.

[0015] In some embodiments, the present disclosure provides a composition comprising a particle (*e.g.*, a nanoparticle or polymer core), said particle comprising a plurality of enzyme or protein binding or incorporation substrates, wherein the enzyme or protein binding or incorporation substrates bind with one or more enzymes or proteins to form one or more binding or incorporation complexes (*e.g.*, a multivalent binding or incorporation complex), and wherein said binding or incorporation may be monitored or identified by observation of the location, presence, or persistence of the one or more binding or incorporation complexes. In some embodiments, said particle may comprise a polymer, branched polymer, dendrimer, liposome, micelle, nanoparticle, or quantum dot. In some embodiments, said substrate may comprise a nucleotide, a nucleoside, a nucleotide analog, or a nucleoside analog. In some embodiments, the enzyme or protein binding or incorporation substrate may comprise an agent that can bind with a polymerase. In some embodiments, the enzyme or protein may comprise a polymerase. In some embodiments, said observation of the location, presence, or persistence of one or more binding or incorporation complexes may comprise fluorescence detection. In some embodiments, the present disclosure provides a composition comprising multiple distinct particles as disclosed herein, wherein each particle comprises a single type of nucleoside or nucleoside analog, and wherein each nucleoside or nucleoside analog is associated with a fluorescent label of a detectably different emission or excitation wavelength. In some embodiments, the present disclosure provides said composition further comprising one or more labels, *e.g.*, fluorescence labels, on the particle. In some embodiments, the present disclosure provides said composition wherein the composition comprises at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, or more than 20 tethered nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs tethered to the particle. In some embodiments, the present disclosure provides said composition wherein the nucleoside or nucleoside analog is present at a surface density of between 0.001 and 1,000,000 per μm^2, between 0.01 and 1,000,000 per $\mu m^{2,}$, between 0.1 and 1,000,000 per μm^2, between 1 and 1,000,000 per μm^2, between 10 and 1,000,000 per μm^2, between 100 and 1,000,000 per μm^2, between 1,000 and 1,000,000 per μm^2, between 1,000 and 100,000 per μm^2, between 10,000 and 100,000 per μm^2, or between 50,000 and 100,000 per μm^2, or within a range defined by any two of the foregoing

values. In some embodiments, the present disclosure provides said composition wherein the nucleoside or nucleoside analog is present within a nucleotide or nucleotide analog. In some embodiments, the present disclosure provides said composition wherein the composition comprises or incorporates a nucleotide or nucleotide analog that is modified so as to prevent its incorporation into an extending nucleic acid chain during a polymerase reaction. In some embodiments, the present disclosure provides said composition wherein the composition comprises or incorporates a nucleotide or nucleotide analog that is reversibly modified so as to prevent its incorporation into an extending nucleic acid chain during a polymerase reaction. In some embodiments, the present disclosure provides said composition wherein one or more labels comprise a fluorescent label, a FRET donor, and/or a FRET acceptor. In some embodiments, the present disclosure provides said composition wherein the substrate (*e.g.*, nucleotide, nucleotide analog, nucleoside, or nucleoside analog) is attached to the particle through a linker. In some embodiments, the present disclosure provides said composition wherein at least one nucleotide or nucleotide analog is a nucleotide that has been modified to inhibit elongation during a polymerase reaction or a sequencing reaction, such as, for example, a nucleotide that lacks a 3' hydroxyl group; a nucleotide that has been modified to contain a blocking group at the 3' position; a nucleotide that has been modified with a 3'-O-azido group, a 3'-O-azidomethyl group, , a 3'-O-alkyl hydroxylamino group, a 3'-phosphorothioate group, a 3'-O-malonyl group, or a 3'-O-benzyl group; and/or a nucleotide that has not been modified at the 3' position.

[0016] In some embodiments, the present disclosure provides a method of determining the sequence of a nucleic acid molecule comprising the steps, without regard to order, of 1) providing a nucleic acid molecule comprising a template strand and a complementary strand that is at least partially complementary to the template strand; 2) contacting the nucleic acid molecule with the one or more nucleic acid binding or incorporation composition according to any of the embodiments disclosed herein; 3) detecting binding or incorporation of the nucleic acid binding or incorporation composition to the nucleic acid molecule, and 4) determining an identity of a terminal nucleotide to be incorporated into said complementary strand of said nucleic acid molecule. In some embodiments, said method may further comprise incorporating said terminal nucleotide into said complementary strand, and repeating said contacting, detecting, and incorporating steps for one or more additional iterations, thereby determining the sequence of said template strand of said nucleic acid molecule. In some embodiments, the present disclosure provides said method wherein said nucleic acid molecule has been clonally-amplified on a solid support. In some embodiments, the present disclosure provides said method wherein the clonal amplification comprises the use of a polymerase chain reaction (PCR), multiple displacement amplification (MDA), transcription-mediated amplification

(TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, bridge amplification, isothermal bridge amplification, rolling circle amplification, circle-to-circle amplification, helicase-dependent amplification, recombinase-dependent amplification, single-stranded binding (SSB) protein-dependent amplification, or any combination thereof. In some embodiments, the present disclosure provides said method wherein a sequencing reaction cycle comprising the contacting, detecting, and incorporating steps is performed in less than 30 minutes, less than 20 minutes, or in less than 10 minutes. In some embodiments, the present disclosure provides said method wherein an average Q-score for base calling accuracy over a sequencing run is greater than or equal to 30, or greater than or equal to 40. In some embodiments, the present disclosure provides said method wherein at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the terminal nucleotides identified have a Q-score of greater than 30; or greater than 40. In some embodiments, the present disclosure provides said method wherein at least 95% of the terminal nucleotides identified have a Q-score of greater than 30.

[0017] In some embodiments, the present disclosure provides a reagent comprising one or more nucleic acid binding or incorporation compositions as disclosed herein, and a buffer. In some embodiments, the present disclosure provides said reagent, wherein said reagent comprises 1, 2, 3, 4, or more nucleic acid binding or incorporation compositions, wherein each nucleic acid binding or incorporation composition comprises a single type of nucleotide or nucleotide analog, and wherein said nucleotide or nucleotide analog comprises a nucleotide, nucleotide analog, nucleoside, or nucleoside analog. In some embodiments, the present disclosure provides said method wherein said reagent comprises 1, 2, 3, 4, or more nucleic acid binding or incorporation compositions, wherein each nucleic acid binding or incorporation composition comprises a single type of nucleotide or nucleotide analog, and wherein said nucleotide or nucleotide analog may respectively correspond to one or more from the group consisting of ATP, ADP, AMP, dATP, dADP, and dAMP; one or more from the group consisting of TTP, TDP, TMP, dTTP, dTDP, dTMP, UTP, UDP, UMP, dUTP, dUDP, and dUMP; one or more from the group consisting of CTP, CDP, CMP, dCTP, dCDP, and dCMP; and one or more from the group consisting of GTP, GDP, GMP, dGTP, dGDP, and dGMP. In some embodiments, the present disclosure provides said method wherein said reagent comprises 1, 2, 3, 4, or more nucleic acid binding or incorporation compositions, wherein each nucleic acid binding or incorporation composition comprises a single type of nucleotide or nucleotide analog, and wherein said nucleotide or nucleotide analog may respectively correspond to one or more from the group consisting of ATP, ADP, AMP, dATP, dADP, dAMP TTP, TDP, TMP, dTTP, dTDP, dTMP, UTP, UDP, UMP, dUTP, dUDP, dUMP, CTP, CDP, CMP, dCTP, dCDP, dCMP, GTP, GDP, GMP, dGTP, dGDP, and dGMP.

[0018] In some embodiments, the present disclosure provides a kit comprising any of the compositions disclosed herein; and/or any of the reagents disclosed herein; one or more buffers; and instructions for the use thereof.

[0019] In some embodiments, the present disclosure provides a system for performing any of the methods disclosed herein; wherein said methods may comprise use of any of the compositions as disclosed herein; and/or any of the reagents as disclosed herein; one or more buffers, and one or more nucleic acid molecules optionally tethered or attached to a solid support, wherein said system is configured to iteratively perform for the sequential contacting of said nucleic acid molecules with said composition and/or said reagent; and for the detection of binding or incorporation of the nucleic acid binding or incorporation compositions to the one or more nucleic acid molecules.

[0020] In some embodiments, the present disclosure provides a composition as disclosed herein for use in increasing the contrast to noise ratio (CNR) of a labeled nucleic acid complex bound to or associated with a surface.

[0021] In some embodiments, the present disclosure provides a composition as disclosed herein for use in establishing or maintaining control over the persistence time of a signal from a labeled nucleic acid complex bound to or associated with a surface.

[0022] In some embodiments, the present disclosure provides a composition as disclosed herein for use in establishing or maintaining control over the persistence time of a fluorescence, luminescence, electrical, electrochemical, colorimetric, radioactive, magnetic, or electromagnetic signal from a labeled nucleic acid complex bound to or associated with a surface.

[0023] In some embodiments, the present disclosure provides a composition as disclosed herein for use in increasing the specificity, accuracy, or read length of a nucleic acid sequencing and/or genotyping application.

[0024] In some embodiments, the present disclosure provides a composition as disclosed herein for use in increasing the specificity, accuracy, or read length in a sequencing by binding or incorporation, sequencing by synthesis, single molecule sequencing, or ensemble sequencing method.

[0025] In some embodiments, the present disclosure provides a reagent as disclosed herein for use in increasing the contrast to noise ratio (CNR) of a labeled nucleic acid complex bound to or associated with a surface.

[0026] In some embodiments, the present disclosure provides a reagent as disclosed herein for use in establishing or maintaining control over the persistence time of a signal from a labeled nucleic acid complex bound to or associated with a surface.

[0027] In some embodiments, the present disclosure provides a reagent as disclosed herein for use in establishing or maintaining control over the persistence time of a fluorescence, luminescence,

electrical, electrochemical, colorimetric, radioactive, magnetic, or electromagnetic signal from a labeled nucleic acid complex bound to or associated with a surface.

[0028] In some embodiments, the present disclosure provides a reagent as disclosed herein for use in increasing the specificity, accuracy, or read length of a nucleic acid sequencing and/or genotyping application.

[0029] In some embodiments, the present disclosure provides a reagent as disclosed herein for use in increasing the specificity, accuracy, or read length in a sequencing by binding or incorporation, sequencing by synthesis, single molecule sequencing, or ensemble sequencing method.

INCORPORATION BY REFERENCE

[0030] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference in its entirety. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0032] The novel features of the inventive concepts disclosed herein are set forth with particularity in the appended claims. A better understanding of the features and advantages of the disclosed compositions, methods, and systems will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the inventive concepts are utilized, and the accompanying drawings of which:

[0033] FIGS. 1A-1H illustrate the steps utilizing a non-limiting examples of multivalent binding composition for sequencing a target nucleic acid: **FIG. 1A** illustrates a non-limiting example 4-of attaching target nucleic acid to a surface; **FIG. 1B** illustrates clonally the target nucleic acid to form clusters of amplified target nucleic acid molecules; **FIG. 1C** illustrates a non-limiting example of priming the target nucleic acid to produce a primed target nucleic acid; **FIG. 1D** illustrates a non-limiting example of contacting the primed target nucleic acid to the multivalent binding composition and polymerase to form a binding complex; **FIG. 1E** illustrates a non-limiting example of the images of the binding complex captured on the surface; **FIG. 1F** illustrates a non-limiting example of extending the primer strand by one nucleotide; **FIG. 1G** illustrates a non-limiting example of another cycle of contacting the primed target nucleic acid to the multivalent binding composition and

polymerase to form a binding complex; and **FIG. 1H** illustrates non-limiting examples of the images of binding complex captured on the surface in subsequent sequencing cycles.

[0034] FIG. 2 shows a flow chart outlining the steps for sequencing a target nucleic acid and extending the primer strand through a single base addition.

[0035] FIG. 3 shows a flow chart outlining the steps for sequencing a target nucleic acid and extending the primer strand through incorporating the nucleotide on the particle-nucleotide conjugate.

[0036] FIGS. 4A-4B illustrate a non-limiting example of detecting target nucleic acid using the polymer-nucleotide conjugates. **FIG. 4A** shows the step of contacting the polymerase and polymer-nucleotide conjugates to some nucleic acid molecules; **FIG 4B** shows the binding complex formed between the polymerase, polymer-nucleotide conjugates, and the target nucleic acid molecules.

[0037] FIGS. 5A-5C show schematic representations of non-limiting examples of varying configurations of the polymer-nucleotide conjugates: **FIG. 5A** shows polymer-nucleotide conjugates having various multi-arm configurations; **FIG. 5B** shows a polymer-nucleotide conjugate having the polymer branch radiating from the center; and **FIG. 5C** shows polymer-nucleotide conjugates having the binding moiety biotin.

[0038] FIG. 6 shows a generalized graphical depiction of the increase in signal intensity that has been observed during binding, persistence, and washing and removal of multivalent substrates.

[0039] FIGS. 7A-7J show fluorescence images of the steps in a sequencing reaction using multivalent PEG-substrate compositions. **FIG. 7A**. Red and green fluorescent images post exposure of DNA RCA templates (G and A first base) to 500 nM base labeled nucleotides (A-Cy3 and G-Cy5) in exposure buffer containing 20 nM Klenow polymerase and 2.5 mM Sr^{+2}. Images were collected after washing with imaging buffer with the same composition as the exposure buffer but containing no nucleotides or polymerase. Contrast was scaled to maximize visualization of the dimmest signals, but no signals persisted following washing with imaging buffer (**FIG. 7A**, inset). **FIGS. 7B-7E**: fluorescence images showing multivalent PEG-nucleotide (base-labeled) ligands PB1 (**FIG. 7B**), PB2 (**FIG. 7C**), PB3 (**FIG. 7D**), and PB5 (**FIG. 7E**) having an effective nucleotide concentration of 500 nM after mixing in the exposure buffer and imaging in the imaging buffer as described above. **FIG. 7F**: fluorescence image showing multivalent PEG-nucleotide (base-labeled) ligand PB5 at 2.5uM after mixing in the exposure buffer and imaging in the imaging buffer as above. **FIGS. 7G-7I**: Fluorescence images showing further base discrimination by exposure of the multivalent binding composition to inactive mutants of klenow polymerase (**FIG. 7G**. D882H; **FIG. 7H**. D882E; **FIG. 7I**. D882A) vs. the wild type Klenow (control) enzyme (**FIG. 7J**).

[0040] FIGS. 8A-8B show the efficacy of the multivalent reporter compositions in determining the base sequence of a DNA sequence over 5 sequencing cycles: **FIG. 8A** shows images and expected

sequences for templates taken after each sequencing cycle; and **FIG. 8B** shows aligned sequencing results utilizing the images taken in **FIG. 8A**.

[0041] FIGS. 9A-9J show fluorescence images of multivalent polyethylene glycol (PEG) polymer-nucleotide (base-labeled) conjugates, having an effective nucleotide concentration of 500 nM and varying PEG branch length, after contacting to a support surface comprising DNA templates (comprising G or A as the first base; prepared using rolling circle amplification (RCA)) in an exposure buffer comprising 20 nM Klenow polymerase and 2.5 mM Sr^{+2}. Images were acquired after washing with an imaging buffer having the same composition as the exposure buffer but lacking nucleotides and polymeras. Panels show images obtained using multivalent PEG-nucleotide ligands with arm lengths as follows. **FIG. 9A**: 1K PEG. **FIG. 9B**: 2K PEG. **FIG. 9C**: 3K PEG. **FIG. 9D**: 5K PEG. **FIG. 9E**: 10K PEG. **FIG. 9F**: 20K PEG. **FIG. 9G** shows images obtained using 10K PEG and an inactive klenow polymerase comprising the mutation D882H. **FIG. 7H** shows images obtained using 10K PEG and an inactive klenow polymerase comprising the mutation D882E. **FIG. 7I** shows images obtained using 10K PEG and an inactive klenow polymerase comprising the mutation D882A. **FIG. 7J** shows images obtained using 10K PEG and an active wild type klenow polymerase.

[0042] FIG. 10 shows a quantitative representation of the fluorescence intensities in the images shown in **FIGS. 9A-9F,** separated by color value, with orange trace corresponding to the red label (Cy3 label; A bases) and blue trace corresponding to the green label (Cy5 label; G bases).

[0043] FIG. 11 shows normalized fluorescence from multivalent substrates bound to DNA clusters as described for **FIGS. 7A-7J**, with the substrate complexes formed in the presence (condition B) and absence (condition A) of Triton-X100 (0.016%).

[0044] FIGS. 12A-12B show plots of normalized fluorescence intensity measured for multivalent polymer-nucleotide conjugates and free nucleotides. **FIG. 12A**: Two replicates of a multivalent polymer-nucleotide conjugate bound to DNA clusters (Conditions A and B) vs. binding complexes formed using labeled free nucleotides (Condition C) after 1 minute; **FIG. 12B**: Time course of fluorescence from multivalent substrate complexes over the course of 60 min.

DETAILED DESCRIPTION

I. Definitions

[0045] As used herein, “nucleic acid” (also referred to as a “polynucleotide”, “oligonucleotide”, ribonucleic acid (RNA), or deoxyribonucleic acid (DNA)) is a linear polymer of two or more nucleotides joined by covalent internucleosidic linkages, or variants or functional fragments thereof. In naturally occurring examples of nucleic acids, the internucleoside linkage is a phosphodiester bond. However, other examples optionally comprise other internucleoside linkages, such as

phosphorothiolate linkages and may or may not comprise a phosphate group. Nucleic acids include double- and single-stranded DNA, as well as double- and single-stranded RNA, DNA/RNA hybrids, peptide-nucleic acids (PNAs), hybrids between PNAs and DNA or RNA, and may also include other types of nucleic acid modifications.

[0046] As used herein, a "nucleotide" refers to a nucleotide, nucleoside, or analog thereof. The nucleotide refers to both naturally occurring and chemically modified nucleotides and can include but are not limited to a nucleoside, a ribonucleotide, a deoxyribonucleotide, a protein-nucleic acid residue, or derivatives. Examples of the nucleotide includes an adenine, a thymine, a uracil, a cytosine, a guanine, or residue thereof; a deoxyadenine, a deoxythymine, a deoxyuracil, a deoxycytosine, a deoxyguanine, or residue thereof; a adenine PNA, a thymine PNA, a uracil PNA, a cytosine PNA, a guanine PNA, or residue or equivalents thereof, an N- or C-glycoside of a purine or pyrimidine base (*e.g.*, a deoxyribonucleoside containing 2-deoxy-D-ribose or ribonucleoside containing D-ribose).

[0047] "Complementary," as used herein, refers to the topological compatibility or matching together of interacting surfaces of a ligand molecule and its receptor. Thus, the receptor and its ligand can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other.

[0048] "Branched polymer", as used herein, refers to a polymer having a plurality of functional groups that help conjugate a biologically active molecule such as a nucleotide, and the functional group can be either on the side chain of the polymer or directly attaches to a central core or central backbone of the polymer. The branched polymer can have linear backbone with one or more functional groups coming off the backbone for conjugation. The branched polymer can also be a polymer having one or more sidechains, wherein the side chain has a site suitable for conjugation. Examples of the functional group include but are limited to hydroxyl, ester, amine, carbonate, acetal, aldehyde, aldehyde hydrate, alkenyl, acrylate, methacrylate, acrylamide, active sulfone, hydrazide, thiol, alkanoic acid, acid halide, isocyanate, isothiocyanate, maleimide, vinylsulfone, dithiopyridine, vinylpyridine, iodoacetamide, epoxide, glyoxal, dione, mesylate, tosylate, and tresylate.

[0049] "Polymerase," as used herein, refers to an enzyme that contains a nucleotide binding moiety and helps formation of a binding complex between a target nucleic acid and a complementary nucleotide. The polymerase can have one or more activities including, but not limited to, base analog detection activities, DNA polymerization activity, reverse transcriptase activity, DNA binding or incorporation, strand displacement activity, and nucleotide binding or incorporation and recognition. The polymerase can include catalytically inactive polymerase, catalytically active polymerase, reverse transcriptase, and other enzymes containing a nucleotide binding or incorporation moiety.

[0050] "Persistence time," as used herein, refers to the length of time that a binding complex, which is formed between the target nucleic acid, a polymerase, a conjugated or unconjugated nucleotide, remains stable without any binding component dissociates from the binding complex. The persistence time is indicative of the stability of the binding complex and strength of the binding interactions. Persistence time can be measured by observing the onset and/or duration of a binding complex, such as by observing a signal from a labeled component of the binding complex. For example, a labeled nucleotide or a labeled reagent comprising one or more nucleotides may be present in a binding complex, thus allowing the signal from the label to be detected during the persistence time of the binding complex. One non-limiting example of label is a fluorescent label.

II. Method of Analyzing Target Nucleic Acid

[0051] Disclosed herein are multivalent binding or incorporation compositions and their use in analyzing nucleic acid molecules, including in sequencing or other bioassay applications. An increase in binding or incorporation of a nucleotide to an enzyme (*e.g.*, polymerase) or an enzyme complex can be affected by increasing the effective concentration of the nucleotide. The increase can be achieved by increasing the concentration of the nucleotide in free solution, or by increasing the amount of the nucleotide in proximity to the relevant binding or incorporation site. The increase can also be achieved by physically restricting a number of nucleotides into a limited volume thus resulting in a local increase in concentration, and such as structure may thus bind or incorporate to the binding or incorporation site with a higher apparent avidity than would be observed with unconjugated, untethered, or otherwise unrestricted individual nucleotide. One non-limiting means of effecting such restriction is by providing a multivalent binding or incorporation composition in which multiple nucleotides are bound to a particle such as a polymer, a branched polymer, a dendrimer, a micelle, a liposome, a microparticle, a nanoparticle, a quantum dot, or other suitable particle known in the art.

[0052] The multivalent binding or incorporation composition disclosed herein can include at least one particle-nucleotide conjugate, and the particle-nucleotide conjugate has a plurality of copies of the same nucleotide attached to the particle. When the nucleotide is complementary to the target nucleic acid, the particle-nucleotide conjugate forms a binding or incorporation complex with the polymerase and the target nucleic acid, and the binding or incorporation complex exhibits increased stability and longer persistence time than the binding or incorporation complex formed using a single unconjugated or untethered nucleotide. Each of the nucleotide moieties of the multivalent binding composition may bind to a complementary N+1 nucleotide of a primed target nucleic acid molecule, thereby forming a multivalent binding complex comprising two or more target nucleic acid molecules, two or more polymerase (or other enzyme) molecules, and the multivalent binding composition (*e.g.*, the polymer-nucleotide conjugate). Each of the nucleotide moieties of the multivalent binding

composition may bind to a complementary N nucleotide of a primed target nucleic acid molecule, thereby forming a multivalent binding complex comprising two or more target nucleic acid molecules, two or more polymerase (or other enzyme) molecules, and the multivalent binding composition (*e.g.*, the polymer-nucleotide conjugate). From this bound complex the nucleotide can interrogate the complementary base prior to incorporation of a modified reversibly blocked nucleotide that elongates the replicating strand by 1 base. In addition, it is possible to imagine interrogation of the N nucleotide with a bound complex, stepping forward with a reversibly terminated nucleotide, and subsequently probing the N+1 base to pre and post deblocking. In this way you could perform error checking and improve the overall accuracy of base-calling by reading the interrogated twice. The important discriminating factor from traditional methods is the binding is used to interrogate the matched base, while the stepping or incorporation step is used only to move forward on the elongating strand.

[0053] The multivalent binding or incorporation composition can be used to localize detectable signals to active regions of biochemical interactions, such as sites of protein-nucleic acid interactions, nucleic acid hybridization reactions, or enzymatic reactions, such as polymerase reactions. For instance, the multivalent binding or incorporation composition described herein can be utilized to identify sites of base incorporation in elongating nucleic acid chains during polymerase reactions and to provide base discrimination for sequencing and array-based applications. The increased binding or incorporation between the target nucleic acid and the nucleotide in the multivalent binding or incorporation composition, when the nucleotide is complementary to the target nucleic acid, provides enhanced signal that greatly improve base call accuracy and shorten imaging time.

[0054] In addition, the use of multivalent binding composition allows sequencing signals from a given sequence to originate within cluster regions containing multiple copies of the target sequence. Sequencing methods incorporating multiple copies of a target sequence have the advantage that signals can be amplified due to the presence of multiple simultaneous sequencing reactions within the defined region, each providing its own signal. The presence of multiple signals within a defined area also reduces the impact of any single skipped cycle, due to the fact that the signal from a large number of correct base calls can overwhelm the signal from a smaller number of skipped or incorrect base calls, therefore providing methods for reducing phasing errors and/or to improve read length in sequencing reactions.

