REAGENTS FOR MASSIVELY PARALLEL NUCLEIC ACID SEQUENCING

CROSS REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 63/108,207, filed October 30, 2020, which is hereby incorporated by reference in its entirety.

BACKGROUND

[0002] Sequences of nucleic acid molecules may be determined using massively parallel sequencing. Massively parallel sequencing may be performed using sequencing by synthesis in which, in a primer extension reaction, a polymerizing enzyme adds nucleotides sequentially to a growing strand to yield a strand that is complementary to a template strand, and the nucleotides being incorporated are detected. Such sequences may be used in various applications, such as, for example, disease (e.g., cancer) diagnostics.

SUMMARY

[0002a] In one aspect, the present disclosure refers to a method of analyzing a nucleic acid sequence, the method comprising: (a) providing a fluorescently-labeled nucleotide conjugate comprising (i) a core, (ii) a plurality of nucleotide moieties coupled to the core, and (iii) one or more fluorophores coupled to the core; (b) bringing the nucleic acid sequence in contact with the fluorescently-labeled nucleotide conjugate and a polymerizing enzyme under conditions sufficient to form a binding complex comprising (i) the polymerizing enzyme and (ii) a nucleotide of the nucleic acid sequence bound to a nucleotide moiety of the plurality of nucleotide moieties of the fluorescently-labeled nucleotide conjugate, wherein the binding complex persists for a persistence time of 1 second or more before it is removed; (c) contacting the binding complex with an imaging reagent, wherein the imaging reagent comprises a photobleaching reducing agent; and (d) obtaining a fluorescent image of the binding complex in a presence of the imaging reagent.

[0003] Aspects disclosed herein provide methods of analyzing a nucleic acid, the method comprising: (a) bringing a primed nucleic acid sequence into contact with a fluorescently-labeled nucleotide conjugate under conditions sufficient to form a binding complex comprising a first nucleotide of the primed nucleic acid molecule bound to a second nucleotide of the fluorescently-labeled nucleotide conjugate; (b) contacting the binding complex with an imaging reagent; and (c) obtaining an image of the binding complex in a

presence of the imaging reagent, thereby reducing a risk of photo-bleaching of the fluorescently-labeled nucleotide conjugate as compared to a risk of photo-bleaching under like conditions in absence of the imaging reagent.

[0004] In some embodiments, the method further comprises identifying the first nucleotide by analyzing the image obtained in (c). In some embodiments, the imaging reagent comprises ascorbic acid. In some embodiments, the ascorbic acid comprises sodium ascorbate. In some embodiments, the concentration of the ascorbic acid is at least 20 mM. In some embodiments, the concentration of the ascorbic acid is between about 10mM and about 100 mM. In some embodiments, the concentration of the ascorbic acid is about 50 mM. In some embodiments, the fluorescently-labeled nucleotide conjugate comprises: (i) a core, (ii) a plurality of the second nucleotide coupled thereto, and (ii) one or more fluorophores directly coupled to the core. In some embodiments, the fluorescently-labeled nucleotide conjugate

CLAIMS

What is claimed:

1. A method of analyzing a nucleic acid sequence, the method comprising:

(a) ~~bringing a primed nucleic acid sequence into contact with a~~ providing a fluorescently-labeled nucleotide conjugate comprising (i) a core, (ii) a plurality of nucleotide moieties coupled to the core, and (iii) one or more fluorophores coupled to the core;

(b) bringing the nucleic acid sequence in contact with the fluorescently-labeled nucleotide conjugate and a polymerizing enzyme under conditions sufficient to form a binding complex comprising ~~a first nucleotide of the primed nucleic acid molecule bound to a second~~ (i) the polymerizing enzyme and (ii) a nucleotide of the nucleic acid sequence bound to a nucleotide moiety of the plurality of nucleotide moieties of the fluorescently-labeled nucleotide conjugate, wherein the binding complex persists for a persistence time of 1 second or more before it is removed;

(~~b~~) c) contacting the binding complex with an imaging reagent, wherein the imaging reagent comprises a photobleaching reducing agent; and

(~~c~~) d) obtaining ~~an~~a fluorescent image of the binding complex in a presence of the imaging reagent~~, thereby reducing a risk of photo-bleaching of the fluorescently-labeled nucleotide conjugate as compared to a risk of photo-bleaching under like conditions in absence of the imaging reagent~~.

~~2. The method of claim 1, further comprising identifying the first nucleotide by analyzing the image obtained in (c).~~

~~3. The method of claim 1, wherein the imaging reagent comprises ascorbic acid.~~

~~4. The method of claim 3, wherein the ascorbic acid comprises sodium ascorbate.~~

~~5. The method of claim 3, wherein the concentration of the ascorbic acid is at least 20 mM.~~

~~6. The method of claim 3, wherein the concentration of the ascorbic acid is between about 10mM and about 100 mM.~~

~~7. The method of claim 3, wherein the concentration of the ascorbic acid is about 50 mM.~~

~~8. The method of claim 1, wherein the fluorescently-labeled nucleotide conjugate comprises: (i) a core, (ii) a plurality of the second nucleotide coupled thereto, and (ii) one or more fluorophores directly coupled to the core.~~

2. The method of claim 1, wherein the photobleaching reducing agent comprises (i) ascorbate, (ii) Trolox, or (iii) a combination of the ascorbate and the Trolox.

3. The method of claim 2, wherein in (d), the fluorescent image exhibits no residual fluorescent signal from the fluorescently-labeled nucleotide conjugate of the binding complex when (i) the photobleaching reducing agent comprises 50 millimolar (mM) of the ascorbate and 2 mM of the Trolox, wherein the Trolox is non-aged Trolox and (ii) the obtaining comprises performing a 90 second exposure by a laser having a power of between 75-200 milliwatt (mW).

4. The method of claim 2, wherein the photobleaching reducing agent comprises ascorbate.

5. The method of claim 4, wherein a concentration of the ascorbate is at least 20 millimolar (mM).

6. The method of claim 4, wherein a concentration of the ascorbate is between about 10 millimolar (mM) and about 100 mM.

7. The method of claim 4, wherein a concentration of the ascorbate is about 50 millimolar (mM).

8. The method of claim 1, further comprising bringing the nucleic acid sequence into contact with a second fluorescently-labeled nucleotide conjugate under conditions sufficient to form a second binding complex comprising a second nucleotide of the nucleic acid sequence bound to a second nucleotide moiety of the fluorescently-labeled nucleotide conjugate, wherein the nucleotide is adjacent to the second nucleotide in the nucleic acid sequence.

9. The method of claim 1