[0055] The multivalent binding compositions and their use disclosed herein lead to one or more of: (i) stronger signal for better base-calling accuracy compared to conventional nucleic acid amplification and sequencing methodologies; ii) allow greater discrimination of sequence-specific signal from background signals; (iii) reduced requirements for the amount of starting material

necessary, (iv) increased sequencing rate and shortened sequencing time; (v) reducing phasing errors, and (vi) improving read length in sequencing reactions.

[0056] In some embodiments, the target nucleic acid can refer to a target nucleic acid sample having one or more nucleic acid molecules. In some embodiments, the target nucleic acid can include a plurality of nucleic acid molecules. In some embodiments, the target nucleic acid can include two or more nucleic acid molecules. In some embodiments, the target nucleic acid can include two or more nucleic acid molecules having the same sequences.

A. Sequencing Target Nucleic Acid

[0057] **FIG. 1A-1H** illustrate one exemplified method in which the multivalent binding composition is used for sequencing a target nuclei acid. As shown in **FIG. 1A**, the target nucleic acid 102 can be tethered to a solid support surface 101. The target nucleic acid can be attached to the surface either directly or indirectly. Although not shown in **FIG. 1A**, the target nucleic acid 102 can be hybridized to an adapter, which is attached to the surface through a covalent or noncovalent bond. When one or more adapters are used to attach the target nucleic acid to the surface, the target surface can comprise a fragment that is complementary to the adapter and thus hybridize to the adaptor. In some instances, one adapter sequence may be tethered to the surface. In some instances, a plurality of adapter sequences may be tethered to the surface. In some instances, the target nucleic acid 102 can also be attached directly to the solid-support surface without the use of an adapter. The solid support can be a low non-specific binding surface.

[0058] In **FIG. 1B**, after the initial step of attaching the target nucleic acid to the surface of a solid support surface (*e.g.*, through hybridization to adapters), the target nucleic acid is then clonally-amplified to form clusters of amplified nucleic acids. When the target nucleic acid is attached to the surface through an adapter, the surface density of clonally-amplified nucleic acid sequences hybridized to adapter on the support surface may span the same range as the surface density of tethered adapters (or primers). The clonal amplification may be performed using a polymerase chain reaction (PCR), multiple displacement amplification (MDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), real-time SDA, bridge amplification, isothermal bridge amplification, rolling circle amplification, circle-to-circle amplification, helicase-dependent amplification, recombinase-dependent amplification, single-stranded binding (SSB) protein-dependent amplification, or any combination thereof.

[0059] **FIG. 1C** illustrates a non-limiting step of annealing a primer 103 to the target nucleic acid 102 to form a primed target nucleic acid 104. **FIG. 1B** only shows one primer being used in the

annealing step, but more than one primer can be used depending on the types of target nucleic acid. In some instances, the adapter that is used to attach the target nucleic acid to the surface has the same sequence as the primer used to prepare the primed target nucleic acid. The primer may comprise forward amplification primers, reverse amplification primers, sequencing primers, and/or molecular barcoding sequences, or any combination thereof. In some instances, one primer sequence may be used in the hybridization step. In some instances, a plurality of different primer sequences may be used in the hybridization step.

[0060] As shown in **FIG. 1D**, the primed target nucleic acid 104 is combined with a multivalent binding or incorporation composition and a polymerase 106 to form a binding or incorporation complex. The non-limiting example of multivalent binding or incorporation composition in **FIG. 1D** comprises four particle-nucleotide conjugates 105a, 105b, 105c, and 105d. Each particle-nucleotide conjugate has multiple copies of a nucleotide attached to the particle, and the four particle-nucleotide conjugates cover four types of nucleotide respectively. The particle-nucleotide conjugate having a nucleotide that is complementary to the next base on the primed target nucleic acid will form a binding or incorporation complex with the polymerase and the target nucleic acid. In some instances, the multivalent binding or incorporation composition may include one, two or three particle-nucleotide conjugates. In some embodiments, each different type of particle-nucleotide conjugate can be labeled with a separate label. In some embodiments, three of four types of nucleotide conjugates can be labeled, with a fourth either unlabeled or conjugated to an undetectable label. In some embodiments, 1, 2, 3, or 4 particle-nucleotide conjugates can be labeled, either with the same label, or each with a label corresponding to the identity of its conjugated nucleotide, with, respectively, 3, 2, 1, or no particle-nucleotide conjugates that may be either left unlabeled or conjugated to an undetectable label. In some embodiments, detection of a polymerase complex incorporating a particle-nucleotide conjugate may be carried out using four-color detection, such that conjugates corresponding to all four nucleotides are present in a sample, each conjugate having a separate label corresponding to the nucleotide conjugated thereto. In some embodiments, the four particle-nucleotide conjugates may be exposed to or contacted with the target nucleic acid at the same time; in some other embodiments, the four particle-nucleotide conjugates may be exposed to or contacted with the target nucleic acid sequentially, either individually, or in groups of two or three. In some embodiments, detection of a polymerase complex incorporating a particle-nucleotide conjugate may be carried out using three-color detection, such that conjugates corresponding to three of the four nucleotides are present in a sample, with three conjugates having a separate label corresponding to the nucleotide conjugated thereto and one conjugate having no label or being conjugated to an undetectable label. In some embodiments, only three types of conjugates are

provided, such that conjugates corresponding to three of the four nucleotides are present in a sample, with three conjugates having a separate label corresponding to the nucleotide conjugated thereto and one conjugate being absent. In some embodiments, the identity of nucleotides corresponding to an unlabeled or absent nucleotide conjugate can be determined with respect to the location and/or identity of "dark" spots or locations of known target nucleic acids showing no fluorescence signal. In some embodiments, the present disclosure provides said method, wherein the detection of the binding or incorporation complex is performed in the absence of unbound or solution-borne polymer nucleotide conjugates.

[0061] In some embodiments where three of the four particle-nucleotide conjugates are labeled, or where only three of the four particle-nucleotide conjugates are present, the identity of the nucleotide corresponding to the unlabeled or absent conjugate may be established by the absence of a signal or by monitoring of the presence of unlabeled complexes such as by the identification of "dark" spots or unlabeled regions in a sequencing reaction. In some embodiments, detection of a polymerase complex incorporating a particle-nucleotide conjugate may be carried out using two-color detection, such that conjugates corresponding to two of the four nucleotides are present in a sample, with two conjugates having a separate label corresponding to the nucleotide conjugated thereto and two conjugates having no label or being conjugated to an undetectable label. In some embodiments, only two of the four particle-nucleotide conjugates are labeled. In some embodiments where two of the four particle-nucleotide conjugates are labeled, the identity of the nucleotide corresponding to the unlabeled conjugate or conjugates may be established by the absence of a signal or by monitoring of the presence of unlabeled complexes such as by the identification of "dark" spots or unlabeled regions in a sequencing reaction. In some embodiments where two of the four particle-nucleotide conjugates are labeled, the four particle-nucleotide conjugates may be exposed to or contacted with the target nucleic acid sequentially, either individually, or in groups of two or three. In some embodiments two of the four particle-nucleotide conjugates may share a common label, and the four particle-nucleotide conjugates may be exposed to or contacted with the target nucleic acid sequentially, either individually, or in groups of two or three, wherein each contacting step shows the distinction between two or more different bases, such that after two, three, four, or more such contacting steps the identities of all unknown bases have been determined.

[0062] FIG. 1E illustrates the images captured on the surface after the binding or incorporation complex is formed between the polymerase, the target nucleic acid, and the particle-nucleotide conjugate having a nucleotide commentary to the next base of the primed target nucleic acid. The captured image includes four binding or incorporation complexes 107a, 107b, 107c, and 107d formed on the surface, and each binding or incorporation complex has a different nucleotide which can be

distinguished based on the label (*e.g.*, fluorescence emission color) on the particle-nucleotide conjugate. Because use of the particle-nucleotide conjugate allows binding or incorporation signals from a given sequence to originate within cluster regions containing multiple copies of the target sequence, the sequencing signals is greatly enhanced. Although **FIG. 1E** involves four particle-nucleotide conjugates, each having a different type of nucleotide, some methods can use one, two, or three particle-nucleotide conjugates, each having a different type of nucleotide and label. In some embodiments, each different type of particle-nucleotide conjugate can be labeled either with the same label, or each with a label corresponding to the identity of its conjugated nucleotide. In some embodiments, three of four types of nucleotide conjugates can be labeled, with a fourth either unlabeled or conjugated to an undetectable label. In some embodiments, 1, 2, 3, or 4 particle-nucleotide conjugates can be labeled with a separate label, with, respectively, 3, 2, 1, or no particle-nucleotide conjugates either unlabeled or conjugated to an undetectable label In some embodiments, a detection step can comprise simultaneous and/or serial excitation of up to 4 different excitation wavelengths, such as wherein the fluorescence imaging is carried out by detecting single and/or multiple fluorescence emission bands that uniquely classify each of the possible base pairing (A, G, C, or T). In some embodiments, four different nucleic acid binding or incorporation compositions, each comprising a different nucleotide or nucleotide analog, may be used to determine the identity of the terminal nucleotide, wherein one of the four different nucleic acid binding or incorporation compositions is labeled with a first fluorophore, one is labeled with a second fluorophore, one is labeled with both the first and second fluorophore, and one is not labeled, and wherein the detecting step comprises simultaneous excitation at a first excitation wavelength and a second excitation wavelength and images are acquired at a first fluorescence emission wavelength and a second fluorescence emission wavelength.

[0063] When the multivalent binding or incorporation composition is used in replacement of single unconjugated or untethered nucleotides to form a binding or incorporation complex with the polymerase and the primed target nucleic acid, the local concentration of the nucleotide is increased many-fold, which in turn enhances the signal intensity. The formed binding or incorporation complex also has a longer persistence time which in turn helps shorten the imaging step. The high signal intensity results from the high binding or incorporation avidity of the polymer nucleotide conjugate (which may also comprise multiple fluorophores or other labels) which thus forms a complex which remains stable for the entire binding or incorporation and imaging step. The strong binding or incorporation between the polymerase, the primed target strand, and the polymer-nucleotide or nucleotide analog conjugate also means that the multivalent binding or incorporation complex thus formed will remain stable during washing steps, and the signal intensity will remain high when other

reaction mixture components and unmatched nucleotide analogs are washed away. After the imaging step, the binding or incorporation complex can be destabilized (*e.g.*, by changing the buffer composition) and the primed target nucleic acid can then be extended for one base.

[0064] The sequencing method may further comprise incorporating the N+1 or terminal nucleotide into the primed strand as shown in **FIG. 1F**. In **FIG. 1F**, the primer strand of the primed target nucleic acid 108 can be extended for one base to form an extended nucleic acid 109. The extension step can occur after or concurrently with the destabilization of the multivalent binding or incorporation complex. The primed target nucleic acid 108 can be extended using a complementary nucleotide that is attached to the particle in the particle-nucleotide conjugate or using an unconjugated or untethered free nucleotide that is provided after the multivalent binding or incorporation composition has been removed.

[0065] After the extension step, the contacting step as shown in **FIG. 1G** can be performed again to form binding or incorporation complexes and imitate the next sequencing cycle. The contacting, detecting, and extension steps can be repeated for one or more cycles, thereby determining the sequence of the target nucleic acid molecule. For example, **FIG. 1H** illustrates the surface images obtained after performing multiple sequencing cycles, and the images can then be processed to determine the sequences of the target nucleic acid molecules.

[0066] The extension of the primed target nucleic acid may be prevented or inhibited due to a blocked nucleotide on the strand or the use of polymerase that is catalytically inactive. When the nucleotide in the polymer-nucleotide conjugate has a blocking group that prevents the extension of the nucleic acid, incorporation of a nucleotide may be achieved by the removal of a blocking group from said nucleotide (such as by detachment of said nucleotide from its polymer, branched polymer, dendrimer, particle, or the like). When the extension of the primed target nucleic acid is inhibited due to the use of polymerase that is catalytically inactive, incorporation of a nucleotide may be achieved by the provision of a cofactor or activator such as a metal ion.

[0067] Also disclosed herein are systems configured for performing any of the disclosed nucleic acid sequencing or nucleic acid analysis methods. The system may comprise a fluid flow controller and/or fluid dispensing system configured to sequentially and iteratively contact the primed target nucleic acid molecules attached to a solid support with the disclosed polymerase and multivalent binding or incorporation compositions and/or reagents. The contacting may be performed within one or more flow cells. In some instances, said flow cells may be fixed components of the system. In some instances, said flow cells may be removable and/or disposable components of the system.

[0068] The sequencing system may include an imaging module, *i.e.*, one or more light sources, one or more optical components, and one or more image sensors for imaging and detection of binding or

incorporation of the disclosed nucleic acid binding or incorporation compositions to target nucleic acid molecules tethered to a solid support or the interior of a flow cell. The disclosed compositions, reagents, and methods may be used for any of a variety of nucleic acid sequencing and analysis applications. Examples include, but are not limited to, DNA sequencing, RNA sequencing, whole genome sequencing, targeted sequencing, exome sequencing, genotyping, and the like.

[0069] The sequencing system may also include computer control systems that are programmed to implement methods of the disclosure. The computer system is programmed or otherwise configured to implement methods of the disclosure including nucleic acid sequencing methods, interpretation of nucleic acid sequencing data and analysis of cellular nucleic acids, such as RNA (*e.g.*, mRNA), and characterization of cells from sequencing data. The computer system can be an electronic device of a user or a computer system that is remotely located with respect to the electronic device. The electronic device can be a mobile electronic device.

[0070] FIG.2 is a flowchart outlining the steps in sequencing a target nucleic acid. 201 describes a step of attaching target library sequences to a solid support surface by hybridizing the target nucleic acid molecules to complementary adapters on substrate surface. The target nucleic acid molecules can be single stranded or partially double stranded. Prior to 201, the nucleic acid molecules in the target library may have been prepared to contain fragments complementary to the adaptor sequences through ligation or other methods. 202 describes the step of clonal amplification to generate clusters of target nucleic acid molecules on the surface. 203 describes hybridizing sequencing primers to complementary primer binding or incorporation sequences on the target nucleic acid to form the primed target nucleic acid. 204 describes combining the polymerase, the multivalent binding or incorporation composition, which contains labeled (*e.g.*, fluorescently-labeled) particle-nucleotide conjugates, and the primed target nucleic acid. 204 may also include a step of washing or removing the unbound reagents including polymerase and particle-nucleotide conjugate.

[0071] Again referring to **FIG. 2**, when the nucleotide on the particle-nucleotide conjugate is complementary to the next base of the primed target nucleic acid (205), the particle-nucleotide conjugate, polymerase, and primed target nucleic acid form a ternary binding or incorporation complex, which can be detected by detection methods (*e.g.*, florescence imaging) compatible with the label on the particle-nucleotide conjugate. 205 can also include measuring the persistence time of the ternary binding or incorporation complex. In 206, the binding or incorporation complex is destabilized to remove the binding or incorporation of the particle-nucleotide conjugate and polymerase. The dissociation can be achieved by placing the binding or incorporation complex in a condition (*e.g.*, adding Strontium ions) that will change the conformation of the polymerase and destabilize the binding or incorporation. 206 may also include a step of washing or removing the

dissociated particle-nucleotide conjugate and/or polymerase. 207 describes the step of extending the primed strand of the primed target nucleic acid by a single base addition reaction. After the single base extension, steps 204, 205, 206, and 207 can be repeated in multiple cycles to determine the sequences of the target nucleic acid.

[0072] FIG. 3 is another flowchart outlining the steps in sequencing a target nucleic acid, which includes cleaving a nucleotide from the particle-nucleotide conjugate and incorporating the cleaved nucleotide. 301 describes a step of attaching target library sequences to a solid support surface by hybridizing the target nucleic acid molecules to complementary adapters on substrate surface. The target nucleic acid molecules can be single stranded or partially double stranded. Prior to 301, the nucleic acid molecules in the target library may have been prepared to contain fragments complementary to the adaptor sequences through ligation or other methods. 302 describes the step of clonal amplification to generate clusters of target nucleic acid molecules on the surface. 303 describes hybridizing sequencing primers to complementary primer binding or incorporation sequences on the target nucleic acid to form the primed target nucleic acid. 304 describes combining the polymerase, the multivalent binding or incorporation composition, which contains labeled (*e.g.*, fluorescently-labeled) particle-nucleotide conjugates, and the primed target nucleic acid. In the particle-nucleotide conjugates, the nucleotides are attached to the particle through chemical bonds or interactions that can be later severed. 404 may also include a step of washing or removing the unbound reagents including polymerase and particle-nucleotide conjugate.

[0073] Again referring to **FIG. 3**, when the nucleotide on the particle-nucleotide conjugate is complementary to the next base of the primed target nucleic acid (305), the particle-nucleotide conjugate, polymerase, and primed target nucleic acid form a ternary binding or incorporation complex, which can be detected by detection methods (*e.g.*, florescence imaging) compatible with the label on the particle-nucleotide conjugate. 305 can also include measuring the persistence time of the ternary binding or incorporation complex. In 306, the polymerase is placed in a condition that would make it catalytically active to incorporate a nucleotide. The condition can include exposing the polymerase to Mg or Mn ions in the reaction solution. The nucleotide that is bound to the polymerase and the primed target nucleic acid is then cleaved from the particle and then incorporated into the primed strand of the primed target nucleic acid. The binding or incorporation complex is destabilized. 306 may also include a step of washing or removing the dissociated particle-nucleotide conjugate and/or polymerase. After the extension, steps 304, 305, and 306 can be repeated in multiple cycles to determine the sequences of the target nucleic acid.

B. Detecting Target Nucleic Acid Molecules

[0074] FIGS. A-4B illustrate one exemplified method in which the multivalent binding or incorporation composition is used for detecting a target nuclei acid. As shown in **FIG. 4A**, the polymer-nucleotide conjugate 401 is placed in contact with polymerase 406, a first nucleic acid molecule 404 and a second nucleic acid molecule 405. The polymer-nucleotide conjugate 401 has multiple polymer branches radiating from the core, and some branches are attached to nucleotide or oligonucleotide 402, and some branches are attached to a label 403. When the nucleotide or oligonucleotide 402 on the polymer-nucleotide conjugate 401 is complementary to at least a fraction of the first nucleic acid 404, a multivalent binding or incorporation complex is formed as shown in **FIG. B**, and the strong binding or incorporation signal can help detect target nucleic acid with sequences complementary or partially complementary to the nucleotide or oligonucleotide on the polymer-nucleotide conjugate. In some instances, at least one of the polymerase, nucleic acid molecules, and polymer-nucleotide conjugates is attached to a solid support.

[0075] The multivalent binding or incorporation composition described herein can be used in a method of detecting a target nucleic acid in a sample. Also disclosed herein are systems configured for performing any of the disclosed nucleic acid analysis methods. The system may comprise a fluid flow controller and/or fluid dispensing system configured to sequentially and iteratively contact the nucleic acid molecules with the disclosed polymerase and multivalent binding or incorporation compositions and/or reagents. The contacting may be performed within one or more flow cells. In some instances, said flow cells may be fixed components of the system. In some instances, said flow cells may be removable and/or disposable components of the system. The system may also include a cartridge comprising a sample collection unit and an assay assembly, wherein the sample collection unit is configured to collect a sample, and wherein the assay assembly comprises at least one reaction site containing a multivalent binding or incorporation composition adapted to interact with said analyte, allowing the predetermined portion of sample to react with assay reagents contained within the assay assembly to yield a signal indicative of the presence of the analyte in the sample, and detecting the signal generated from the analyte.

III. Multivalent Binding or incorporation Composition

[0076] The present disclosure relates to multivalent binding or incorporation compositions having a plurality of nucleotides conjugated to a particle (*e.g.*, a polymer, branched polymer, dendrimer, or equivalent structure). Contacting the multivalent binding or incorporation composition with a polymerase and multiple copies of a primed target nucleic acid may result in the formation of a

ternary complex which may be detected and in turn achieve a more accurate determination of the bases of the target nucleic acid.

[0077] When the multivalent binding or incorporation composition is used in replacement of single unconjugated or untethered nucleotide to form a complex with the polymerase and one or more copies of the target nucleic acid, the local concentration of the nucleotide as well as the binding avidity of the complex (in the case that a complex comprising two or more target nucleic acid molecules is formed) is increased many fold, which in turn enhances the signal intensity, particularly the correct signal versus mismatch. The multivalent binding or incorporation composition described herein can include at least one particle-nucleotide conjugate (each particle-nucleotide conjugate comprising multiple copies of a single nucleotide moiety) for interacting with the target nucleic acid. The multivalent composition can also include two, three, or four different particle-nucleotide conjugates, each having a different nucleotide conjugated to the particle.

[0078] The multivalent binding or incorporation composition can comprise 1, 2, 3, 4, or more types of particle-nucleotide conjugates, wherein each particle-nucleotide conjugate comprises a different type of nucleotide. A first type of the particle-nucleotide conjugate can comprise a nucleotide selected from the group consisting of ATP, ADP, AMP, dATP, dADP, and dAMP. A second type of the particle-nucleotide conjugate can comprise a nucleotide selected from the group consisting of TTP, TDP, TMP, dTTP, dTDP, dTMP, UTP, UDP, UMP, dUTP, dUDP, and dUMP. A third type of the particle-nucleotide conjugate can comprise a nucleotide selected from the group consisting of CTP, CDP, CMP, dCTP, dCDP, and dCMP. A fourth type of the particle-nucleotide conjugate can comprise a nucleotide selected from the group consisting of GTP, GDP, GMP, dGTP, dGDP, and dGMP. In some embodiments, each particle-nucleotide conjugate comprises a single type of nucleotide respectively corresponding to one or more nucleotide selected from the group consisting of ATP, ADP, AMP, dATP, dADP, dAMP TTP, TDP, TMP, dTTP, dTDP, dTMP, UTP, UDP, UMP, dUTP, dUDP, dUMP, CTP, CDP, CMP, dCTP, dCDP, dCMP, GTP, GDP, GMP, dGTP, dGDP, and dGMP. Each multivalent binding or incorporation composition may further comprise one or more labels corresponding to the particular nucleotide conjugated to each respective conjugate. Non-limiting examples of labels include fluorescent labels, colorimetric labels, electrochemical labels (such as, for example, glucose or other reducing sugars, or thiols or other redox active moieties), luminescent labels, chemiluminescent labels, spin labels, radioactive labels, steric labels, affinity tags, or the like.

A. Particle-Nucleotide Conjugate

[0079] In a particle-nucleotide conjugate, multiple copies of the same nucleotide may be covalently bound to or noncovalently bound to the particle. Examples of the particle can include a branched polymer; a dendrimer; a cross linked polymer particle such as an agarose, polyacrylamide, acrylate, methacrylate, cyanoacrylate, methyl methacrylate particle; a glass particle; a ceramic particle; a metal particle; a quantum dot; a liposome; an emulsion particle, or any other particle (e.g, nanoparticles, microparticles, or the like) known in the art. In a preferred embodiment, the particle is a branched polymer.

[0080] In some instances, the particle-nucleotide conjugate (*e.g.*, a polymer-nucleotide conjugate) may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 copies of a nucleotide, nucleotide analog, nucleoside, or nucleoside analog tethered to the particle.

[0081] The nucleotide can be linked to the particle through a linker, and the nucleotide can be attached to one end or location of a polymer. The nucleotide can be conjugated to the particle through the 5' end of the nucleotide. In some particle-nucleotide conjugates, one nucleotide attached to one end or location of a polymer. In some particle-nucleotide conjugate, multiple nucleotides are attached to one end or location of a polymer. The conjugated nucleotide is sterically accessible to one or more proteins, one or more enzymes, and nucleotide binding or incorporation moieties. In some embodiments, a nucleotide may be provided separately from a nucleotide binding or incorporation moiety such as a polymerase. In some embodiments, the linker does not comprise a photo emitting or photo absorbing group.

[0082] The particle can also have a binding or incorporation moiety. In some embodiments, particles may self-associate without the use of a separate interaction moiety. In some embodiments, particles may self-associate due to buffer conditions or salt conditions, *e.g.*, as in the case of calcium-mediated interactions of hydroxyapatite particles, lipid or polymer mediated interactions of micelles or liposomes, or salt-mediated aggregation of metallic (such as iron or gold) nanoparticles.

[0083] The particle-nucleotide conjugate can have one or more labels. Examples of the labels include but are not limited to fluorophores, spin labels, metals or metal ions, colorimetric labels, nanoparticles, PET labels, radioactive labels, or other such label as may render said composition detectable by such methods as are known in the art of the detection of macromolecules or molecular interactions. The label may be attached to the nucleotide (*e.g.* by attachment to the 5' phosphate moiety of a nucleotide), to the particle itself (*e.g.*, to the PEG subunits), to an end of the polymer, to a central moiety, or to any other location within said polymer-nucleotide conjugate which would be recognized by one of skill in the art to be sufficient to render said composition, such as a particle, detectable by such

methods as are known in the art or described elsewhere herein. In some embodiments, one or more labels are provided so as to correspond to or differentiate a particular particle-nucleotide conjugate.

[0084] In some embodiments, the label is a fluorophore. Non-limiting examples of fluorescent moieties include, but are not limited to, fluorescein and fluorescein derivatives such as carboxyfluorescein, tetrachlorofluorescein, hexachlorofluorescein, carboxynapthofluorescein, fluorescein isothiocyanate, NHS-fluorescein, iodoacetamidofluorescein, fluorescein maleimide, SAMSA-fluorescein, fluorescein thiosemicarbazide, carbohydrazinomethylthioacetyl-amino fluorescein, rhodamine and rhodamine derivatives such as TRITC, TMR, lissamine rhodamine, Texas Red, rhodamine B, rhodamine 6G, rhodamine 10, NHS-rhodamine, TMR-iodoacetamide, lissamine rhodamine B sulfonyl chloride, lissamine rhodamine B sulfonyl hydrazine, Texas Red sulfonyl chloride, Texas Red hydrazide, coumarin and coumarin derivatives such as AMCA, AMCA-NHS, AMCA-sulfo-NHS, AMCA-HPDP, DCIA, AMCE-hydrazide, BODIPY and derivatives such as BODIPY FL C3-SE, BODIPY 530/550 C3, BODIPY 530/550 C3-SE, BODIPY 530/550 C3 hydrazide, BODIPY 493/503 C3 hydrazide, BODIPY FL C3 hydrazide, BODIPY FL IA, BODIPY 530/551 IA, Br-BODIPY 493/503, Cascade Blue and derivatives such as Cascade Blue acetyl azide, Cascade Blue cadaverine, Cascade Blue ethylenediamine, Cascade Blue hydrazide, Lucifer Yellow and derivatives such as Lucifer Yellow iodoacetamide, Lucifer Yellow CH, cyanine and derivatives such as indolium based cyanine dyes, benzo-indolium based cyanine dyes, pyridium based cyanine dyes, thiozolium based cyanine dyes, quinolinium based cyanine dyes, imidazolium based cyanine dyes, Cy 3, Cy5, lanthanide chelates and derivatives such as BCPDA, TBP, TMT, BHHCT, BCOT, Europium chelates, Terbium chelates, Alexa Fluor dyes, DyLight dyes, Atto dyes, LightCycler Red dyes, CAL Flour dyes, JOE and derivatives thereof, Oregon Green dyes, WellRED dyes, IRD dyes, phycoerythrin and phycobilin dyes, Malachite green, stilbene, DEG dyes, NR dyes, near-infrared dyes and others known in the art such as those described in Haugland, Molecular Probes Handbook, (Eugene, Oreg.) 6th Edition; Lakowicz, Principles of Fluorescence Spectroscopy, 2nd Ed., Plenum Press New York (1999), or Hermanson, Bioconjugate Techniques, 2nd Edition, or derivatives thereof, or any combination thereof. Cyanine dyes may exist in either sulfonated or non-sulfonated forms, and consist of two indolenin, benzo-indolium, pyridium, thiozolium, and/or quinolinium groups separated by a polymethine bridge between two nitrogen atoms. Commercially available cyanine fluorophores include, for example, Cy3, (which may comprise 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium or 1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-2-(3-{1-[6-(2,5-dioxopyrrolidin-1-yloxy)-6-oxohexyl]-3,3-dimethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene}prop-1-en-1-yl)-3,3-dimethyl-3H-indolium-5-sulfonate), Cy5 (which

may comprise 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium or 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-2-((1E,3E)-5-((E)-1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-5-sulfonate), and Cy7 (which may comprise 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium or 1-(5-carboxypentyl)-2-[(1E,3E,5E,7Z)-7-(1-ethyl-5-sulfo-1,3-dihydro-2H-indol-2-ylidene)hepta-1,3,5-trien-1-yl]-3H-indolium-5-sulfonate), where “Cy” stands for 'cyanine', and the first digit identifies the number of carbon atoms between two indolenine groups. Cy2 which is an oxazole derivative rather than indolenin, and the benzo-derivatized Cy3.5, Cy5.5 and Cy7.5 are exceptions to this rule.

[0085] In some embodiments, the detection label can be a FRET pair, such that multiple classifications can be performed under a single excitation and imaging step. As used herein, FRET may comprise excitation exchange (Forster) transfers, or electron-exchange (Dexter) transfers.

B. Polymer-Nucleotide Conjugate

[0086] One example of the particle-nucleotide conjugate is a polymer-nucleotide conjugate. Some non-limiting examples of the polymer-nucleotide conjugates are shown in **FIGS. 5A-5C.** For example, **FIG. 5A** shows polymer-nucleotide conjugates having various configurations, *e.g.*, a “starburst” configuration comprising a fluorescently-labeled streptavidin core and four nucleotides bound to the core via biotinylated, linear PEG linkers of molecular weight ranging from 1K Dalton to 10K Daltons; **FIG. 5B** shows a polymer-nucleotide conjugate having a dendrimer core of, for example, 12, 24, 48, or 96 arms, and linear PEG linkers of molecular weight ranging from 1K Dalton to 10K Daltons radiating from the center; and **FIG. 5C** shows an example of polymer-nucleotide conjugates comprising a network of, *e.g.*, streptavidin cores, linked together by branched PEG linkers comprising a binding or incorporation moiety such as a biotin.

[0087] Examples of suitable linear or branched polymers include linear or branched polyethylene glycol (PEG), linear or branched polypropylene glycol, linear or branched polyvinyl alcohol, linear or branched polylactic acid, linear or branched polyglycolic acid, linear or branched polyglycine, linear or branched polyvinyl acetate, a dextran, or other such polymers, or copolymers incorporating any two or more of the foregoing or incorporating other polymers as are known in the art. In one embodiment, the polymer is a PEG. In another embodiment, the polymer can have PEG branches.

[0088] Suitable polymers may be characterized by a repeating unit incorporating a functional group suitable for derivatization such as an amine, a hydroxyl, a carbonyl, or an allyl group. The polymer

can also have one or more pre-derivatized substituents such that one or more particular subunits will incorporate a site of derivatization or a branch site, whether or not other subunits incorporate the same site, substituent, or moiety. A pre-derivatized substituent may comprise or may further comprise, for example, a nucleotide, a nucleoside, a nucleotide analog, a label such as a fluorescent label, radioactive label, or spin label, an interaction moiety, an additional polymer moiety, or the like, or any combination of the foregoing.

[0089] In the polymer-nucleotide conjugate, the polymer can have a plurality of branches. The branched polymer can have various configurations, including but are not limited to stellate ("starburst") forms, aggregated stellate ("helter skelter") forms, bottle brush, or dendrimer. The branched polymer can radiate from a central attachment point or central moiety, or may incorporate multiple branch points, such as, for example, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more branch points. In some embodiments, each subunit of a polymer may optionally constitute a separate branch point.

[0090] The length and size of the branch can differ based on the type of polymer. In some branched polymers, the branch may have a length of between 1 and 1,000 nm, between 1 and 100 nm, between 1 and 200 nm, between 1 and 300 nm, between 1 and 400 nm, between 1 and 500 nm, between 1 and 600 nm, between 1 and 700 nm, between 1 and 800 nm, or between 1 and 900 nm, or more, or having a length falling within or between any of the values disclosed herein.

[0091] In some polymer-nucleotide conjugates, the polymer core may have a size corresponding to an apparent molecular weight of 1K Da, 2K Da, 3K Da, 4K Da, 5K Da, 10K Da, 15K Da, 20K Da, 30K Da, 50K Da, 80K Da, 100K Da, or any value within a range defined by any two of the foregoing. The apparent molecular weight of a polymer may be calculated from the known molecular weight of a representative number of subunits, as determined by size exclusion chromatography, as determined by mass spectrometry, or as determined by any other method as is known in the art.

[0092] In some branched polymers, the branch may have a size corresponding to an apparent molecular weight of 1K Da, 2K Da, 3K Da, 4K Da, 5K Da, 10K Da, 15K Da, 20K Da, 30K Da, 50K Da, 80K Da, 100K Da, or any value within a range defined by any two of the foregoing. The apparent molecular weight of a polymer may be calculated from the known molecular weight of a representative number of subunits, as determined by size exclusion chromatography, as determined by mass spectrometry, or as determined by any other method as is known in the art. The polymer can have multiple branches. The number of branches in the polymer can be 2, 3, 4, 5, 6, 7, 8, 12, 16, 24, 32, 64, 128 or more, or a number falling within a range defined by any two of these values.

[0093] For polymer-nucleotide conjugates comprising a branched polymer of, for example, a branched PEG comprising 4, 8, 16, 32, or 64 branches, the polymer nucleotide conjugate can have nucleotides attached to the ends of the PEG branches, such that each end has attached thereto 0, 1, 2,

3, 4, 5, 6 or more nucleotides. In one non-limiting example, a branched PEG polymer of between 3 and 128 PEG arms may have attached to the ends of the polymer branches one or more nucleotides, such that each end has attached thereto 0, 1, 2, 3, 4, 5, 6 or more nucleotides or nucleotide analogs. In some embodiments, a branched polymer or dendrimer has an even number of arms. In some embodiments, a branched polymer or dendrimer has an odd number of arms.

[0094] In some instances, the length of the linker (*e.g.*, a PEG linker) may range from about 1 nm to about 1,000 nm. In some instances, the length of the linker may be at least 1 nm, at least 10 nm, at least 25 nm, at least 50 nm, at least 75 nm, at least 100 nm, at least 200 nm, at least 300 nm, at least 400 nm, at least 500 nm, at least 600 nm, at least 700 nm, at least 800 nm, at least 900 nm, or at least 1,000 nm. In some instances, the length of the linker may range between any two of the values in this paragraph. For example, in some instances, the length of the linker may range from about 75 nm to about 400 nm. Those of skill in the art will recognize that in some instances, the length of the linker may have any value within the range of values in this paragraph, *e.g.*, 834 nm.

[0095] In some instances, the length of the linker is different for different nucleotides (including deoxyribonucleotides and ribonucleotides), nucleotide analogs (including deoxyribonucleotide analogs and ribonucleotide analogs), nucleosides (including deoxyribonucleosides or ribonucleosides), or nucleoside analogs (including deoxyribonucleoside analogs or ribonucleoside analogs). In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, deoxyadenosine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, deoxyguanosine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, thymidine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, comprises deoxyuridine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, deoxycytidine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, adenosine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, guanosine, and the length of the linker is between 1 and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, 5-methyl-uridine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises,

for example, uridine, and the length of the linker is between 1 nm and 1,000 nm. In some instances, one of the nucleotides, nucleotide analogs, nucleosides, or nucleoside analogs comprises, for example, cytidine, and the length of the linker is between 1 nm and 1,000 nm.

[0096] In the polymer-nucleotide conjugate, each branch or a subset of branches of the polymer may have attached thereto a moiety comprising a nucleotide (*e.g.*, an adenine, a thymine, a uracil, a cytosine, or a guanine residue or a derivative or mimetic thereof), and the moiety is capable of binding or incorporation to a polymerase, reverse transcriptase, or other nucleotide binding or incorporation domain. Optionally, the moiety may be capable of being incorporated into an elongating nucleic acid chain during a polymerase reaction. In some instances, said moiety may be blocked such that it is not capable of being incorporated into an elongating nucleic acid chain during a polymerase reaction. In some other instances, said moiety may be reversibly blocked such that it is not capable of being incorporated into an elongating nucleic acid chain during a polymerase reaction until such block is removed, after which said moiety is then capable of being incorporated into an elongating nucleic acid chain during a polymerase reaction.

[0097] The nucleotide can be conjugated to the polymer branch through the 5' end of the nucleotide. In some instances, the nucleotide may be modified so as to inhibit or prevent incorporation of the nucleotide into an elongating nucleic acid chain during a polymerase reaction. By way of example, the nucleotide may include a 3' deoxyribonucleotide, a 3' azidonucleotide, a 3'-methyl azido nucleotide, or another such nucleotide as is or may be known in the art, so as to not be capable of being incorporated into an elongating nucleic acid chain during a polymerase reaction. In some embodiments, the nucleotide can include a 3'-O-azido group, a 3'-O-azidomethyl group, a 3'-phosphorothioate group, a 3'-O-malonyl group, a 3'-O-alkyl hydroxylamino group, or a 3'-O-benzyl group. In some embodiments, the nucleotide lacks a 3' hydroxyl group.

[0098] The polymer can further have a binding or incorporation moiety in each branch or a subset of branches. Some examples of the binding or incorporation moiety include but are not limited to biotin, avidin, strepavidin or the like, polyhistidine domains, complementary paired nucleic acid domains, G-quartet forming nucleic acid domains, calmodulin, maltose-binding protein, cellulase, maltose, sucrose, glutathione-S-transferase, glutathione, O-6-methylguanine-DNA methyltransferase, benzylguanine and derivatives thereof, benzylcysteine and derivatives thereof, an antibody, an epitope, a protein A, a protein G. The binding or incorporation moiety can be any interactive molecules or fragment thereof known in the art to bind to or facilitate interactions between proteins, between proteins and ligands, between proteins and nucleic acids, between nucleic acids, or between small molecule interaction domains or moieties.

[0099] In some embodiments, a composition as provided herein may comprise one or more elements of a complementary interaction moiety. Non-limiting examples of complementary interaction moieties include, for example, biotin and avidin; SNAP-benzylguanosine; antibody or FAB and epitope; IgG FC and Protein A, Protein G, ProteinA/G, or Protein L; maltose binding protein and maltose; lectin and cognate polysaccharide; ion chelation moieties, complementary nucleic acids, nucleic acids capable of forming triplex or triple helical interactions; nucleic acids capable of forming G-quartets, and the like. One of skill in the art will readily recognize that many pairs of moieties exist and are commonly used for their property of interacting strongly and specifically with one another; and thus any such complementary pair or set is considered to be suitable for this purpose in constructing or envisioning the compositions of the present disclosure. In some embodiments, a composition as disclosed herein may comprise compositions in which one element of a complementary interaction moiety is attached to one molecule or multivalent ligand, and the other element of the complementary interaction moiety is attached to a separate molecule or multivalent ligand. In some embodiments, a composition as disclosed herein may comprise compositions in which both or all elements of a complementary interaction moiety are attached to a single molecule or multivalent ligand. In some embodiments, a composition as disclosed herein may comprise compositions in which both or all elements of a complementary interaction moiety are attached to separate arms of, or locations on, a single molecule or multivalent ligand. In some embodiments, a composition as disclosed herein may comprise compositions in which both or all elements of a complementary interaction moiety are attached to the same arm of, or locations on, a single molecule or multivalent ligand. In some embodiments, compositions comprising one element of a complementary interaction moiety and compositions comprising another element of a complementary interaction moiety may be simultaneously or sequentially mixed. In some embodiments, interactions between molecules or particles as disclosed herein allow for the association or aggregation of multiple molecules or particles such that, for example, detectable signals are increased. In some embodiments, fluorescent, colorimetric, or radioactive signals are enhanced. In other embodiments, other interaction moieties as disclosed herein or as are known in the art are contemplated. In some embodiments, a composition as provided herein may be provided such that one or more molecules comprising a first interaction moiety such as, for example, one or more imidazole or pyridine moieties, and one or more additional molecules comprising a second interaction moiety such as, for example, histidine residues, are simultaneously or sequentially mixed. In some embodiments, said composition comprises 1, 2, 3, 4, 5, 6, or more imidazole or pyridine moieties. In some embodiments, said composition comprises 1, 2, 3, 4, 5, 6, or more histidine residues. In such embodiments, interaction between the molecules or particles as provided may be

facilitated by the presence of a divalent cation such as nickel, manganese, magnesium, calcium, strontium, or the like. In some embodiments, for example, a $(His)_3$ group may interact with a $(His)_3$ group on another molecule or particle via coordination of a nickel or manganese ion.

[0100] The multivalent binding or incorporation composition may comprise one or more buffers, salts, ions, or additives. In some embodiments, representative additives may include, but are not limited to, betaine, spermidine, detergents such as Triton X-100, Tween 20, SDS, or NP-40, ethylene glycol, polyethylene glycol, dextran, polyvinyl alcohol, vinyl alcohol, methylcellulose, heparin, heparan sulfate, glycerol, sucrose, 1,2-propanediol, DMSO, N,N,N-trimethylglycine, ethanol, ethoxyethanol, propylene glycol, polypropylene glycol, block copolymers such as the Pluronic (r) series polymers, arginine, histidine, imidazole, or any combination thereof, or any substance known in the art as a DNA "relaxer" (a compound, with the effect of altering the persistence length of DNA, altering the number of within-polymer junctions or crossings, or altering the conformational dynamics of a DNA molecule such that the accessibility of sites within the strand to DNA binding or incorporation moieties is increased).

[0101] The multivalent binding or incorporation composition may include zwitterionic compounds as additives. Further representative additives may be found in Lorenz, T.C. J. Vis. Exp. (63), e3998, doi:10.3791/3998 (2012), which is hereby incorporated by reference with respect to its disclosure of additives for the facilitation of nucleic acid binding or dynamics, or the facilitation of processes involving the manipulation, use, or storage of nucleic acids. In some embodiments, representative cations may include, but are not limited to, sodium, magnesium, strontium, potassium, manganese, calcium, lithium, nickel, cobalt, or other such cations as are known in the art to facilitate nucleic acid interactions, such as self-association, secondary or tertiary structure formation, base pairing, surface association, peptide association, protein binding, or the like.

IV. Binding Between Target Nucleic Acid and Multivalent Binding or Incorporation Composition

[0102] When the multivalent binding or incorporation composition is used in replacement of single unconjugated or untethered nucleotide to form a complex with the polymerase and one or more copies of the target nucleic acid, the local concentration of the nucleotide as well as the binding avidity of the complex (in the case that a complex comprising two or more target nucleic acid molecules is formed) is increased many-fold, which in turn enhances the signal intensity, particularly the correct signal versus mismatch. The present disclosure contemplates contacting the multivalent binding or incorporation composition with a polymerase and a primed target nucleic acid to determine the formation of a ternary binding or incorporation complex.

[0103] FIG. 6 illustrates the use of the disclosed polymer-nucleotide conjugates for achieving increased signal intensity during binding, persistence, and washing/removal steps. Because of the increased local concentration of the nucleotide on the polymer-nucleotide conjugate and/or the formation of non-covalent bonds with two or more primed target nucleic acid molecules, the binding between the polymerase, the primed target strand, and the polymer-conjugated nucleotide, when the nucleotide is complementary to the next base of the target nucleic acid, becomes more favorable. The formed binding complex has a longer persistence time which in turn helps increase signal and shorten the imaging step. The high signal intensity resulting from the use of the disclosed polymer nucleotide conjugates remains stable for the entire binding and imaging steps. The strong binding between the polymerase, the primed target strand, and the polymer-conjugated nucleotide or nucleotide analog also means that the binding complex thus formed will remain stable during wash steps as other reaction mixture components and unmatched nucleotide analogs are washed away. After the imaging step, the binding complex can be destabilized (*e.g.*, by changing the buffer composition) and the primed target nucleic acid can then be extended for one base. After the extension, the binding and imaging steps can be repeated with the use of the disclosed polymer nucleotide conjugates to determine the identity of the next base.

[0104] As an example, a graphical depiction of the increase in signal intensity during binding, persistence, and washing/removal of a multivalent substrate as described herein is provided in **FIG. 6**, which is representative of the changes in signal intensity that have been observed experimentally. Therefore, the compositions and methods of the present disclosure provide a robust and controllable means of establishing and maintaining a ternary enzyme complex, as well as providing vastly improved means by which the presence of said complex may be identified and/or measured, and a means by which the persistence of said complex may be controlled. This provides important solutions to problems such as that of determining the identity of the N+1 base in nucleic acid sequencing applications.

[0105] Without intending to be bound by any particular theory, it has been observed that multivalent binding compositions disclosed herein associate with polymerase nucleotide complexes in order to form a ternary binding complexes with a rate that is time-dependent, though substantially slower than the rate of association known to be obtainable by nucleotides in free solution. Thus, the on-rate (K_{on}) is substantially and surprisingly slower than the on rate for single nucleotides or nucleotides not attached to multivalent ligand complexes. Importantly, however, the off rate (K_{off}) of the multivalent ligand complex is substantially slower than that observed for nucleotides in free solution. Therefore, the multivalent ligand complexes of the present disclosure provide a surprising and beneficial improvement of the persistence of ternary polymerase-polynucleotide-nucleotide complexes

(especially over such complexes that are formed with free nucleotides) allowing, for example, significant improvements in imaging quality for nucleic acid sequencing applications over currently available methods and reagents. Importantly, this property of the multivalent binding compositions disclosed herein renders the formation of visible ternary complexes controllable, such that subsequent visualization, modification, or processing steps may be undertaken essentially without regard to the dissociation of the complex—that is, the complex can be formed, imaged, modified, or used in other ways as necessary, and will remain stable until a user carries out an affirmative dissociation step, such as exposing the complexes to a dissociation buffer.

[0106] In some instances, the persistence times for the multivalent binding complexes formed using the disclosed particle-nucleotide or polymer-nucleotide conjugates may range from about 0.1 second to about 600 second under non-destabilizing conditions. In some instances, the persistence time may be at least 0.1 second, at least 1 second, at least 2 seconds, at least 3 second, at least 4 second, at least 5 seconds, at least 6 seconds, at least 7 seconds, at least 8 seconds, at least 9 seconds, at least 10 seconds, at least 20 seconds, at least 30 second, at least 40 second, at least 50 seconds, at least 60 seconds, at least 120 seconds, at least 180 seconds, at least 240 seconds, at least 300 seconds, at least 360 seconds, at least 420 seconds, at least 480 seconds, at least 540 seconds, or at least 600 seconds. In some instances, the persistence time may range between any two of the values specified in this paragraph. For example, in some instances, the persistence time may range from about 10 seconds to about 360 seconds. Those of skill in the art will recognize that in some instances, the persistence time may have any value within the range of values specified in this paragraph, *e.g.*, 78 seconds.

[0107] In various embodiments, polymerases suitable for the binding or incorporation interaction describe herein include may include any polymerase as is or may be known in the art. It is, for example, known that every organism encodes within its genome one or more DNA polymerases. Examples of suitable polymerases may include but are not limited to: Klenow DNA polymerase, Thermus aquaticus DNA polymerase I (Taq polymerase), KlenTaq polymerase,and bacteriophage T7 DNA polymerase; human alpha, delta and epsilon DNA polymerases; bacteriophage polymerases such as T4, RB69 and phi29 bacteriophage DNA polymerases, Pyrococcus furiosus DNA polymerase (Pfu polymerase); Bacillus subtilis DNA polymerase III, and E. coli DNA polymerase III alpha and epsilon; 9 degree N polymerase, reverse transcriptases such as HIV type M or O reverse transcriptases, avian myeloblastosis virus reverse transcriptase, or Moloney Murine Leukemia Virus (MMLV) reverse transcriptase, or telomerase. Further non-limiting examples of DNA polymerases can include those from various Archaea genera, such as, Aeropyrum, Archaeglobus, Desulfurococcus, Pyrobaculum, Pyrococcus, Pyrolobus, Pyrodictium, Staphylothermus, Stetteria, Sulfolobus, Thermococcus, and Vulcanisaeta and the like or variants thereof, including such polymerases as are

known in the art such as Vent ™, Deep Vent ™, Pfu, KOD, Pfx, Therminator™, and Tgo polymerases. In some embodiments, the polymerase is a klenow polymerase.

[0108] The ternary complex has longer persistence time when the nucleotide on the polymer-nucleotide conjugate is complementary to the target nucleic acid than when a non-complementary nucleotide. The ternary complex also has longer persistence time when the nucleotide on the polymer-nucleotide conjugate is complementary to the target nucleic acid than a complementary nucleotide that is not conjugated or tethered. For example, in some embodiments, said ternary complexes may have a persistence time of less than 1s, greater than 1s, greater than 2s, greater than 3s, greater than 5s, greater than 10s, greater than 15s, greater than 20s, greater than 30s, greater than 60s, greater than 120s, greater than 360s, greater than 3600s, or more, or for a time lying within a range defined by any two or more of these values.

[0109] The persistence time can be measured, for example, by observing the onset and/or duration of a binding complex, such as by observing a signal from a labeled component of the binding complex. For example, a labeled nucleotide or a labeled reagent comprising one or more nucleotides may be present in a binding complex, thus allowing the signal from the label to be detected during the persistence time of the binding complex.

[0110] It has been observed that different ranges of persistence times are achievable with different salts or ions, showing, for example, that complexes formed in the presence of, for example, magnesium ions (Mg^{2+}) form more quickly than complexes formed with other ions. It has also been observed that complexes formed in the presence of, for example, strontium ions (Sr^{2+}), form readily and dissociate completely or with substantial completeness upon withdrawal of the ion or upon washing with buffer lacking one or more components of the present compositions, such as, *e.g.*, a polymer and/or one or more nucleotides, and/or one or more interaction moieties, or a buffer containing, for example, a chelating agent which may cause or accelerate the removal of a divalent cation from the multivalent reagent containing complex. Thus, in some embodiments, a composition of the present disclosure comprises Mg^{2+}. In some embodiments, a composition of the present disclosure comprises Ca^{2+}. In some embodiments, a composition of the present disclosure comprises Sr^{2+}. In some embodiments, a composition of the present disclosure comprises cobalt ions (Co^{2+}). In some embodiments, a composition of the present disclosure comprises $MgCl_2$. In some embodiments, a composition of the present disclosure comprises $CaCl_2$. In some embodiments, a composition of the present disclosure comprises $SrCl_2$. In some embodiments, a composition of the present disclosure comprises $CoCl_2$. In some embodiments, the composition comprises no, or substantially no magnesium. In some embodiments, the composition comprises no, or substantially no calcium. In some embodiments, the methods of the present disclosure provide for the contacting

of one or more nucleic acids with one or more of the compositions disclosed herein wherein said composition lacks either one of calcium or magnesium or lacks both calcium or magnesium.

[0111] The dissociation of ternary complexes can be controlled by changing the buffer conditions. After the imaging step, a buffer with increased salt content is used to cause dissociation of the ternary complexes such that labeled polymer-nucleotide conjugates can be washed out, providing a means by which signals can be attenuated or terminated, such as in the transition between one sequencing cycle and the next. This dissociation may be affected, in some embodiments, by washing the complexes with a buffer lacking a necessary metal or cofactor. In some embodiments, a wash buffer may comprise one or more compositions for the purpose of maintaining pH control. In some embodiments, a wash buffer may comprise one or more monovalent cations, such as sodium. In some embodiments, a wash buffer lacks or substantially lacks a divalent cation, for example, having no or substantially no strontium, calcium, magnesium, or manganese. In some embodiments, a wash buffer further comprises a chelating agent, such as, for example, EDTA, EGTA, nitrilotriacetic acid, polyhistidine, imidazole, or the like. In some embodiments, a wash buffer may maintain the pH of the environment at the same level as for the bound complex. In some embodiments, a wash buffer may raise or lower the pH of the environment relative to the level seen for the bound complex. In some embodiments, the pH may be within a range from 2-4, 2-7, 5-8, 7-9, 7-10, or lower than 2, or higher than 10, or a range defined by any two of the values provided herein.

[0112] Addition of a particular ion may affect the binding of the polymerase to a primed target nucleic acid, the formation of a ternary complex, the dissociation of a ternary complex, or the incorporation of one or more nucleotides into an elongating nucleic acid such as during a polymerase reaction. In some embodiments, relevant anions may comprise chloride, acetate, gluconate, sulfate, phosphate, or the like. In some embodiments, an ion may be incorporated into the compositions of the present disclosure by the addition of one or more acids, bases, or salts, such as $NiCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $SrCl_2$, $CaCl_2$, $CaSO_4$, $SrCO_3$, $BaCl_2$ or the like. Representative salts, ions, solutions and conditions may be found in Remington: The Science and Practice of Pharmacy, 20th. Edition, Gennaro, A.R., Ed. (2000), which is hereby incorporated by reference in its entirety, and especially with respect to Chapter 17 and related disclosure of salts, ions, salt solutions, and ionic solutions.

[0113] The present disclosure contemplates contacting the multivalent binding or incorporation composition comprising at least one particle-nucleotide conjugate with one or more polymerases. The contacting can be optionally done in the presence of one or more target nucleic acids. In some embodiments, said target nucleic acids are single stranded nucleic acids. In some embodiments, said target nucleic acids are primed single stranded nucleic acids. In some embodiments, said target nucleic acids are double stranded nucleic acids. In some embodiments, said contacting comprises

contacting the multivalent binding or incorporation composition with one polymerase. In some embodiments, said contacting comprises the contacting of said composition comprising one or more nucleotides with multiple polymerases. The polymerase can be bound to a single nucleic acid molecule.

[0114] The binding between target nucleic acid and multivalent binding composition may be provided in the presence of a polymerase that has been rendered catalytically inactive. In one embodiment, the polymerase may have been rendered catalytically inactive by mutation. In one embodiment, the polymerase may have been rendered catalytically inactive by chemical modification. In some embodiments, the polymerase may have been rendered catalytically inactive by the absence of a necessary substrate, ion, or cofactor. In some embodiments, the polymerase enzyme may have been rendered catalytically inactive by the absence of magnesium ions.

[0115] The binding between target nucleic acid and multivalent binding composition occur in the presence of a polymerase wherein the binding solution, reaction solution, or buffer lacks magnesium or manganese. Alternatively, the binding between target nucleic acid and multivalent binding composition occur in the presence of a polymerase wherein the binding solution, reaction solution, or buffer comprises calcium or strontium.

[0116] When the catalytically inactive polymerases are used to help a nucleic acid interact with a multivalent binding composition, the interaction between said composition and said polymerase stabilizes a ternary complex so as to render the complex detectable by fluorescence or by other methods as disclosed herein or otherwise known in the art. Unbound polymer-nucleotide conjugates may optionally be washed away prior to detection of the ternary binding complex.

[0117] Contacting of one or more nucleic acids with the polymer-nucleotide conjugates disclosed herein in a solution containing either one of calcium or magnesium or containing both calcium and magnesium. Alternatively, the contacting of one or more nucleic acids with the polymer-nucleotide conjugates disclosed herein in a solution lacking either one of calcium or magnesium, or lacking both calcium or magnesium, and in a separate step, without regard to the order of the steps, adding to the solution one of calcium or magnesium, or both calcium and magnesium. In some embodiments, the contacting of one or more nucleic acids with the polymer-nucleotide conjugates disclosed herein in a solution lacking strontium, and comprises in a separate step, without regard to the order of the steps, adding to the solution strontium.

V. Use of Multivalent Binding or Incorporation Compositions in Combination with Low Non-specific Binding Surface

[0118] Disclosed herein are solid supports comprising low non-specific binding surface compositions that enable improved nucleic acid hybridization and amplification performance. In general, the disclosed supports may comprise a substrate (or support structure), one or more layers of a covalently or non-covalently attached low-binding, chemical modification layers, *e.g.*, silane layers, polymer films, and one or more covalently or non-covalently attached primer sequences that may be used for tethering single-stranded target nucleic acid(s) to the support surface. In some instances, the formulation of the surface, *e.g.*, the chemical composition of one or more layers, the coupling chemistry used to cross-link the one or more layers to the support surface and/or to each other, and the total number of layers, may be varied such that non-specific binding of proteins, nucleic acid molecules, and other hybridization and amplification reaction components to the support surface is minimized or reduced relative to a comparable monolayer. Often, the formulation of the surface may be varied such that non-specific hybridization on the support surface is minimized or reduced relative to a comparable monolayer. The formulation of the surface may be varied such that non-specific amplification on the support surface is minimized or reduced relative to a comparable monolayer. The formulation of the surface may be varied such that specific amplification rates and/or yields on the support surface are maximized. Amplification levels suitable for detection are achieved in no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, or more than 30 amplification cycles in some cases disclosed herein.

[0119] Examples of materials from which the substrate or support structure may be fabricated include, but are not limited to, glass, fused-silica, silicon, a polymer (*e.g.*, polystyrene (PS), macroporous polystyrene (MPPS), polymethylmethacrylate (PMMA), polycarbonate (PC), polypropylene (PP), polyethylene (PE), high density polyethylene (HDPE), cyclic olefin polymers (COP), cyclic olefin copolymers (COC), polyethylene terephthalate (PET)), or any combination thereof. Various compositions of both glass and plastic substrates are contemplated.

[0120] The substrate or support structure may be rendered in any of a variety of geometries and dimensions known to those of skill in the art, and may comprise any of a variety of materials known to those of skill in the art. For example, in some instances the substrate or support structure may be locally planar (*e.g.*, comprising a microscope slide or the surface of a microscope slide). Globally, the substrate or support structure may be cylindrical (*e.g.*, comprising a capillary or the interior surface of a capillary), spherical (*e.g.*, comprising the outer surface of a non-porous bead), or irregular (*e.g.*, comprising the outer surface of an irregularly-shaped, non-porous bead or particle). In some instances, the surface of the substrate or support structure used for nucleic acid hybridization and

amplification may be a solid, non-porous surface. In some instances, the surface of the substrate or support structure used for nucleic acid hybridization and amplification may be porous, such that the coatings described herein penetrate the porous surface, and nucleic acid hybridization and amplification reactions performed thereon may occur within the pores.

[0121] The substrate or support structure that comprises the one or more chemically-modified layers, *e.g.*, layers of a low non-specific binding polymer, may be independent or integrated into another structure or assembly. For example, in some instances, the substrate or support structure may comprise one or more surfaces within an integrated or assembled microfluidic flow cell. The substrate or support structure may comprise one or more surfaces within a microplate format, *e.g.*, the bottom surface of the wells in a microplate. As noted above, in some preferred embodiments, the substrate or support structure comprises the interior surface (such as the lumen surface) of a capillary. In alternate preferred embodiments the substrate or support structure comprises the interior surface (such as the lumen surface) of a capillary etched into a planar chip.

[0122] As noted, the low non-specific binding supports of the present disclosure exhibit reduced non-specific binding of proteins, nucleic acids, and other components of the hybridization and/or amplification formulation used for solid-phase nucleic acid amplification. The degree of non-specific binding exhibited by a given support surface may be assessed either qualitatively or quantitatively. For example, in some instances, exposure of the surface to fluorescent dyes (*e.g.*, cyanines such as Cy3, or Cy5, *etc.*, fluoresceins, coumarins, rhodamines, *etc.* or other dyes disclosed herein), fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (*e.g.* polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging may be used as a qualitative tool for comparison of non-specific binding on supports comprising different surface formulations. In some instances, exposure of the surface to fluorescent dyes, fluorescently-labeled nucleotides, fluorescently-labeled oligonucleotides, and/or fluorescently-labeled proteins (*e.g.* polymerases) under a standardized set of conditions, followed by a specified rinse protocol and fluorescence imaging may be used as a quantitative tool for comparison of non-specific binding on supports comprising different surface formulations – provided that care has been taken to ensure that the fluorescence imaging is performed under conditions where fluorescence signal is linearly related (or related in a predictable manner) to the number of fluorophores on the support surface (*e.g.*, under conditions where signal saturation and/or self-quenching of the fluorophore is not an issue) and suitable calibration standards are used. In some instances, other techniques known to those of skill in the art, for example, radioisotope labeling and counting methods may be used for quantitative assessment of the degree to which non-specific binding is exhibited by the different support surface formulations of the present disclosure.

[0123] Some surfaces disclosed herein exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein. Some surfaces disclosed herein exhibit a ratio of specific to nonspecific fluorescence of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

[0124] As noted, in some instances, the degree of non-specific binding exhibited by the disclosed low-binding supports may be assessed using a standardized protocol for contacting the surface with a labeled protein (*e.g.*, bovine serum albumin (BSA), streptavidin, a DNA polymerase, a reverse transcriptase, a helicase, a single-stranded binding protein (SSB), *etc.*, or any combination thereof), a labeled nucleotide, a labeled oligonucleotide, *etc.*, under a standardized set of incubation and rinse conditions, followed be detection of the amount of label remaining on the surface and comparison of the signal resulting therefrom to an appropriate calibration standard. In some instances, the label may comprise a fluorescent label. In some instances, the label may comprise a radioisotope. In some instances, the label may comprise any other detectable label known to one of skill in the art. In some instances, the degree of non-specific binding exhibited by a given support surface formulation may thus be assessed in terms of the number of non-specifically bound protein molecules (or other molecules) per unit area. In some instances, the low-binding supports of the present disclosure may exhibit non-specific protein binding (or non-specific binding of other specified molecules, (*e.g.*, cyanine dyes such as Cy3, or Cy5, *etc.*, fluoresceins, coumarins, rhodamines, *etc.*, or other dyes disclosed herein)) of less than 0.001 molecule per μm^2, less than 0.01 molecule per μm^2, less than 0.1 molecule per μm^2, less than 0.25 molecule per μm^2, less than 0.5 molecule per μm^2, less than 1molecule per μm^2, less than 10 molecules per μm^2, less than 100 molecules per μm^2, or less than 1,000 molecules per μm^2. Those of skill in the art will realize that a given support surface of the present disclosure may exhibit non-specific binding falling anywhere within this range, for example, of less than 86 molecules per μm^2. For example, some modified surfaces disclosed herein exhibit nonspecific protein binding of less than 0.5 molecule / μm^2 following contact with a 1 μM solution of Cy3 labeled streptavidin (GE Amersham) in phosphate buffered saline (PBS) buffer for 15 minutes, followed by 3 rinses with deionized water. Some modified surfaces disclosed herein exhibit nonspecific binding of Cy3 dye molecules of less than 0.25 molecules per μm^2. In independent nonspecific binding assays, 1 μM labeled Cy3 SA (ThermoFisher), 1 μM Cy5 SA dye (ThermoFisher), 10 μM Aminoallyl-dUTP - ATTO-647N (Jena Biosciences), 10 μM Aminoallyl-dUTP - ATTO-Rho11 (Jena Biosciences), 10 μM Aminoallyl-dUTP - ATTO-Rho11 (Jena Biosciences), 10 μM 7-Propargylamino-7-deaza-dGTP - Cy5 (Jena Biosciences, and 10 μM 7-

Propargylamino-7-deaza-dGTP – Cy3 (Jena Biosciences) were incubated on the low binding substrates at 37°C for 15 minutes in a 384 well plate format. Each well was rinsed 2-3 x with 50 ul deionized RNase/DNase Free water and 2-3 x with 25 mM ACES buffer pH 7.4. The 384 well plates were imaged on a GE Typhoon instrument using the Cy3, AF555, or Cy5 filter sets (according to dye test performed) as specified by the manufacturer at a PMT gain setting of 800 and resolution of 50-100 μm. For higher resolution imaging, images were collected on an Olympus IX83 microscope (Olympus Corp., Center Valley, PA) with a total internal reflectance fluorescence (TIRF) objective (100X, 1.5 NA, Olympus), a CCD camera (*e.g.*, an Olympus EM-CCD monochrome camera, Olympus XM-10 monochrome camera, or an Olympus DP80 color and monochrome camera), an illumination source (*e.g.*, an Olympus 100W Hg lamp, an Olympus 75W Xe lamp, or an Olympus U-HGLGPS fluorescence light source), and excitation wavelengths of 532 nm or 635 nm. Dichroic mirrors were purchased from Semrock (IDEX Health & Science, LLC, Rochester, New York), *e.g.*, 405, 488, 532, or 633 nm dichroic reflectors/beamsplitters, and band pass filters were chosen as 532 LP or 645 LP concordant with the appropriate excitation wavelength. Some modified surfaces disclosed herein exhibit nonspecific binding of dye molecules of less than 0.25 molecules per μm^2.

[0125] In some instances, the surfaces disclosed herein exhibit a ratio of specific to nonspecific binding of a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein. In some instances, the surfaces disclosed herein exhibit a ratio of specific to nonspecific fluorescence signals for a fluorophore such as Cy3 of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 75, 100, or greater than 100, or any intermediate value spanned by the range herein.

[0126] The low-background surfaces consistent with the disclosure herein may exhibit specific dye attachment (*e.g.*, Cy3 attachment) to non-specific dye adsorption (*e.g.*, Cy3 dye adsorption) ratios of at least 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1, or more than 50 specific dye molecules attached per molecule nonspecifically adsorbed. Similarly, when subjected to an excitation energy, low-background surfaces consistent with the disclosure herein to which fluorophores, *e.g.*, Cy3, have been attached may exhibit ratios of specific fluorescence signal (*e.g.*, arising from Cy3-labeled oligonucleotides attached to the surface) to non-specific adsorbed dye fluorescence signals of at least 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1, or more than 50:1.

[0127] In some instances, the degree of hydrophilicity (or "wettability" with aqueous solutions) of the disclosed support surfaces may be assessed, for example, through the measurement of water contact angles in which a small droplet of water is placed on the surface and its angle of contact with

the surface is measured using, *e.g.*, an optical tensiometer. In some instances, a static contact angle may be determined. In some instances, an advancing or receding contact angle may be determined. In some instances, the water contact angle for the hydrophilic, low-binding support surfaced disclosed herein may range from about 0 degrees to about 30 degrees. In some instances, the water contact angle for the hydrophilic, low-binding support surfaced disclosed herein may no more than 50 degrees, 40 degrees, 30 degrees, 25 degrees, 20 degrees, 18 degrees, 16 degrees, 14 degrees, 12 degrees, 10 degrees, 8 degrees, 6 degrees, 4 degrees, 2 degrees, or 1 degree. In many cases the contact angle is no more than 40 degrees. Those of skill in the art will realize that a given hydrophilic, low-binding support surface of the present disclosure may exhibit a water contact angle having a value of anywhere within this range.

[0128] In some instances, the hydrophilic surfaces disclosed herein facilitate reduced wash times for bioassays, often due to reduced nonspecific binding of biomolecules to the low-binding surfaces. In some instances, adequate wash steps may be performed in less than 60, 50, 40, 30, 20, 15, 10, or less than 10 seconds. For example, in some instances adequate wash steps may be performed in less than 30 seconds.

[0129] Some low-binding surfaces of the present disclosure exhibit significant improvement in stability or durability to prolonged exposure to solvents and elevated temperatures, or to repeated cycles of solvent exposure or changes in temperature. For example, in some instances, the stability of the disclosed surfaces may be tested by fluorescently labeling a functional group on the surface, or a tethered biomolecule (*e.g.*, an oligonucleotide primer) on the surface, and monitoring fluorescence signal before, during, and after prolonged exposure to solvents and elevated temperatures, or to repeated cycles of solvent exposure or changes in temperature. In some instances, the degree of change in the fluorescence used to assess the quality of the surface may be less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% over a time period of 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 15 hours, 20 hours, 25 hours, 30 hours, 35 hours, 40 hours, 45 hours, 50 hours, or 100 hours of exposure to solvents and/or elevated temperatures (or any combination of these percentages as measured over these time periods). In some instances, the degree of change in the fluorescence used to assess the quality of the surface may be less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, or 25% over 5 cycles, 10 cycles, 20 cycles, 30 cycles, 40 cycles, 50 cycles, 60 cycles, 70 cycles, 80 cycles, 90 cycles, 100 cycles, 200 cycles, 300 cycles, 400 cycles, 500 cycles, 600 cycles, 700 cycles, 800 cycles, 900 cycles, or 1,000 cycles of repeated exposure to solvent changes and/or changes in temperature (or any combination of these percentages as measured over this range of cycles).

[0130] In some instances, the surfaces disclosed herein may exhibit a high ratio of specific signal to nonspecific signal or other background. For example, when used for nucleic acid amplification, some surfaces may exhibit an amplification signal that is at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, or greater than 100-fold greater than a signal of an adjacent unpopulated region of the surface. Similarly, some surfaces exhibit an amplification signal that is at least 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 75, 100, or greater than 100-fold greater than a signal of an adjacent amplified nucleic acid population region of the surface.

[0131] In some instances, fluorescence images of the disclosed low background surfaces when used in nucleic acid hybridization or amplification applications to create clusters of hybridized or clonally-amplified nucleic acid molecules (*e.g.*, that have been directly or indirectly labeled with a fluorophore) exhibit contrast-to-noise ratios (CNRs) of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 20, 210, 220, 230, 240, 250, or greater than 250.

[0132] One or more types of primer may be attached or tethered to the support surface. In some instances, the one or more types of adapters or primers may comprise spacer sequences, adapter sequences for hybridization to adapter-ligated target library nucleic acid sequences, forward amplification primers, reverse amplification primers, sequencing primers, and/or molecular barcoding sequences, or any combination thereof. In some instances, 1 primer or adapter sequence may be tethered to at least one layer of the surface. In some instances, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 different primer or adapter sequences may be tethered to at least one layer of the surface.

[0133] In some instances, the tethered adapter and/or primer sequences may range in length from about 10 nucleotides to about 100 nucleotides. In some instances, the tethered adapter and/or primer sequences may be at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides in length. In some instances, the tethered adapter and/or primer sequences may be at most 100, at most 90, at most 80, at most 70, at most 60, at most 50, at most 40, at most 30, at most 20, or at most 10 nucleotides in length. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the length of the tethered adapter and/or primer sequences may range from about 20 nucleotides to about 80 nucleotides. Those of skill in the art will recognize that the length of the tethered adapter and/or primer sequences may have any value within this range, *e.g.*, about 24 nucleotides.

[0134] In some instances, the resultant surface density of primers on the low binding support surfaces of the present disclosure may range from about 100 primer molecules per μm^2 to about 100,000 primer molecules per μm^2. In some instances, the resultant surface density of primers on the low

binding support surfaces of the present disclosure may range from about 1,000 primer molecules per μm^2 to about 1,000,000 primer molecules per μm^2. In some instances, the surface density of primers may be at least 1,000, at least 10,000, at least 100,000, or at least 1,000,000 molecules per μm^2. In some instances, the surface density of primers may be at most 1,000,000, at most 100,000, at most 10,000, or at most 1,000 molecules per μm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of primers may range from about 10,000 molecules per μm^2 to about 100,000 molecules per μm^2. Those of skill in the art will recognize that the surface density of primer molecules may have any value within this range, *e.g.*, about 455,000 molecules per μm^2. In some instances, the surface density of target library nucleic acid sequences initially hybridized to adapter or primer sequences on the support surface may be less than or equal to that indicated for the surface density of tethered primers. In some instances, the surface density of clonally-amplified target library nucleic acid sequences hybridized to adapter or primer sequences on the support surface may span the same range as that indicated for the surface density of tethered primers.

[0135] Local densities as listed above do not preclude variation in density across a surface, such that a surface may comprise a region having an oligo density of, for example, 500,000 / μm^2, while also comprising at least a second region having a substantially different local density.

VI. Illustrative Alternative Embodiments

[0136] The disclosed methods of determining the sequence of a target nucleic acid comprise: a) contacting a double-stranded or partially double-stranded target nucleic acid molecule comprising the template strand to be sequenced and a primer strand to be elongated with one or more of the disclosed nucleic acid binding compositions; and b) detecting the binding of a nucleic acid binding composition to the nucleic acid molecule, thereby determining the presence of one of said one or more nucleic acid binding compositions on said nucleic acid molecule and the identity of the next nucleotide (*i.e.*, the N+1 or terminal nucleotide) to be incorporated into the complementary strand.

[0137] The sequencing method may further comprise incorporating the N+1 or terminal nucleotide into the primer strand, and then repeating the contacting, detecting, and incorporating steps for one or more additional iterations, thereby determining the sequence of the template strand of the nucleic acid molecule. After the step of detecting the ternary binding complex, the primed strand of the primed target nucleic acid is extended for one base before another round of analysis is performed. The primed target nucleic acid can be extended using the conjugated nucleotide that is attached to the polymer in the multivalent binding composition or using an unconjugated or untethered free nucleotide that is provided after the multivalent binding composition has been removed.

[0138] The extension of the primed target nucleic acid may be prevented or inhibited due to a blocked nucleotide on the strand or the use of polymerase that is catalytically inactive. When the nucleotide in the polymer-nucleotide conjugate has a blocking group that prevents the extension of the nucleic acid, incorporation of a nucleotide may be achieved by the removal of a blocking group from said nucleotide (such as by detachment of said nucleotide from its polymer, branched polymer, dendrimer, particle, or the like). When the extension of the primed target nucleic acid is inhibited due to the use of polymerase that is catalytically inactive, incorporation of a nucleotide may be achieved by the provision of a cofactor or activator such as a metal ion.

[0139] Detection of the ternary complex is achieved prior to, concurrently with, or following the incorporation of the nucleotide residue. In some embodiments, a primed target nucleic acid may comprise a target nucleic acid with multiple primed locations for the attachment of polymerases and/or nucleic acid binding moieties. In some embodiments, multiple polymerases may be attached to a single target nucleic acid molecule, such as at multiple sites within a target nucleic acid molecule. In some embodiments, multiple polymerases may be bound to a multivalent binding composition disclosed herein comprising multiple nucleotides. In some embodiments, a target nucleic acid molecule may be a product of a strand displacement synthesis, a rolling circle amplification, a concatenation or fusion of multiple copies of a query sequence, or other such methods as are known in the art or as are disclosed elsewhere herein to produce nucleic acid molecules comprising multiple copies of an identical sequence. Therefore, in some embodiments, multiple polymerases may be attached at multiple identical or substantially identical locations within a target nucleic acid which comprises multiple identical or substantially identical copies of a query sequence. In some embodiments, said multiple polymerases may then be involved in interactions with one or more multivalent binding complexes; however, in preferred embodiments, the number of binding sites within a target nucleic acid is at least two, and the number of nucleotides or substrate moieties present on a particle-nucleotide conjugate such as a polymer-nucleotide conjugate is also greater than or equal to two.

[0140] It may be advantageous to provide the multivalent binding compositions in combination with other elements such as to provide optimized signals, for example to provide identification of a nucleotide at a particular position in a nucleic acid sequence. In some embodiments, the compositions disclosed herein are provided in combination with a surface providing low background binding or low levels of protein binding, especially a hydrophilic or polymer coated surface. Representative surfaces may be found, for example, in U.S. Patent Application No. 16/363,842, the contents of which are hereby incorporated by reference in their entirety.

[0141] In some instances, the nucleic acid molecule is tethered to the surface of a solid support, *e.g.*, through hybridization of the template strand to an adapter nucleic acid sequence or primer nucleic acid sequence that is tethered to the solid support. In some instances, the solid support comprises a glass, fused-silica, silicon, or polymer substrate. In some instances, the solid support comprises a low non-specific binding coating comprising one or more hydrophilic polymer layers (*e.g.* PEG layers) where at least one of the hydrophilic polymer layers comprises a branched polymer molecule (*e.g.*, a branched PEG molecule comprising 4, 8, 16, or 32 branches).

[0142] The solid support comprises oligonucleotide adapters or primers tethered to at least one hydrophilic polymer layer at a surface density ranging from about 1,000 primer molecules per μm^2 to about 1,000,000 primer molecules per μm^2. In some instances, the surface density of oligonucleotide primers may be at least 1,000, at least 10,000, at least 100,000, or at least 1,000,000 molecules per μm^2. In some instances, the surface density of oligonucleotide primers may be at most 1,000,000, at most 100,000, at most 10,000, or at most 1,000 molecules per μm^2. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the surface density of primers may range from about 10,000 molecules per μm^2 to about 100,000 molecules per μm^2. Those of skill in the art will recognize that the surface density of primer molecules may have any value within this range, *e.g.*, about 455,000 molecules per μm^2.

[0143] One of ordinary skill would recognize that in a series of iterative sequencing reactions, occasionally one or more sites will fail to incorporate a nucleotide during a given cycle, thus leading one or more sites to be unsynchronized with the bulk of the elongating nucleic acid chains. Under conditions in which sequencing signals are derived from reactions occurring on single copies of a target nucleic acid, these failures to incorporate will yield discrete errors in the output sequence. It is an object of the present disclosure to describe methods for reducing this type of error in sequencing reactions. For example, the use of multivalent substrates that are capable of incorporation into the elongating strand, by providing increased probabilities of rebinding upon premature dissociation of a ternary polymerase complex, can reduce the frequency of "skipped" cycles in which a base is not incorporated. Thus, in some embodiments, the present disclosure contemplates the use of multivalent substrates as disclosed herein in which the nucleoside moiety is comprised within a nucleotide having a free, or reversibly modified, 5' phosphate, diphosphate, or triphosphate moiety, and wherein the nucleotide is connected to the particle or polymer as disclosed herein, through a labile or cleavable linkage. In some embodiments, the present disclosure contemplates a reduction in the intrinsic error rate due to skipped incorporations as a result of the use of the multivalent substrates disclosed herein.

[0144] The present disclosure also contemplates sequencing reactions in which sequencing signals from or relating to a given sequence are derived from or originate within definable regions containing multiple copies of the target sequence. Sequencing methods incorporating multiple copies of a target sequence have the advantage that signals can be amplified due to the presence of multiple simultaneous sequencing reactions within the defined region, each providing its own signal. The presence of multiple signals within a defined area also reduces the impact of any single skipped cycle, due to the fact that the signal from a large number of correct base calls can overwhelm the signal from a smaller number of skipped or incorrect base calls. The present disclosure further contemplates the inclusion of free, unlabeled nucleotides during elongation reactions, or during a separate part of the elongation cycle, in order to provide incorporation at sites that may have been skipped in previous cycles. For example, during or following an incorporation cycle, unlabeled blocked nucleotides may be added such that they may be incorporated at skipped sites. The unlabeled blocked nucleotides may be of the same type or types as the nucleotide attached to the multivalent binding substrate or substrates that are or were present during a particular cycle, or a mixture of 1, 2, 3, 4 or more types of unlabeled blocked nucleotides may be included.

[0145] When each sequencing cycle proceeds perfectly, each reaction within the defined region will provide an identical signal. However, as noted elsewhere herein, in a series of iterative sequencing reactions, occasionally one or more sites will fail to incorporate a nucleotide during a given cycle, thus leading one or more sites to be unsynchronized with the bulk of the elongating nucleic acid chains. This issue, referred to as "phasing," leads to degradation of the sequencing signal as the signal is contaminated with spurious signals from sites having skipped one or more cycles. This, in turn, creates the potential for errors in base identification. The progressive accumulation of skipped cycles through multiple cycles also reduces the effective read length, due to progressive degradation of the sequencing signal with each cycle. It is a further object of this disclosure to provide methods for reducing phasing errors and/or to improve read length in sequencing reactions.

[0146] The sequencing method can include contacting a target nucleic acid or multiple target nucleic acids, comprising multiple linked or unlinked copies of a target sequence, with the multivalent binding compositions described herein. Contacting said target nucleic acid, or multiple target nucleic acids comprising multiple linked or unlinked copies of a target sequence, with one or more particle-nucleotide conjugates may provide a substantially increased local concentration of the correct nucleotide being interrogated in a given sequencing cycle, thus suppressing signals from improper incorporations or phased nucleic acid chains (*i.e.*, those elongating nucleic acid chains which have had one or more skipped cycles).

[0147] Methods of obtaining nucleic acid sequence information can include contacting a target nucleic acid, or multiple target nucleic acids, wherein said target nucleic acid or multiple target nucleic acids comprise multiple linked or unlinked copies of a target sequence, with one or more particle-nucleotide conjugates. This method results in a reduction in the error rate of sequencing as indicated by reduction in the misidentification of bases, the reporting of nonexistent bases, or the failure to report correct bases. In some embodiments, said reduction in the error orate of sequencing may comprise a reduction of 5%, 10%, 15%, 20% 25%, 50%, 75%, 100%, 150%, 200%, or more compared to the error rate observed using monovalent ligands, including free nucleotides, labeled free nucleotides, protein or peptide bound nucleotides, or labeled protein or peptide bound nucleotides.

[0148] The method of obtaining nucleic acid sequence information can include contacting a target nucleic acid, or multiple target nucleic acids, wherein said templet nucleic acid or multiple target nucleic acids comprise multiple linked or unlinked copies of a target sequence, with one or more particle-nucleotide conjugates. This method results in an increase in average read length of 5%, 10%, 15%, 20% 25%, 50%, 75%, 100%, 150%, 200%, 300%, or more compared to the average read length observed using monovalent ligands, including free nucleotides, labeled free nucleotides, protein or peptide bound nucleotides, or labeled protein or peptide bound nucleotides.

[0149] Disclosed herein are methods of obtaining nucleic acid sequence information, said methods comprising contacting a target nucleic acid, or multiple target nucleic acids, wherein said target nucleic acid or multiple target nucleic acids comprise multiple linked or unlinked copies of a target sequence, with one or more particle-nucleotide conjugates. This method results in an increase in average read length of 10 nucleotides (NT), 20 NT, 25 NT, 30 NT, 50 NT, 75 NT, 100 NT, 125 NT, 150 NT, 200 NT, 250 NT, 300 NT, 350 NT, 400 NT, 500 NT , or more compared to the average read length observed using monovalent ligands, including free nucleotides, labeled free nucleotides, protein or peptide bound nucleotides, or labeled protein or peptide bound nucleotides.

[0150] In some instances, the disclosed compositions and methods may result in average read lengths for sequencing applications that range from 100 nucleotides to 1,000 nucleotides. In some instances, the average read length may be at least 100 nucleotides, at least 200 nucleotides, at least 225 nucleotides, at least 250 nucleotides, at least 275 nucleotides, at least 300 nucleotides, at least 325 nucleotides, at least 350 nucleotides, at least 375 nucleotides, at least 400 nucleotides, at least 425 nucleotides, at least 450 nucleotides, at least 475 nucleotides, at least 500 nucleotides, at least 525 nucleotides, at least 550 nucleotides, at least 575 nucleotides, at least 600 nucleotides, at least 625 nucleotides, at least 650 nucleotides, at least 675 nucleotides, at least 700 nucleotides, at least 725 nucleotides, at least 750 nucleotides, at least 775 nucleotides, at least 800 nucleotides, at least 825

nucleotides, at least 850 nucleotides, at least 875 nucleotides, at least 900 nucleotides, at least 925 nucleotides, at least 950 nucleotides, at least 975 nucleotides, or at least 1,000 nucleotides. In some instances, the average read length may be a range bounded by any two of the values within this range, *e.g.*, an average read length ranging from 375 nucleotides to 825 nucleotides. Those of skill in the art will recognize that in some instances, the average read length may have any value within the range specified in this paragraph, *e.g.*, 523 nucleotides.

[0151] The use of multivalent binding composition for sequencing effectively shortens the sequencing time. The sequencing reaction cycle comprising the contacting, detecting, and incorporating steps is performed in a total time ranging from about 5 minutes to about 60 minutes. In some instances, the sequencing reaction cycle is performed in at least 5 minutes, at least 10 minutes, at least 20 minutes, at least 30 minutes, at least 40 minutes, at least 50 minutes, or at least 60 minutes. In some instances, the sequencing reaction cycle is performed in at most 60 minutes, at most 50 minutes, at most 40 minutes, at most 30 minutes, at most 20 minutes, at most 10 minutes, or at most 5 minutes. Any of the lower and upper values described in this paragraph may be combined to form a range included within the present disclosure, for example, in some instances the sequencing reaction cycle may be performed in a total time ranging from about 10 minutes to about 30 minutes. Those of skill in the art will recognize that the sequencing cycle time may have any value within this range, *e.g.*, about 16 minutes.

[0152] In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide an average base-calling accuracy of at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 96%, at least 98%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% correct over the course of a sequencing run. In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide an average base-calling accuracy of at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 96%, at least 98%, at least 99%, at least 99.5%, at least 99.8%, or at least 99.9% correct per every 1,000 bases, 10,0000 bases, 25,000 bases, 50,000 bases, 75,000 bases, or 100,000 bases called.

[0153] The use of multivalent binding composition for sequencing provides more accurate base readout. The disclosed compositions and methods for nucleic acid sequencing will provide an average Q-score for base-calling accuracy over a sequencing run that ranges from about 20 to about 50. In some instances, the average Q-score is at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50. Those of skill in the art will recognize that the average Q-score may have any value within this range, *e.g.*, about 32.

[0154] In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide a Q-score of greater than 30 for at least 50%, at least 60%, at least 70%, at least 80%, at least

85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide a Q-score of greater than 35 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide a Q-score of greater than 40 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide a Q-score of greater than 45 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified. In some instances, the disclosed compositions and methods for nucleic acid sequencing will provide a Q-score of greater than 50 for at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% of the terminal (or N+1) nucleotides identified.

[0155] The disclosed low non-specific binding supports and associated nucleic acid hybridization and amplification methods may be used for the analysis of nucleic acid molecules derived from any of a variety of different cell, tissue, or sample types known to those of skill in the art. For example, nucleic acids may be extracted from cells, or tissue samples comprising one or more types of cells, derived from eukaryotes (such as animals, plants, fungi, protista), archaebacteria, or eubacteria. In some cases, nucleic acids may be extracted from prokaryotic or eukaryotic cells, such as adherent or non-adherent eukaryotic cells. Nucleic acids are variously extracted from, for example, primary or immortalized rodent, porcine, feline, canine, bovine, equine, primate, or human cell lines. Nucleic acids may be extracted from any of a variety of different cell, organ, or tissue types (*e.g.*, white blood cells, red blood cells, platelets, epithelial cells, endothelial cells, neurons, glial cells, astrocytes, fibroblasts, skeletal muscle cells, smooth muscle cells, gametes, or cells from the heart, lungs, brain, liver, kidney, spleen, pancreas, thymus, bladder, stomach, colon, or small intestine). Nucleic acids may be extracted from normal or healthy cells. Alternately or in combination, nucleic acids are extracted from diseased cells, such as cancerous cells, or from pathogenic cells that are infecting a host. Some nucleic acids may be extracted from a distinct subset of cell types, *e.g.*, immune cells (such as T cells, cytotoxic (killer) T cells, helper T cells, alpha beta T cells, gamma delta T cells, T cell progenitors, B cells, B-cell progenitors, lymphoid stem cells, myeloid progenitor cells, lymphocytes, granulocytes, Natural Killer cells, plasma cells, memory cells, neutrophils, eosinophils, basophils, mast cells, monocytes, dendritic cells, and/or macrophages, or any combination thereof), undifferentiated human stem cells, human stem cells that have been induced to differentiate, rare

cells (*e.g.*, circulating tumor cells (CTCs), circulating epithelial cells, circulating endothelial cells, circulating endometrial cells, bone marrow cells, progenitor cells, foam cells, mesenchymal cells, or trophoblasts). Nucleic acids may further comprise nucleic acids derived from viral samples and from subviral pathogens, such as viroids and infectious RNAs. Nucleic acids may be derived from clinical or other samples, such as sputum, saliva, ocular fluid, synovial fluid, blood, feces, urine, tissue exudate, sweat, pus, drainage fluid or the like. Nucleic acids may further be derived from plant or fungal samples, such as leaf, cambium, root, meristem, pollen, ovum, seed, spore, inflorescence, mycelium, or the like. Nucleic acids may also be derived from environmental or industrial samples, such as water, air, dust, food, or the like. Other cells, tissues, and samples are contemplated and consistent with the disclosure herein.

[0156] Nucleic acid extraction from cells or other biological samples may be performed using any of a number of techniques known to those of skill in the art. For example, a DNA extraction procedure may comprise (i) collection of the cell sample or tissue sample from which DNA is to be extracted, (ii) disruption of cell membranes (*i.e.*, cell lysis) to release DNA and other cytoplasmic components, (iii) treatment of the lysed sample with a concentrated salt solution to precipitate proteins, lipids, and RNA, followed by centrifugation to separate out the precipitated proteins, lipids, and RNA, and (iv) purification of DNA from the supernatant to remove detergents, proteins, salts, or other reagents used during the cell membrane lysis step.

[0157] A variety of suitable commercial nucleic acid extraction and purification kits are consistent with the disclosure herein. Examples include, but are not limited to, the QIAamp kits (for isolation of genomic DNA from human samples) and DNAeasy kits (for isolation of genomic DNA from animal or plant samples) from Qiagen (Germantown, MD), or the Maxwell® and ReliaPrep™ series of kits from Promega (Madison, WI).

VII. Systems

[0158] *System modules:* As noted above, also disclosed herein are systems configured for performing any of the disclosed nucleic acid sequencing or nucleic acid detection and analysis methods. In some instances, the disclosed systems may comprise one or more of the multivalent binding compositions described herein, one or more buffers, and/or one or more nucleic acid molecules tethered to a solid support.

[0159] In some instances, the system may further comprise a fluid flow controller and/or fluid dispensing system configured to sequentially and iteratively contact template nucleic acid molecules hybridized to nucleic acid molecules (*e.g.*, adapters or primers) tethered to a solid support with the disclosed multivalent binding compositions and/or reagents. In some instances, said contacting may

be performed within one or more flow cells. In some instances, said flow cells may be fixed components of the system. In some instances, said flow cells may be removable and/or disposable components of the system.

[0160] In some instances, the system may further comprise an imaging module, where the imaging module comprises, *e.g.*, one or more light sources, one or more optical components (*e.g.*, lenses, mirrors, prisms, optical filters, colored glass filters, narrowband interference filters, broadband interference filters, dichroic reflectors, diffraction gratings, apertures, optical fibers, or optical waveguides and the like), and one or more image sensors (*e.g.*, charge-coupled device (CCD) sensors or cameras, complementary metal–oxide–semiconductor (CMOS) image sensors or cameras, or negative-channel metal-oxide semiconductor (NMOS) image sensors or cameras) for imaging and detection of binding of the disclosed multivalent binding compositions to target (or template) nucleic acid molecules tethered to a solid support or the interior of a flow cell.

[0161] *Processors and computer systems:* One or more processors may be employed to implement the systems for nucleic acid sequencing or other nucleic acid detection and analysis methods disclosed herein. The one or more processors may comprise a hardware processor such as a central processing unit (CPU), a graphic processing unit (GPU), a general-purpose processing unit, or computing platform. The one or more processors may be comprised of any of a variety of suitable integrated circuits (*e.g.*, application specific integrated circuits (ASICs) designed specifically for implementing deep learning network architectures, or field-programmable gate arrays (FPGAs) to accelerate compute time, *etc.*, and/or to facilitate deployment), microprocessors, emerging next-generation microprocessor designs (*e.g.*, memristor-based processors), logic devices and the like. Although the disclosure is described with reference to a processor, other types of integrated circuits and logic devices may also be applicable. The processor may have any suitable data operation capability. For example, the processor may perform 512 bit, 256 bit, 128 bit, 64 bit, 32 bit, or 16 bit data operations. The one or more processors may be single core or multi core processors, or a plurality of processors configured for parallel processing.

[0162] The one or more processors or computers used to implement the disclosed methods may be part of a larger computer system and/or may be operatively coupled to a computer network (a "network") with the aid of a communication interface to facilitate transmission of and sharing of data. The network may be a local area network, an intranet and/or extranet, an intranet and/or extranet that is in communication with the Internet, or the Internet. The network in some cases is a telecommunication and/or data network. The network may include one or more computer servers, which in some cases enables distributed computing, such as cloud computing. The network, in some

cases with the aid of the computer system, may implement a peer-to-peer network, which may enable devices coupled to the computer system to behave as a client or a server.

[0163] The computer system may also include memory or memory locations (*e.g.*, random-access memory, read-only memory, flash memory, Intel® Optane™ technology), electronic storage units (*e.g.*, hard disks), communication interfaces (*e.g.*, network adapters) for communicating with one or more other systems, and peripheral devices, such as cache, other memory, data storage and/or electronic display adapters. The memory, storage units, interfaces and peripheral devices may be in communication with the one or more processors, *e.g.*, a CPU, through a communication bus, *e.g.*, as is found on a motherboard. The storage unit(s) may be data storage unit(s) (or data repositories) for storing data.

[0164] The one or more processors, *e.g.*, a CPU, execute a sequence of machine-readable instructions, which are embodied in a program (or software). The instructions are stored in a memory location. The instructions are directed to the CPU, which subsequently program or otherwise configure the CPU to implement the methods of the present disclosure. Examples of operations performed by the CPU include fetch, decode, execute, and write back. The CPU may be part of a circuit, such as an integrated circuit. One or more other components of the system may be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

[0165] The storage unit stores files, such as drivers, libraries and saved programs. The storage unit stores user data, *e.g.*, user-specified preferences and user-specified programs. The computer system in some cases may include one or more additional data storage units that are external to the computer system, such as located on a remote server that is in communication with the computer system through an intranet or the Internet.

[0166] Some aspects of the methods and systems provided herein may be implemented by way of machine (*e.g.*, processor) executable code stored in an electronic storage location of the computer system, such as, for example, in the memory or electronic storage unit. The machine-executable or machine-readable code may be provided in the form of software. During use, the code is executed by the one or more processors. In some cases, the code is retrieved from the storage unit and stored in the memory for ready access by the one or more processors. In some situations, the electronic storage unit is precluded, and machine-executable instructions are stored in memory. The code may be pre-compiled and configured for use with a machine having one or more processors adapted to execute the code or may be compiled at run time. The code may be supplied in a programming language that is selected to enable the code to execute in a pre-compiled or as-compiled fashion.

[0167] Various aspects of the technology may be thought of as "products" or "articles of manufacture", *e.g.*, "computer program or software products", often in the form of machine- (or

processor-) executable code and/or associated data that is stored in a type of machine readable medium, where the executable code comprises a plurality of instructions for controlling a computer or computer system in performing one or more of the methods disclosed herein. Machine-executable code may be stored in an optical storage unit comprising an optically readable medium such as an optical disc, CD-ROM, DVD, or Blu-Ray disc. Machine-executable code may be stored in an electronic storage unit, such as memory (*e.g.*, read-only memory, random-access memory, flash memory) or on a hard disk. "Storage" type media include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memory chips, optical drives, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software that encodes the methods and algorithms disclosed herein.

[0168] All or a portion of the software code may at times be communicated via the Internet or various other telecommunication networks. Such communications, for example, enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, other types of media that are used to convey the software encoded instructions include optical, electrical and electromagnetic waves, such as those used across physical interfaces between local devices, through wired and optical landline networks, and over various atmospheric links. The physical elements that carry such waves, such as wired or wireless links, optical links, or the like, are also considered media that convey the software encoded instructions for performing the methods disclosed herein. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

[0169] The computer system often includes, or may be in communication with, an electronic display for providing, for example, images captured by a machine vision system. The display is often also capable of providing a user interface (UI). Examples of UI's include but are not limited to graphical user interfaces (GUIs), web-based user interfaces, and the like.

[0170] *System control software:* In some instances, the disclosed systems may comprise a computer (or processor) and computer-readable media that includes code for providing a user interface as well as manual, semi-automated, or fully-automated control of all system functions, *e.g.* control of a fluid flow controller and/or fluid dispensing system (or sub-system), a temperature control system (or sub-system), an imaging system (or sub-system), *etc.* In some instances, the system computer or processor may be an integrated component of the instrument system (*e.g.* a microprocessor or mother board embedded within the instrument). In some instances, the system computer or processor may be a stand-alone module, for example, a personal computer or laptop computer. Examples of fluid flow control functions that may be provided by the instrument control software include, but are not

limited to, volumetric fluid flow rates, fluid flow velocities, the timing and duration for sample and reagent additions, rinse steps, and the like. Examples of temperature control functions that may be provided by the instrument control software include, but are not limited to, specifying temperature set point(s) and control of the timing, duration, and ramp rates for temperature changes. Examples of imaging system control functions that may be provided by the instrument control software include, but are not limited to, autofocus capability, control of illumination or excitation light exposure times and intensities, control of image acquisition rate, exposure time, data storage options, and the like.

[0171] *Image processing software:* In some instances of the disclosed systems, the system may further comprise computer-readable media that includes code for providing image processing and analysis capability. Examples of image processing and analysis capability that may be provided by the software include, but are not limited to, manual, semi-automated, or fully-automated image exposure adjustment (*e.g.* white balance, contrast adjustment, signal-averaging and other noise reduction capability, *etc.*), manual, semi-automated, or fully-automated edge detection and object identification (*e.g.*, for identifying clusters of amplified template nucleic acid molecules on a substrate surface), manual, semi-automated, or fully-automated signal intensity measurements and/or thresholding in one or more detection channels (*e.g.*, one or more fluorescence emission channels), manual, semi-automated, or fully-automated statistical analysis (*e.g.*, for comparison of signal intensities to a reference value for base-calling purposes).

[0172] In some instances, the system software may provide integrated real-time image analysis and instrument control, so that sample loading, reagent addition, rinse, and/or imaging / base-calling steps may be prolonged, modified, or repeated as necessary until, *e.g.*, optimal base-calling results are achieved. Any of a variety of image processing and analysis algorithms known to those of skill in the art may be used to implement real-time or post-processing image analysis capability. Examples include, but are not limited to, the Canny edge detection method, the Canny-Deriche edge detection method, first-order gradient edge detection methods (*e.g.* the Sobel operator), second order differential edge detection methods, phase congruency (phase coherence) edge detection methods, other image segmentation algorithms (*e.g.* intensity thresholding, intensity clustering methods, intensity histogram-based methods, *etc.*), feature and pattern recognition algorithms (*e.g.* the generalized Hough transform for detecting arbitrary shapes, the circular Hough transform, *etc.*), and mathematical analysis algorithms (*e.g.* Fourier transform, fast Fourier transform, wavelet analysis, auto-correlation, *etc.*), or combinations thereof.

[0173] In some instances, the system control and image processing/analysis software may be written as separate software modules. In some instances, the system control and image processing/analysis software may be incorporated into an integrated software package.

VIII. Examples

1. Preparation of Multivalent Binding Composition

[0174] One type of multi-armed substrate, as shown in **FIG. 5A** were made by reacting propargylamine dNTPs with Biotin-PEG-NHS. This aqueous reaction was driven to completion and purified; resulting in a pure Biotin-PEG-dNTP species. In separate reactions, several different PEG lengths were used, corresponding to average molecular weights varying from 1K Da to 20K Da. The Biotin-PEG-dNTP species were mixed with either freshly prepared or commercially-sourced dye-labeled streptavidin (SA) using a Dye:SA ratio of 3-5:1. Mixing of Biotin-PEG-dNTP with dye-labeled streptavidin was done in the presence of excess biotin-PEG-dNTP to ensure saturation of the biotin binding sites on each streptavidin tetramer. Complete complexes were purified away from excess biotin-PEG-dNTP by size exclusion chromatography. Each nucleotide type was conjugated and purified separately, then mixed together to create a four-base mix for sequencing.

[0175] Another type of multi-armed substrate as shown in **FIG. 5A** was made in a single pot by reacting multi-arm PEG NHS with excess Dye-NH_2 and propargylamine dNTPs. Various multi-arm PEG NHS variants were used ranging from 4-16 arms and ranging in molecular weight from 5K Da to 40K Da. After reacting, excess small molecule dye and dNTP were removed by size exclusion chromatography. Each nucleotide type was conjugated and purified independently then mixed together to create a four-base mix for sequencing.

[0176] Class II substrates as shown in **FIG. 5B** were made using one pot reactions to simultaneously conjugate dye and dNTP. Alkyne-PEG-NHS was reacted with excess propargylamine dNTP. This product (Alkyne-PEG-dNTP) was then purified to homogeneity by chromatography. Multiple PEG lengths were used, with average molecular weights varying between 1K Da and 20K Da. Dendrimer cores containing a variable, discrete number (12, 24, 48, 96) of azide conjugation sites were used. Conjugation of Alkyne-Dye and Alkyne-PEG-dNTP to the dendrimer core occurred in a one pot reaction containing excess dye and dNTP species via copper-mediated click chemistry. After reacting, excess small molecule dye and dNTP were removed by size exclusion chromatography. Each nucleotide type was conjugated and purified independently then mixed together to create a four-base mix for sequencing. We note that this scheme allows the ready substitution of alternative cores, such as dextrans, other polymers, proteins, *etc.*

[0177] Class III polymer-nucleotide conjugates as shown in **FIG. 5C** were constructed by reacting 4- or 8-arm PEG NHS with a saturating mixture of biotin and propargylamine dNTP. This reaction was then purified by size exclusion chromatography. The result of this reaction was a multi-arm PEG containing a discrete distribution of biotin and nucleotide. This heterogeneous population was then reacted with dye-labeled streptavidin and purified by size exclusion chromatography. Each

nucleotide type was conjugated and purified independently then mixed together to create a four-base mix for sequencing. We note that the distribution of biotin and nucleotide is tunable by the input ration of Biotin-NH_2 to propargylamine dNTP.

2. Detection of Ternary Complex

[0178] Binding reactions using the multivalent binding composition having PEG polymer-nucleotide conjugates were analyzed to detect possible formation of ternary binding complex, and the fluorescence images of the various steps are illustrated in **FIGS. 7A-7J**. In **FIG. 7A**, red and green fluorescent images post exposure of DNA rolling circle application (RCA) templates (G and A first base) to 500 nM base labeled nucleotides (A-Cy3 and G-Cy5) in exposure buffer containing 20 nM Klenow polymerase and 2.5 mM Sr^{+2}. Multivalent PEG-substrate compositions were prepared using varying ratios of 4-armed PEG-amine (4ArmPEG-NH), biotin-PEG-amine (Biotin-PEG-NH), and nucleotide (Nuc) as follows: Samples PB1 and PB5, 4ArmPEG-NH: Biotin-PEG-NH: Nuc = 0.25: 1: 0.5; Sample PB2, 4ArmPEG-NH: Biotin-PEG-NH: Nuc =0.125: 0.5: 0.25; Sample PB3, 4ArmPEG-NH: Biotin-PEG-NH: Nuc = 0.25: 1: 0.5. Images were collected after washing with imaging buffer with the same composition as the exposure buffer but containing no nucleotides or polymerase.

[0179] Contrast was scaled to maximize visualization of the dimmest signals, but no signals persisted following washing with imaging buffer (**FIG. 7A**, inset). In **FIGS. 7B-7E**, the fluorescence images showing multivalent PEG-nucleotide (base-labeled) ligands at 500 nM after mixing in the exposure buffer and imaging in the imaging buffer as above (**FIG. 7B**: PB1; **FIG. 7C**: PB2; **FIG. 7D**: PB3; **FIG. 7E**: PB5). **FIG. 7F**: fluorescence image showing multivalent PEG-nucleotide (base-labeled) ligand PB5 at 2.5uM after mixing in the exposure buffer and imaging in the imaging buffer as above. In **FIGS. 7G-7I**, the fluorescence images showing further base discrimination by exposure of multivalent ligands to inactive mutants of Klenow polymerase (**FIG. 7G**: D882H; **FIG. 7H**: D882E; **FIG. 7I**: D882A, and the wild type Klenow (control) enzyme is shown in **FIG. 7J)**.

[0180] Using multivalent ligands formulations, the base discrimination can be enabled by providing polymerase-ligand interactions having increased avidity. In addition, it is shown that increased concentration of multivalent ligands can generate higher signals as well as various Klenow mutations that knock out catalytic activity can be used for avidity-based sequencing.

3. Sequencing of Target Nucleic Acid Molecules Using Ternary Complexes

[0181] In order to demonstrate sequencing based on multivalent ligand reporters, 4 known templates were amplified using RCA methods on a low binding substrate. Successive cycles were exposed to

exposure buffer containing 20 nM Klenow polymerase and 2.5 mM $Sr^{+2}$and washed with imaging buffer and imaged. After imaging, the substrates were washed with wash buffer (EDTA and high salt) and blocked nucleotides were added to proceed to the next base. The cycle was repeated for 5 cycles. Spots were detected using standard imaging processing and spot detection and the sequences were called using a two-color green and red scheme (G-Cy3 and A-Cy5) to identify the templates being cycled. As shown in **FIG. 8A** and **FIG. 8B**, multivalent ligands are able to provide base discrimination through all 5 sequencing cycles.

4. Control of Nucleotide Dissociation from Ternary Complex

[0182] Ternary complexes are prepared and imaged as in Example 2. The complexes are imaged over varying lengths of time to demonstrate the persistence of the ternary complex, *e.g.*, as long as 60 seconds. After a length of time, the complexes are washed with a buffer identical to the buffer used for the formation of the complexes, only lacking any divalent cation, *e.g.*, 10mM Tris pH 8.0, 0.5mM EDTA, 50mM NaCl, 0.016% Triton X100 (without SrOAc), or, alternatively, the complexes are washed with a buffer identical to the buffer used for the formation of the complexes, which contains a chelating agent but otherwise lacks any divalent cation, *e.g.*, 10mM Tris pH 8.0, 0.5mM EDTA, 50mM NaCl, 0.016% Triton X100 (without SrOAc), with 100nm-100mM EDTA. The fluorescence from the complexes is observed over time allowing observation and quantitation of the dissociation of the ternary complexes. A representative time course of this dissolution is shown in **FIG. 6**.

5. Extension of Target Nucleic Acid Complementary Sequence

[0183] After preparing, imaging, and dissociating ternary complexes as in Example 4, a deblocking solution is flowed into the chamber containing the bound DNA molecules, sufficient to remove the blocking moiety, such as an O-azidomethyl group, an O-alkyl hydroxylamino group, or an O-amino group, from the 3' end of the elongating DNA strand. Either following or concurrently with this, an extension solution is flowed into the chamber containing the bound DNA molecules. The extension solution contains a buffer, a divalent cation sufficient to support polymerase activity, an active polymerase, and an appropriate amount of all four nucleotides, where the nucleotides are blocked such that they are incapable of supporting further elongation after the addition of a single nucleotide to the elongating DNA strand, such as by incorporation of a 3'-O-azidomnethyl group, a 3'-O-alkyl hydroxylamino group, or a 3'-O-amino group. The elongating strand is thus extended by one and only one base, and the binding of catalytically inactive polymerase and multivalent binding substrate can be used to call the next base in the cycle.

[0184] Alternatively, the nucleotides attached to the multivalent substrate may be attached through a labile bond, such that a buffer may be flowed into the chamber containing the bound DNA molecules containing a divalent cation or other cofactor sufficient to render the polymerase catalytically active. Prior to, after, or concurrently with this, conditions may be provided that are sufficient to cleave the base from the multivalent substrate such that it may be incorporated into the elongating strand. This cleavage and incorporation results in the dissociation of the label and the polymer backbone of the multivalent substrate while extending the elongating DNA strand by exactly one base. Washing to remove used polymer backbone is carried out, and new multivalent substrate is flowed into the chamber containing the bound DNA molecules, allowing the new base to be called as in Example 1.

6. Use of Polymer-Nucleotide Conjugates with Various Lengths of PEG Branch

[0185] The polymer-nucleotide conjugates having varying PEG arm lengths described in Example 3 were subjected to a single sequencing cycle and imaged as described in Example 1. As shown in **FIGS. 9A-9J**, increasing the length of the PEG branches led to increased signal up to a length corresponding to an apparent average PEG MW of 5K Da (**FIGS. 9A-9D**). The use of longer PEG arms than this led to decreases in the fluorescence signal for both Cy3-A and Cy5-G (**FIG. 9E-9G**). Quantitative measurements of signal intensity are shown graphically in **FIG. 10**.

7. Enhancement of Multivalent Substrate Binding by Addition of Detergent

[0186] Multivalent substrates were prepared and assembled into binding complexes in the presence and absence of detergent: one set using 10mM Tris pH 8.0, 0.5mM EDTA, 50mM NaCl, 5mM SroAc, 0% TritonX100 (Condition A), and one set using 10mM Tris pH 8.0, 0.5mM EDTA, 50mM NaCl, 5mM SroAc, 0.016% Triton X100. **Fig. 11** shows normalized fluorescence from these multivalent substrates bound to DNA clusters, with the substrate complexes formed in the presence (condition B) of Triton-X100 (0.016%) showing clearly enhanced fluorescence intensity.

8. Evaluation of Multivalent Substrate Binding Time Courses

[0187] Multivalent substrates were prepared and assembled into binding complexes as in Example 2. Complexes were also formed under identical buffer conditions using free labeled nucleotides. Complexes were imaged over the course of 60 min. to characterize the persistence time of the complexes. **FIGS. 12A-12B** shows representative results. Multivalent binding complexes are stable over timescales of >60 minutes (**FIG. 12B**) while labeled free nucleotides dissociate in less than one minute (**FIG. 12A**).

VIII. Conclusion

[0188] The present disclosure provides greatly improved methods and compositions for DNA sequencing and biosensor applications. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. By way of example, the inventive concepts have been described primarily with reference to the use of polymer-nucleotide conjugates, but it will be readily recognized by those of skill in the art that other types of particle-nucleotide conjugates could also be used. For example, in some embodiments it may be desirable to use particle-nucleotide conjugates which include quantum dot; a liposome; or an emulsion particle. Alternatively, the conjugation could be achieved by noncovalent bond such as hydrogen bond or other interactions. The scope of the disclosed inventive concepts should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of determining an identity of a nucleotide in a target nucleic acid sequence comprising:

a. providing a composition comprising:

i. two or more copies of said target nucleic acid sequence;

ii. two or more primer nucleic acid molecules that are complementary to one or more regions of said target nucleic acid sequence; and

iii. two or more polymerase molecules;

b. contacting said composition with a polymer nucleotide conjugate under conditions sufficient to allow a multivalent binding complex to be formed between said polymer-nucleotide conjugate and said two or more copies of said target nucleic acid sequence in said composition of (a), wherein the polymer-nucleotide conjugate comprises two or more copies of a nucleotide moiety and optionally one or more detectable labels; and

c. detecting said multivalent binding complex, thereby determining the identity of said nucleotide in the target nucleic acid sequence.

2. The method of claim 1, wherein the target nucleic acid sequence is DNA.

3. The method of claim 1 or claim 2, wherein the detection of the multivalent binding complex is performed in the absence of unbound or solution-borne polymer nucleotide conjugates.

4. The method of any one of claims 1 to 3, wherein the target nucleic acid sequence has been replicated or amplified or has been produced by replication or amplification.

5. The method of any one of claims 1 to 4, wherein the one or more detectable labels are fluorescent labels.

6. The method of any one of claims 1 to 5, wherein detecting the multivalent complex comprises a fluorescence measurement.

7. The method of any one of claims 1 to 6, wherein the contacting comprises use of one type of polymer-nucleotide conjugate.

8. The method of any one of claims 1 to 7, wherein the contacting comprises use of two or more types of polymer-nucleotide conjugates.

9. The method of claim 8, wherein each type of the two or more types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety.

10. The method of claim 9, wherein the contacting comprises use of three types of polymer-nucleotide conjugate and wherein each type of the three types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety.

11. The method of any one of claims 1 to 10, wherein the polymer-nucleotide conjugate comprises a blocked nucleotide moiety.

12. The method of claim 11, wherein the blocked nucleotide is a 3 '-0-azidomethyl nucleotide, a 3 '-0-methyl nucleotide, or a 3 '-0-alkyl hydroxylamine nucleotide.

13. The method of any one of claims 1 to 12, wherein said contacting occurs in the presence of an ion that stabilizes said multivalent binding complex.

14. The method of any one of claims 1 to 13, wherein the contacting is done in the presence of strontium ions, magnesium ions, calcium ions, or any combination thereof.

15. The method of any one of claims 1 to 14, wherein the polymerase molecules are catalytically inactive.

16. The method of any one of claims 1 to 15, wherein the polymerase molecules have been rendered catalytically inactive by mutation or chemical modification.

17. The method of any one of claims 1 to 16, wherein the polymerase molecules have been rendered catalytically inactive by the absence of a necessary ion or cofactor.

18. The method of any one of claims 1 to 17, wherein the polymerase molecules are catalytically active.

19. The method of any one of claims 1 to 18, wherein the polymer-nucleotide conjugate does not comprise a blocked nucleotide moiety.

20. The method of any one of claims 1 to 19, wherein the multivalent binding complex has a persistence time of greater than 2 seconds.

21. The method of any one of claims 1 to 20, wherein the method can be carried out at a temperature within a range of 25°C to 62°C.

22. The method of any one of claims 1 to 21, wherein the polymer-nucleotide conjugate further comprises one or more fluorescent labels and the two or more copies of the target nucleic acid sequence are deposited on, attached to, or hybridized to a surface, wherein a fluorescence image of

the multivalent binding complex on the surface has a contrast to noise ratio in the detecting step of greater than 20.

23. The method of any one of claims 1 to 22, wherein the composition of (a) is deposited on a surface using a buffer that incorporates a polar aprotic solvent.

24. The method of any one of claims 1 to 23, wherein the contacting is performed under a condition that stabilizes said multivalent binding complex when said nucleotide moiety is complementary to a next base of said target nucleic acid sequence and destabilizes said multivalent binding complex when said nucleotide moiety is not complementary to said next base of said target nucleic acid sequence.

25. The method of any one of claims 1 to 24, wherein said polymer-nucleotide conjugate comprises a polymer having a plurality of branches and said two or more nucleotide moieties are attached to said branches.

26. The method of claim 25, wherein said polymer has a star, comb, cross-linked, bottle brush, or dendrimer configuration.

27. The method of any one of claims 1 to 26, wherein said polymer-nucleotide conjugate comprises one or more binding groups selected from the group consisting of an avidin, a biotin, an affinity tag, and combinations thereof.

28. The method of any one of claims 1 to 27, further comprising a dissociation step that destabilizes said multivalent binding complex formed between the composition of (a) and the polymer-nucleotide conjugate, said dissociation step enabling removal of said polymer-nucleotide conjugate.

29. The method of claim 28, further comprising an extension step to incorporate a nucleotide that is complementary to a next base of the target nucleic acid sequence into said two or more primer nucleic acid molecules.

30. The method of claim 29, wherein the extension step occurs concurrently with or after said dissociation step.

31. A method of determining an identity of a nucleotide in a target nucleic acid sequence comprising:

a. providing a composition comprising:

i. two or more copies of said target nucleic acid sequence;

ii. two or more primer nucleic acid molecules that are complementary to one or more regions of said target nucleic acid sequence; and

iii. two or more polymerase molecules;

b. contacting said composition with a polymer nucleotide conjugate under conditions sufficient to allow a multivalent complex to be formed between said polymer-nucleotide conjugate and said two or more copies of said target nucleic acid sequence in said composition of (a), wherein the polymer-nucleotide conjugate comprises two or more copies of a reversibly terminated nucleotide moiety and optionally one or more cleavable detectable labels; and

c. detecting said multivalent complex, thereby determining the identity of said nucleotide in the target nucleic acid sequence.

32. The method of claim 31, wherein the target nucleic acid sequence is DNA.

33. The method of claim 31 or claim 32, further comprising contacting the composition of (a) with reversibly terminated nucleotides or polymer-nucleotide conjugates comprising two or more copies of a reversibly terminated nucleotide following the detection of said multivalent binding complex.

34. The method of any one of claims 31 to 33, wherein the target nucleic acid sequence has been replicated or amplified or has been produced by replication or amplification.

35. The method of any one of claims 31 to 34, wherein the one or more detectable labels are fluorescent labels.

36. The method of any one of claims 31 to 35, wherein detecting the multivalent complex comprises a fluorescence measurement.

37. The method of any one of claims 31 to 36, wherein the contacting comprises use of one type of polymer-nucleotide conjugate.

38. The method of any one of claims 31 to 37, wherein the contacting comprises use of two or more types of polymer-nucleotide conjugates.

39. The method of claim 38, wherein each type of the two or more types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety.

40. The method of claim 39, wherein the contacting comprises use of three types of polymer-nucleotide conjugate and wherein each type of the three types of polymer-nucleotide conjugate comprises a different type of nucleotide moiety.

41. The method of any one of claims 31 to 40, wherein the polymer-nucleotide conjugate comprises a blocked nucleotide moiety.

42. The method of claim 41, wherein the blocked nucleotide is a 3 '-0-azidomethyl, 3 '-0-methyl, or 3 '-0-alkyl hydroxylamine.

43. The method of any one of claims 31 to 42, wherein said contacting occurs in the presence of an ion that stabilizes said multivalent binding complex.

44. The method of any one of claims 31 to 43, wherein the polymerase molecules are catalytically inactive.

45. The method of any one of claims 31 to 44, wherein the polymerase molecules have been rendered catalytically inactive by mutation or chemical modification.

46. The method of any one of claims 31 to 45, wherein the polymerase molecules are catalytically active.

47. The method of any one of claims 31 to 46, wherein the polymer-nucleotide conjugate does not comprise a blocked nucleotide moiety.

48. The method of any one of claims 31 to 47, wherein the method can be carried out at a temperature within a range of 25°C to 80°C.

49. The method of any one of claims 31 to 48, wherein the polymer-nucleotide conjugate further comprises one or more fluorescent labels and the two or more copies of the target nucleic acid sequence are deposited on, attached to, or hybridized to a surface, wherein a fluorescence image of the multivalent binding complex on the surface has a contrast to noise ratio in the detecting step of greater than 20.

50. A system comprising:

a) one or more computer processors individually or collectively programmed to implement a method comprising:

i) contacting a substrate comprising multiple copies of a target nucleic acid sequence tethered to a surface of the substrate with a reagent comprising a polymerase and one or more primer nucleic acid sequences that are complementary to one or more regions of said target nucleic acid sequence to form a primed target nucleic acid sequence;

ii) contacting the substrate surface with a reagent comprising a polymer nucleotide conjugate under conditions sufficient to allow a multivalent binding complex to be formed between said polymer-nucleotide conjugate and two or more copies of said

primed target nucleic acid sequence, wherein the polymer-nucleotide conjugate comprises two or more copies of a known nucleotide moiety and a detectable label;

iii) acquiring and processing an image of the substrate surface to detect said multivalent binding complex, thereby determining the identity of a nucleotide in the target nucleic acid sequence.

51. The system of claim 50, further comprising a fluidics module configured to deliver a series of reagents to the substrate surface in a specified sequence and for specified time intervals.

52. The system of claim 50 or claim 51, further comprising an imaging module configured to acquire images of the substrate surface.

53. The system of any one of claims 50 to 52, wherein (ii) and (iii) are repeated two or more times thereby determining the identity of a series of two or more nucleotides in the target nucleic acid sequence.

54. The system of any one of claims 50 to 53, wherein the series of steps further comprises a dissociation step that destabilizes said multivalent binding complex, said dissociation step enabling removal of said polymer-nucleotide conjugate.

55. The system of claim 54, wherein the series of steps further comprises an extension step to incorporate a nucleotide that is complementary to a next base of the target nucleic acid sequence into said two or more primer nucleic acid molecules.

56. The system of claim 55, wherein the extension step occurs concurrently with or after said dissociation step.

57. The system of any one of claims 50 to 56, wherein the detectable label comprises a fluorophore and the images comprise fluorescence images.

58. The system of claim 57, wherein the fluorescence images of the multivalent binding complex on the substrate surface has a contrast-to-noise ratio of greater than 20 when the fluorophore is cyanine dye 3 (Cy3) and the image is acquired using an inverted fluorescence microscope equipped with a 20X objective, NA = 0.75, dichroic mirror optimized for 532 nm light, a bandpass filter optimized for Cyanine dye-3 emission, and a camera, under non-signal saturating conditions while the surface is immersed in 25 mM ACES, pH 7.4 buffer.

59. The system of any one of claims 50 to 58, wherein the series of steps is completed in less than 60 minutes.

60. The system of any one of claims 50 to 59, wherein the series of steps is completed in less than 30 minutes.

61. The system of any one of claims 50 to 60, wherein the series of steps is completed in less than 10 minutes.

62. The system of any one of claims 50 to 61, wherein an accuracy of base-calling is characterized by a Q-score of greater than 25 for at least 80% of the nucleotide identities determined.

63. The system of any one of claims 50 to 62, wherein an accuracy of base-calling is characterized by a Q-score of greater than 30 for at least 80% of the nucleotide identities determined.

64. The system of any one of claims 50 to 63, wherein an accuracy of base-calling is characterized by a Q-score of greater than 40 for at least 80% of the nucleotide identities determined.

65. A composition comprising:

a) a polymer core; and

b) two or more nucleotide, nucleotide analog, nucleoside, or nucleoside analog moieties attached to the polymer core;

wherein the length of the linker is dependent on the nucleotide, nucleotide analog, nucleoside, or nucleoside analog moiety that is attached to the polymer core.

66. A composition comprising:

a) a mixture of polymer-nucleotide conjugates, wherein each polymer-nucleotide conjugate comprises:

i) a polymer core; and

ii) two or more nucleotide, nucleotide analog, nucleoside, or nucleoside analog moieties attached to the polymer core, wherein the length of the linker is dependent on the nucleotide, nucleotide analog, nucleoside, or nucleoside analog moiety that is attached to the polymer core; and

wherein the mixture comprises polymer-nucleotide conjugates having at least two different types of attached nucleotide, nucleotide analog, nucleoside, or nucleoside analog moiety.

67. The composition of claim 65 or claim 66, wherein the polymer core comprises a polymer having a plurality of branches and the two or more nucleotide, nucleotide analog, nucleoside, or nucleoside analog moieties are attached to said branches.

68. The composition of claim 67, wherein the polymer has a star, comb, cross-linked, bottle brush, or dendrimer configuration.

69. The composition of any one of claims 65 to 68, wherein the polymer-nucleotide conjugate comprises one or more binding groups selected from the group consisting of an avidin, a biotin, an affinity tag, and combinations thereof.

70. The composition of any one of claims 65 to 69, wherein the polymer core comprises a branched polyethylene glycol (PEG) molecule.

71. The composition of any one of claims 65 to 70, wherein the polymer-nucleotide conjugate comprises a blocked nucleotide moiety.

72. The composition of claim 71, wherein the blocked nucleotide is a 3 '-0-azidomethyl nucleotide, a 3 '-0-methyl nucleotide, or a 3'-0-alkyl hydroxylamine nucleotide.

73. The composition of any one of claims 65 to 72, wherein the polymer-nucleotide conjugate further comprises one or more fluorescent labels.

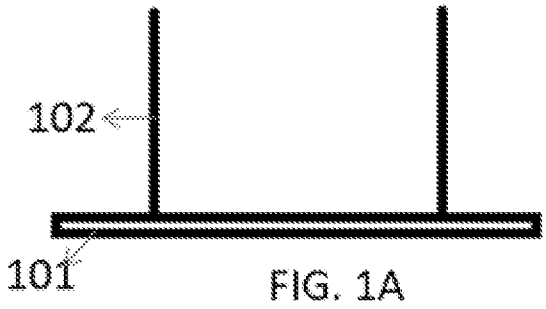


FIG. 1A

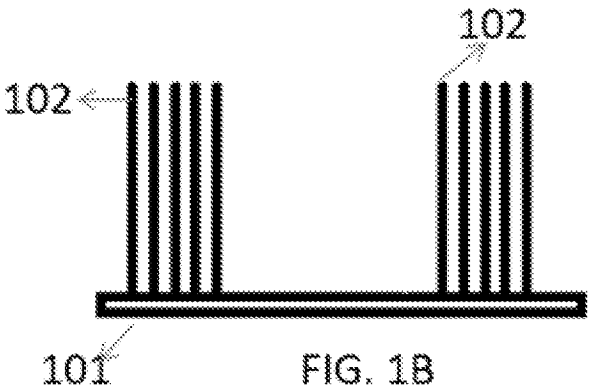


FIG. 1B

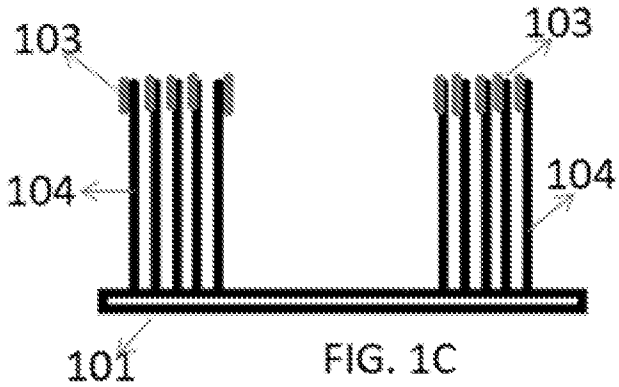


FIG. 1C

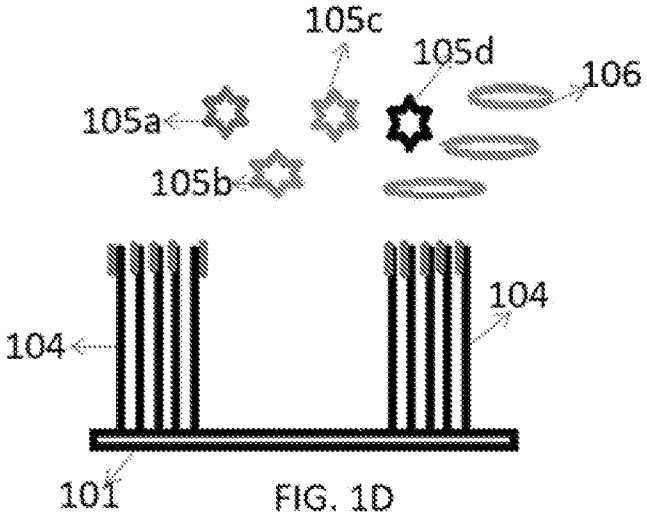


FIG. 1D

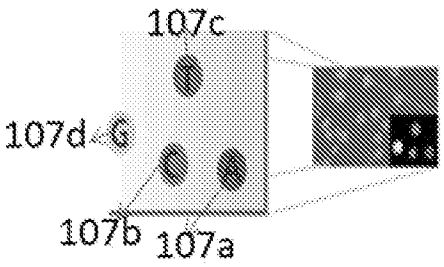


FIG. 1E

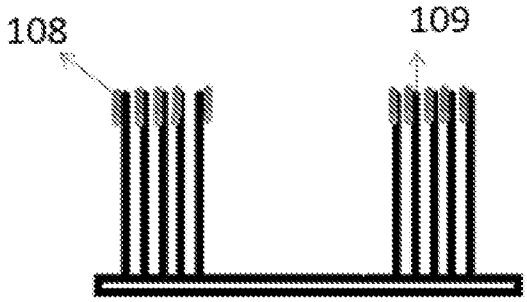


FIG. 1F

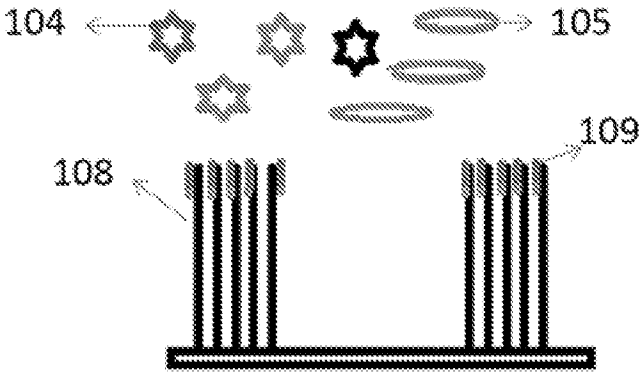


FIG. 1G

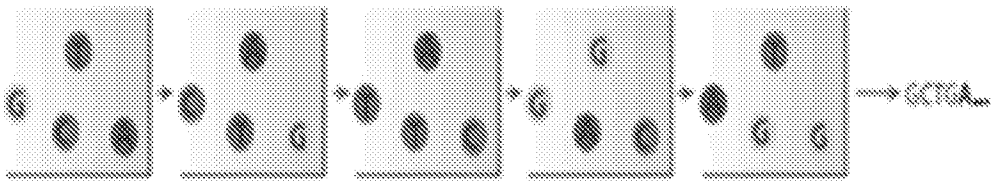


FIG. 1H

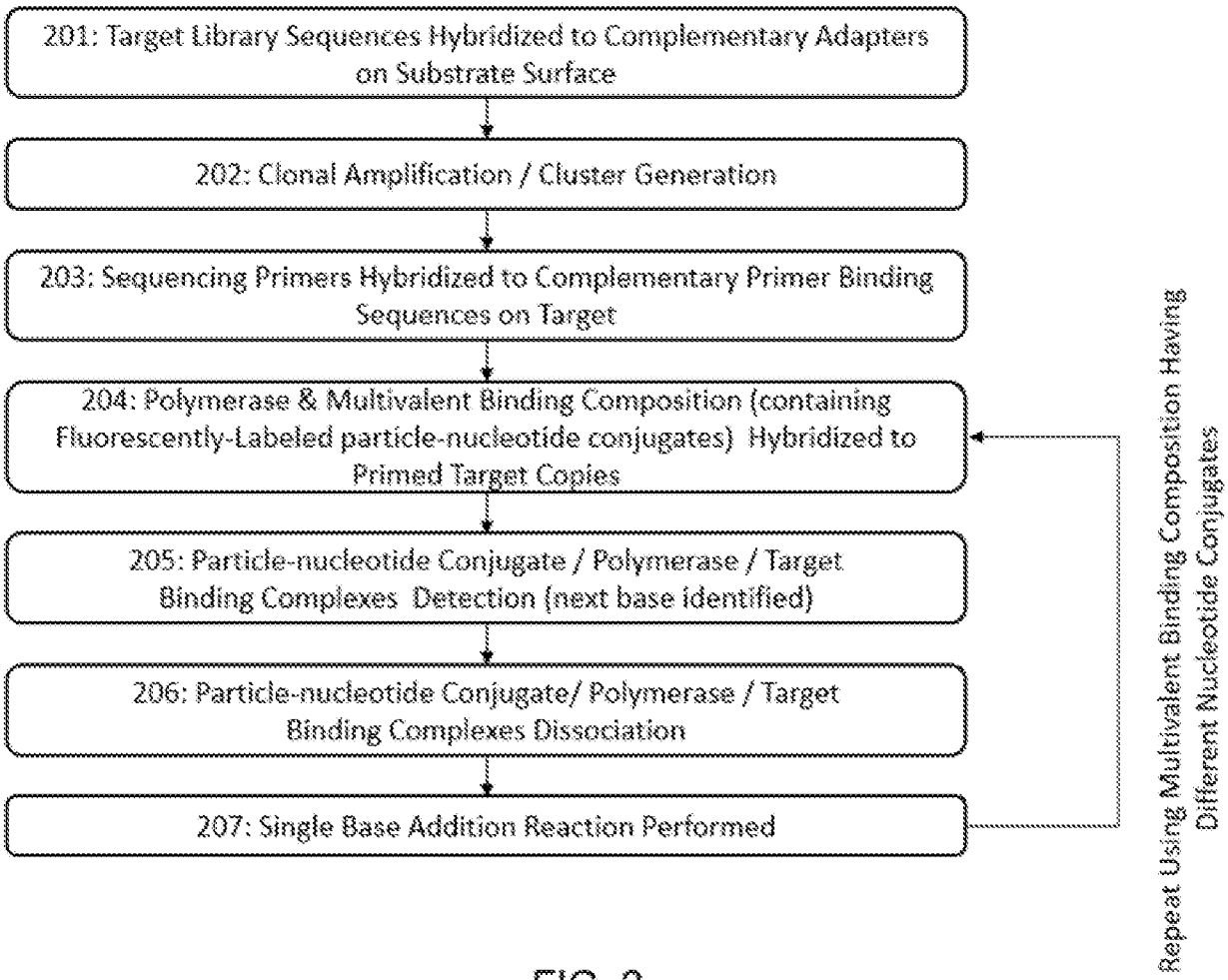

201: Target Library Sequences Hybridized to Complementary Adapters on Substrate Surface
202: Clonal Amplification / Cluster Generation
203: Sequencing Primers Hybridized to Complementary Primer Binding Sequences on Target
204: Polymerase & Multivalent Binding Composition (containing Fluorescently-Labeled particle-nucleotide conjugates) Hybridized to Primed Target Copies
205: Particle-nucleotide Conjugate / Polymerase / Target Binding Complexes Detection (next base identified)
206: Particle-nucleotide Conjugate/ Polymerase / Target Binding Complexes Dissociation
207: Single Base Addition Reaction Performed
Repeat Using Multivalent Binding Composition Having Different Nucleotide Conjugates

FIG. 2

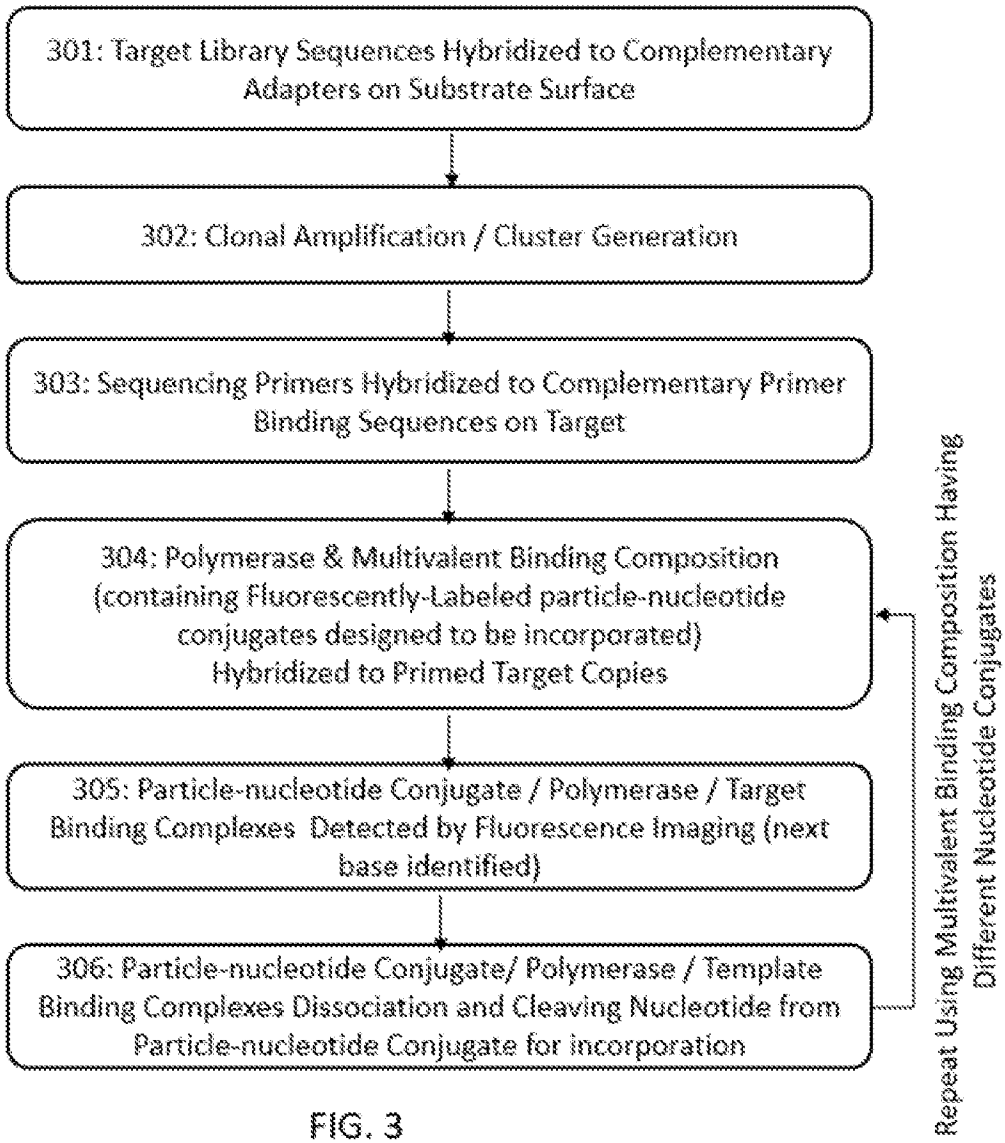

301: Target Library Sequences Hybridized to Complementary Adapters on Substrate Surface
302: Clonal Amplification / Cluster Generation
303: Sequencing Primers Hybridized to Complementary Primer Binding Sequences on Target
304: Polymerase & Multivalent Binding Composition (containing Fluorescently-Labeled particle-nucleotide conjugates designed to be incorporated) Hybridized to Primed Target Copies
305: Particle-nucleotide Conjugate / Polymerase / Target Binding Complexes Detected by Fluorescence Imaging (next base identified)
306: Particle-nucleotide Conjugate/ Polymerase / Template Binding Complexes Dissociation and Cleaving Nucleotide from Particle-nucleotide Conjugate for incorporation
Repeat Using Multivalent Binding Composition Having Different Nucleotide Conjugates

FIG. 3

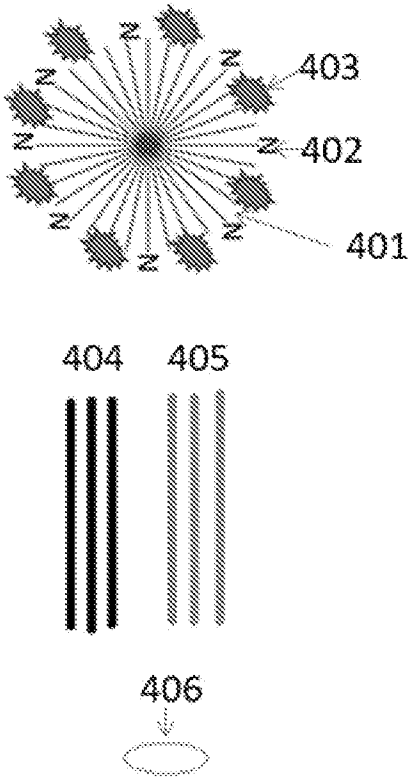


FIG. 4A

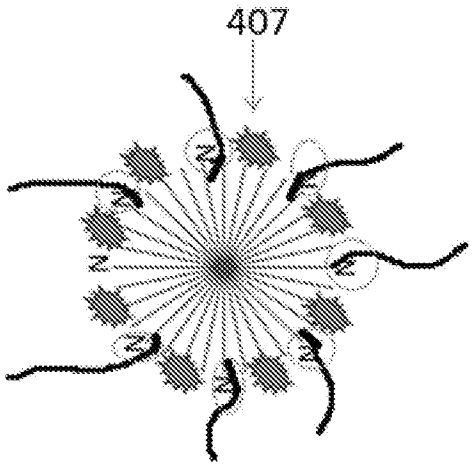


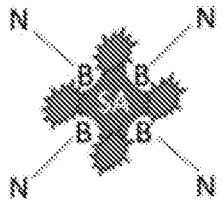
FIG. 4B

Class I

Valency = 0-4

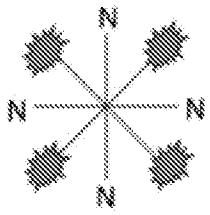
Homogenous population with saturation

"starburst"



PEG length = 1k-10k

"Helter-Skelter"



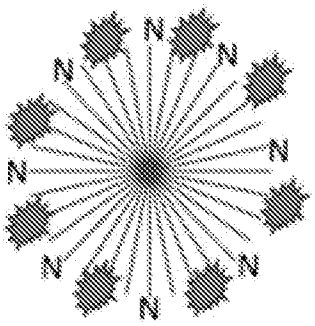
PEG length = 1.25k-5k

FIG. 5A

Class II

Valency >4

Homogenous population with Saturation



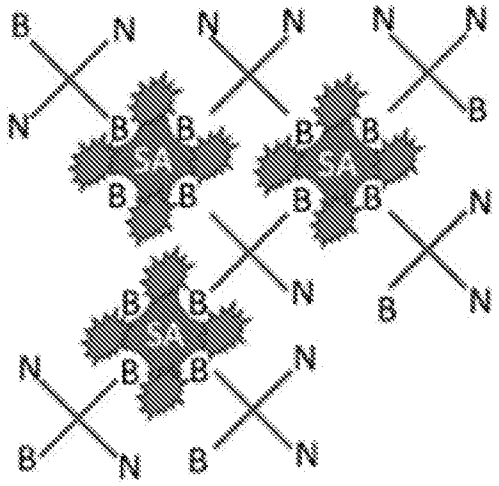
Dendrimer core 12,24,48,96 arms

PEG length = 1K-10K

Other cores = dextrans, other polymers

FIG. 5B

Class III
Valency >4
Distribution of sizes based on construction



8arm PEG-NHS mixed with Biotin-NH2
and dNTP-NH2 then mixed with labeled
streptavidin

FIG. 5C

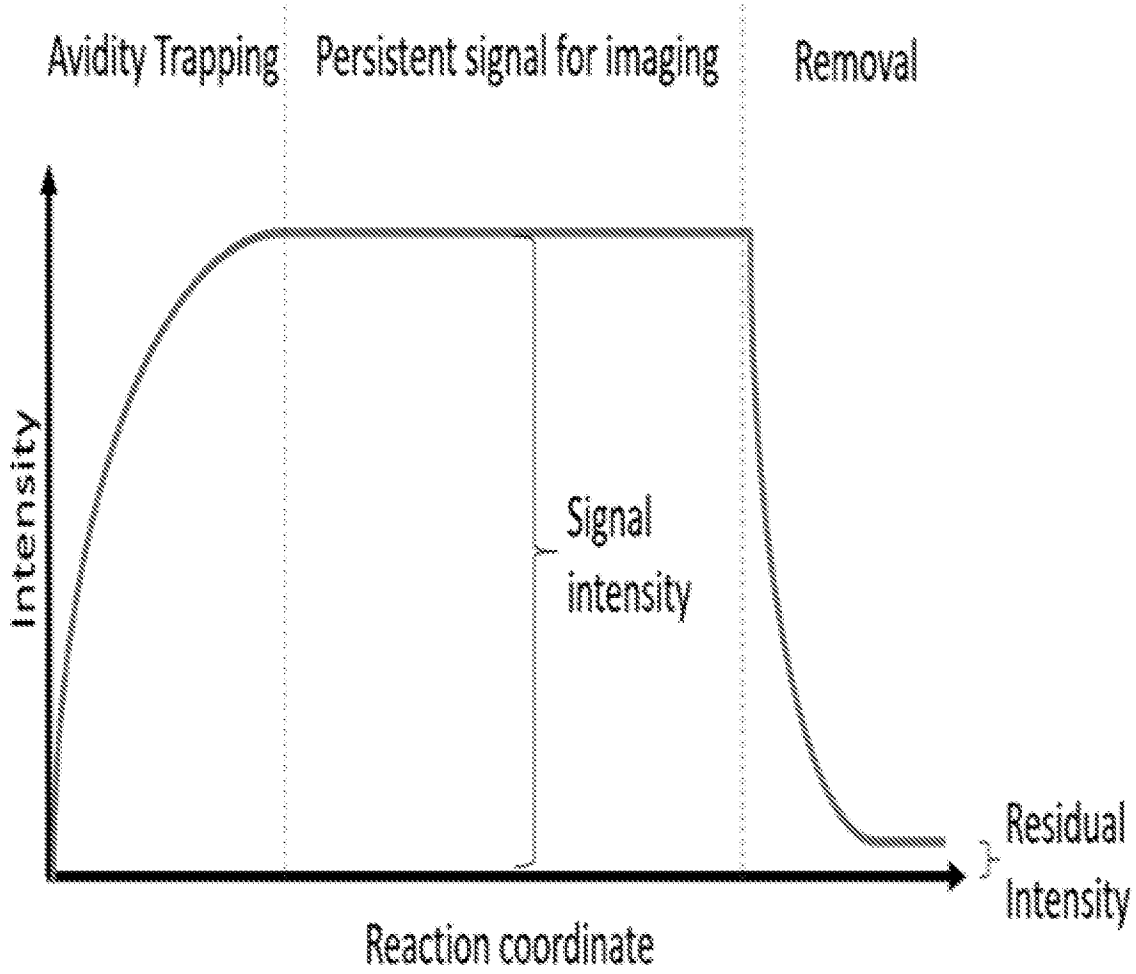
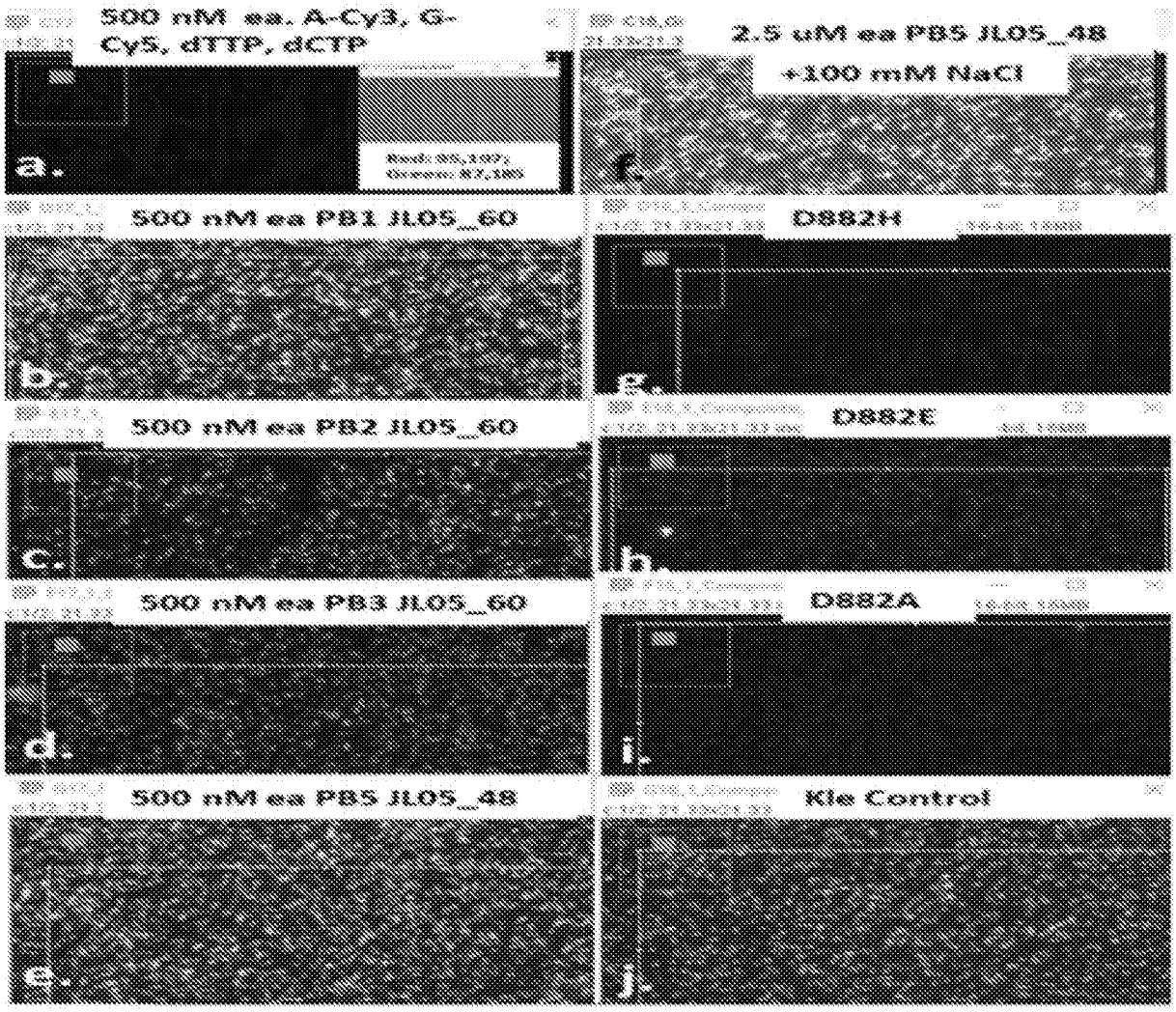

Avidity Trapping
Persistent signal for imaging
Removal
Intensity
Signal intensity
Residual Intensity
Reaction coordinate

FIG. 6


500 nM ea. A-Cy3, G-Cy5, dTTP, dCTP
a.
500 nM ea PB1 JL05_60
b.
500 nM ea PB2 JL05_60
c.
500 nM ea PB3 JL05_60
d.
500 nM ea PB5 JL05_48
e.
2.5 uM ea PB5 JL05_48
+100 mM NaCl
f.
D882H
g.
D882E
h.
D882A
i.
Kle Control
j.

FIGS. 7A-7J

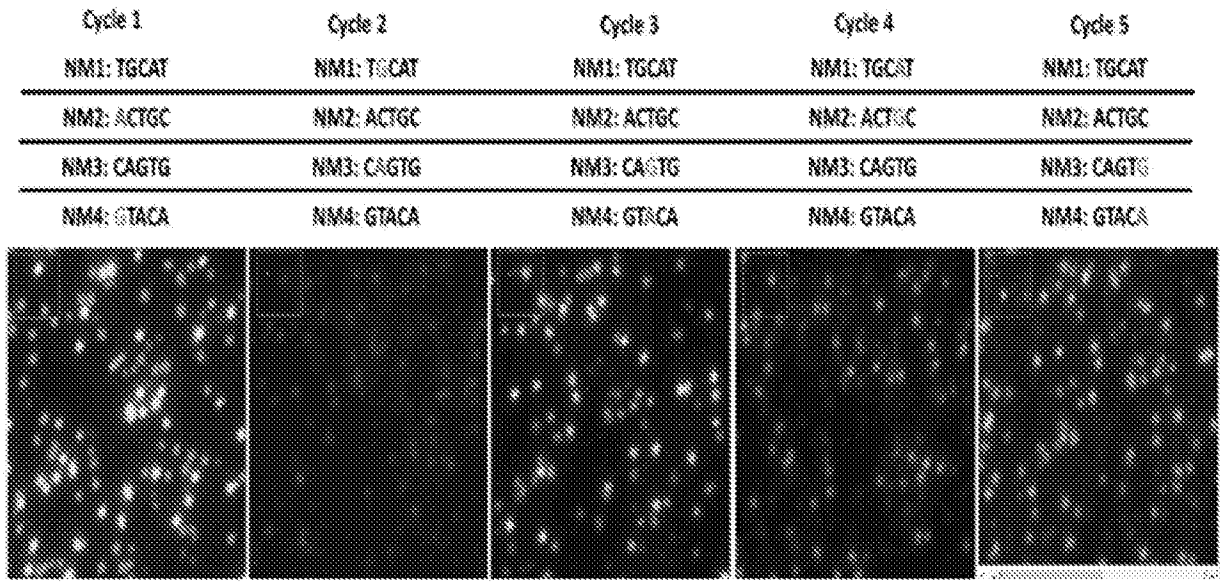
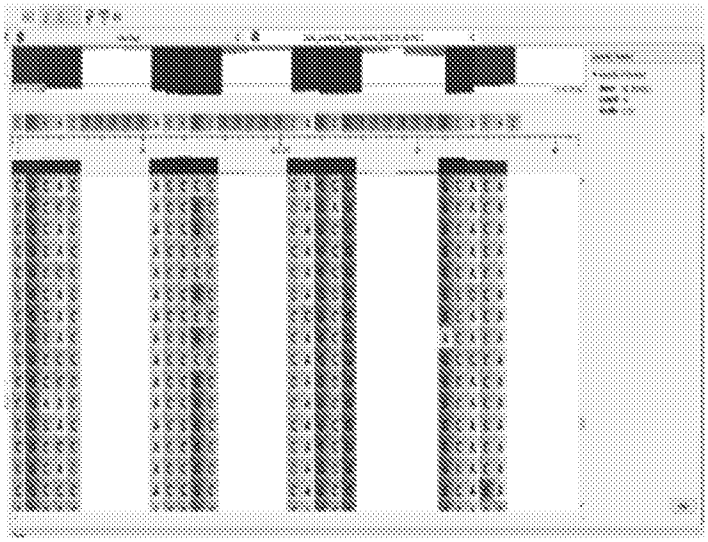

Cycle 1
NM1: TGCAT
NM2: ACTGC
NM3: CAGTG
NM4: GTACA
Cycle 2
NM1: TGCAT
NM2: ACTGC
NM3: CAGTG
NM4: GTACA
Cycle 3
NM1: TGCAT
NM2: ACTGC
NM3: CAGTG
NM4: GTACA
Cycle 4
NM1: TGCAT
NM2: ACTGC
NM3: CAGTG
NM4: GTACA
Cycle 5
NM1: TGCAT
NM2: ACTGC
NM3: CAGTG
NM4: GTACA

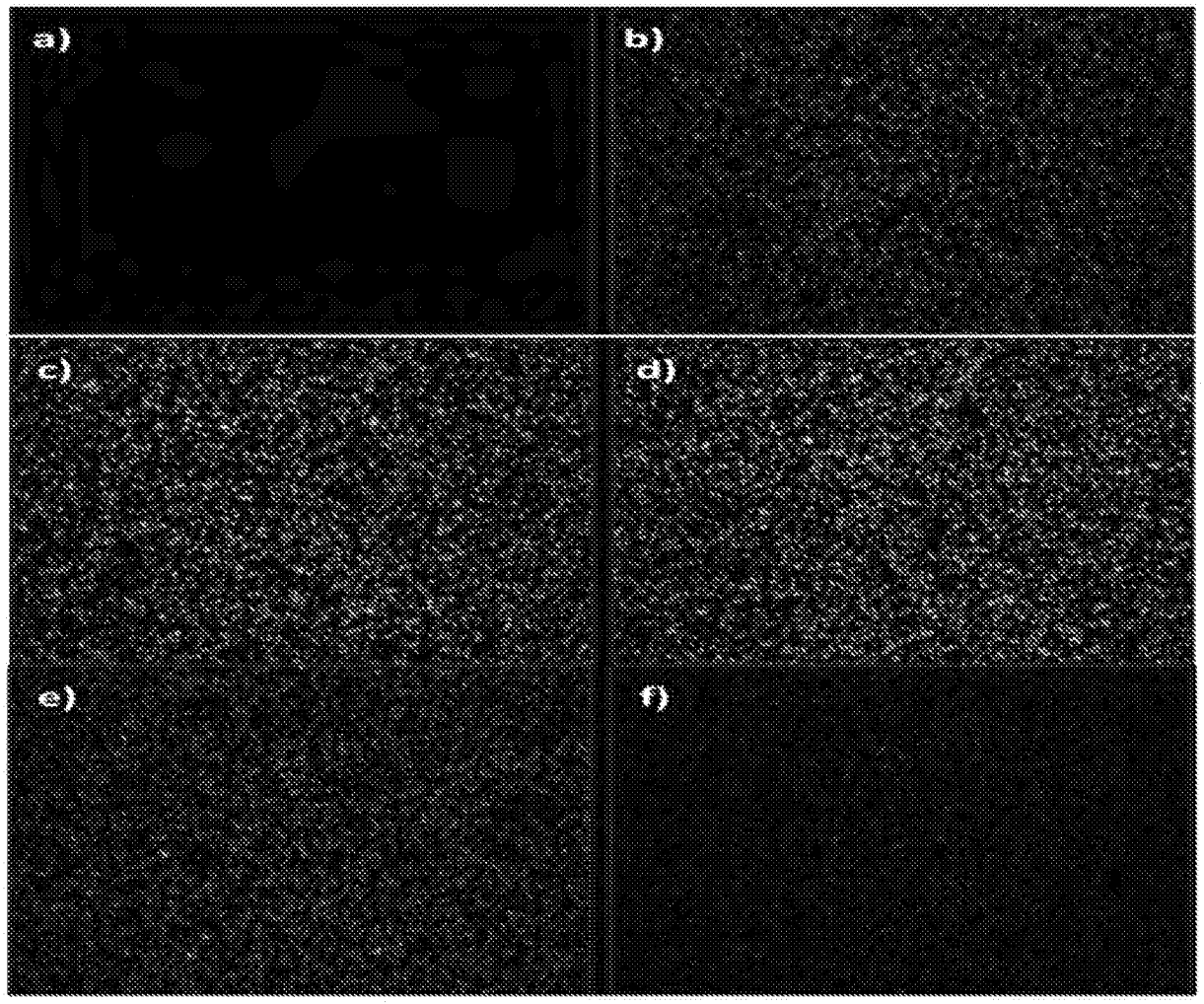
FIG. 8A

- 5 Cycles:
- G: Cy3 (Green)
- A: Cy5 (Red)
- C: dark bases (both C & T)



Expected Seq	#
CGCAC	1484
ACCGC	1466
CAGCG	1515
GCACA	1320
# correct	5785
total reads	10508
#read in FOV	9238
% correct	0.626218
allow 1 error	8727
%mapped	0.83051

FIG.8B



FIGS. 9A-9F

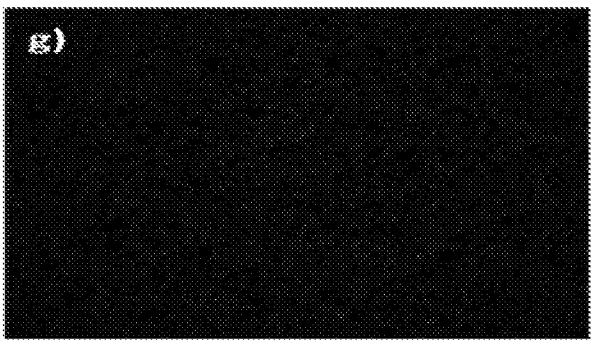


FIG. 9G

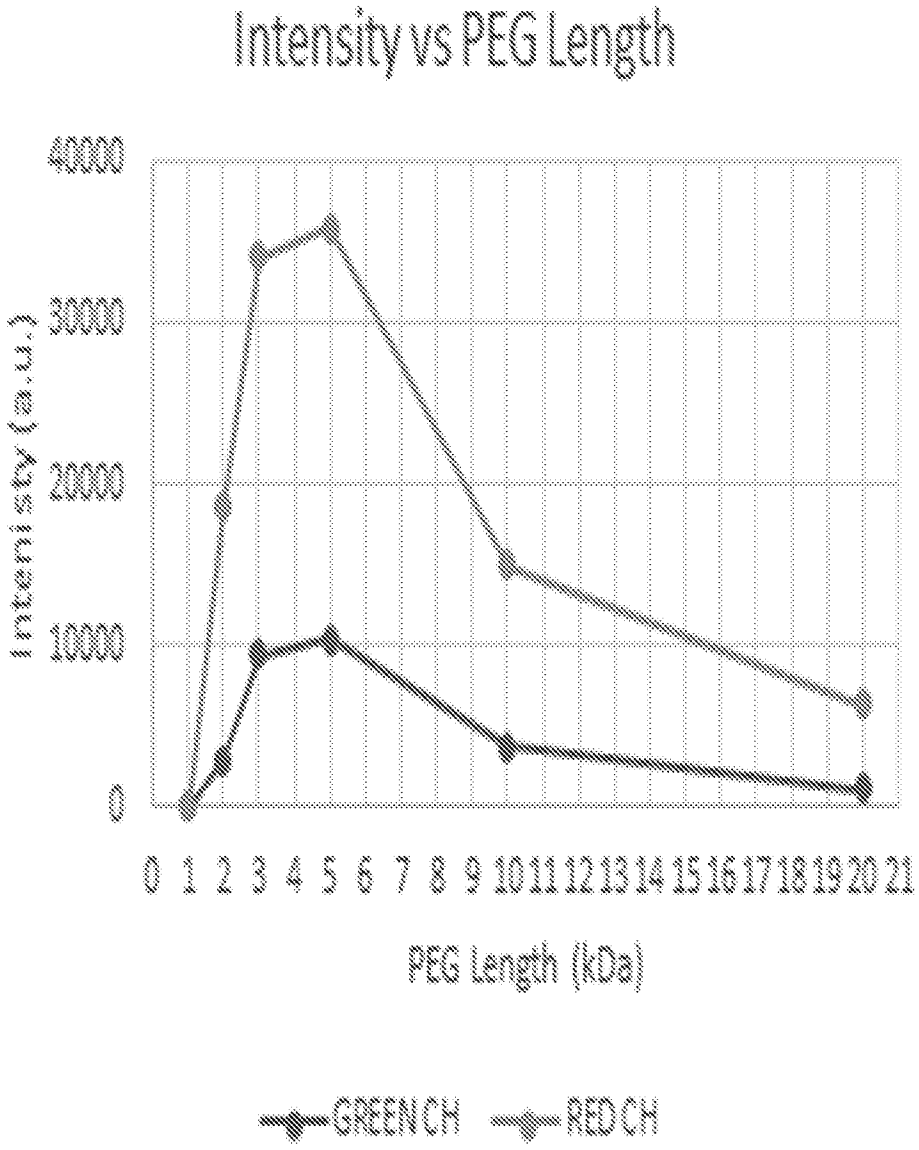

Intensity vs PEG Length
40000
30000
20000
10000
0
Intensity (a.u.)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
PEG Length (kDa)
GREEN CH
RED CH

FIG. 10

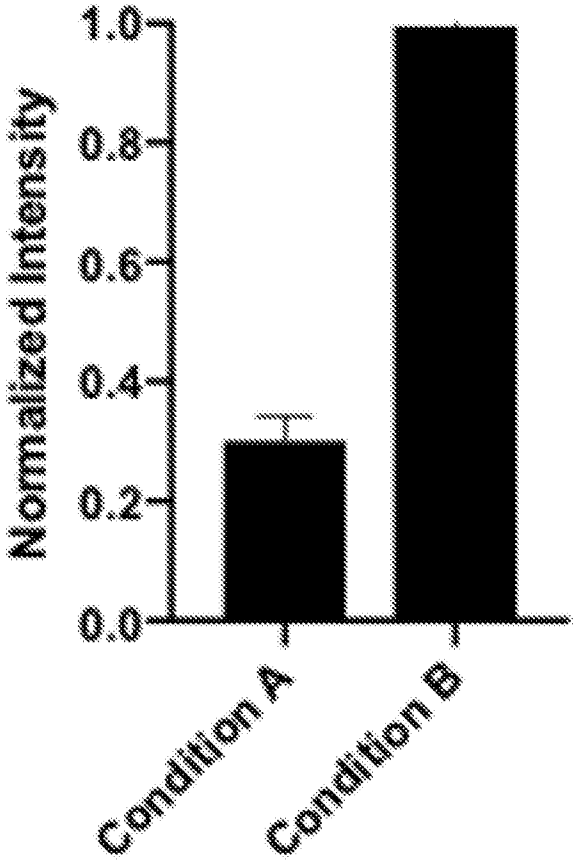

Normalized Intensity
1.0
0.8
0.6
0.4
0.2
0.0
Condition A
Condition B

FIG.11

FIG.12A

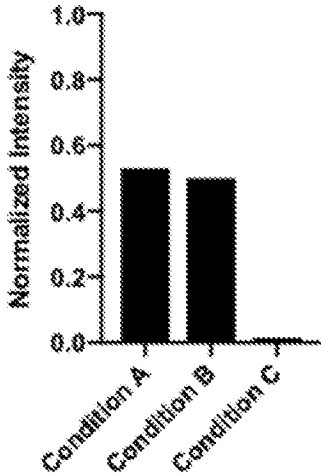
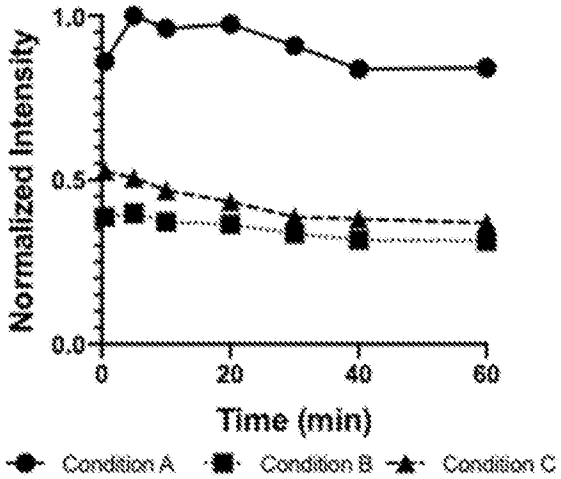


FIG.12B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/034409

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/6869 (2018.01)

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)

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)

CAPLUS, MEDLINE, BIOSIS, EMBASE, BIOTECHABS, EPODOC, PATENW: nucleotide, multivalent, polymer, peg, conjugate and like terms. CPC: C12Q1/6869

DOCDB, DWPI: Applicant and inventor names

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

"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
3 August 2020	03 August 2020
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Felix White AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832565

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2020/034409**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	US 2009/0186343 A1 (Wang et al) 23 July 2009	
X	Fig 13, [0047], [0143], claim 21	65-68, 71-73
Y	as above	1-64
	WO 2012/027625 A2 (PACIFIC BIOSCIENCES OF CALIFORNIA, INC.) 01 March 2012	
X	Fig 24-25, [0087], [0200]	65-66, 71-73
Y	as above	1-64
	US 2010/0093992 A1 (Cherkasov et al.) 15 April 2010	
X	Figs 28 & 32, [44]	65-70, 71-73
Y	as above	1-64
	Anderson, J.P. et al "Fluorescent Structural DNA Nanoballs Functionalized With Phosphate-Linked Nucleotide Triphosphates" Nano Letters 2010 10(3): 788–792. doi:10.1021/nl9039718.	
X	Abstract, second last paragraph, Fig 3A	65-73
Y	As above	1-64
	WO 2005/111240 A2 (LI-COR, INC.) 24 November 2005	
X	Abstract, Figures, [0078]	65-73
Y	As above	1-64
	WO 2009/073201 A2 (PACIFIC BIOSCIENCES OF CALIFORNIA, INC.) 11 June 2009	
X	Fig. 1, [0056], [0065], [0077]	65-73
Y	As above	1-64
	WO 2013/123258 A1 (PACIFIC BIOSCIENCES OF CALIFORNIA, INC.) 22 August 2013	
X	Fig. 27B	65-66, 71-73
Y	As above	1-64
	Technology Spotlight: Illumina® Sequencing, 2010, downloaded from https://www.illumina.com/documents/products/techspotlights/techspotlight_sequencing.pdf on 31/7/2020	
Y	Whole document, esp Fig 3-7	1-64

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2020/034409**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2009/0186343 A1	23 July 2009	US 2009186343 A1	23 Jul 2009
		WO 2009105077 A2	27 Aug 2009
WO 2012/027625 A2	01 March 2012	WO 2012027625 A2	01 Mar 2012
		US 2012058482 A1	08 Mar 2012
		US 8669374 B2	11 Mar 2014
		US 2012052506 A1	01 Mar 2012
		US 8889886 B2	18 Nov 2014
		US 2012077189 A1	29 Mar 2012
		US 8906612 B2	09 Dec 2014
		US 2012052507 A1	01 Mar 2012
		US 8993737 B2	31 Mar 2015
		US 2012058469 A1	08 Mar 2012
		US 9051263 B2	09 Jun 2015
		US 2015072894 A1	12 Mar 2015
		US 9441270 B2	13 Sep 2016
		US 2015197794 A1	16 Jul 2015
		US 9499862 B2	22 Nov 2016
		US 2015307933 A1	29 Oct 2015
		US 9702001 B2	11 Jul 2017
		US 2012058473 A1	08 Mar 2012
		US 9777320 B2	03 Oct 2017
		US 2015344950 A1	03 Dec 2015
		US 9920365 B2	20 Mar 2018
		US 2017183501 A1	29 Jun 2017
		US 10392659 B2	27 Aug 2019
		US 2020232023 A1	23 Jul 2020
		WO 2012027618 A2	01 Mar 2012
		WO 2012027623 A2	01 Mar 2012
US 2010/0093992 A1	15 April 2010	US 2010093992 A1	15 Apr 2010
		US 9035035 B2	19 May 2015
		DE 10356837 A1	30 Jun 2005
		DE 102006012317 A1	18 Jan 2007
		EP 1725572 A2	29 Nov 2006
		EP 1725572 B1	31 May 2017
		EP 1885876 A2	13 Feb 2008

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INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2020/034409**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
		US 2010029494 A1	04 Feb 2010
		US 8637650 B2	28 Jan 2014
		WO 2005044836 A2	19 May 2005
		WO 2006097320 A2	21 Sep 2006

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2020/034409**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2005/111240 A2	24 November 2005	WO 2005111240 A2	24 Nov 2005
		US 2005266456 A1	01 Dec 2005
		US 7462452 B2	09 Dec 2008
		US 2011177496 A1	21 Jul 2011
		US 8592148 B2	26 Nov 2013
		US 9045798 B1	02 Jun 2015
		US 2015086994 A1	26 Mar 2015
		US 10000798 B2	19 Jun 2018
		US 2018258481 A1	13 Sep 2018
		US 10577651 B2	03 Mar 2020
WO 2009/073201 A2	11 June 2009	WO 2009073201 A2	11 Jun 2009
		AU 2008331824 A1	11 Jun 2009
		AU 2008331824 B2	24 Jul 2014
		CA 2707600 A1	11 Jun 2009
		EP 2217928 A2	18 Aug 2010
		EP 2217928 B1	09 Aug 2017
		US 2009208957 A1	20 Aug 2009
WO 2013/123258 A1	22 August 2013	WO 2013123258 A1	22 Aug 2013
		EP 2814953 A1	24 Dec 2014
		EP 2814953 B1	07 Jun 2017
		EP 3222627 A1	27 Sep 2017
		EP 3222627 B1	07 Aug 2019
		EP 3590948 A1	08 Jan 2020
		US 2013316912 A1	28 Nov 2013
		US 9062091 B2	23 Jun 2015
		US 2015330987 A1	19 Nov 2015
		US 10023605 B2	17 Jul 2018
		US 2019010183 A1	10 Jan 2019

End of Annex

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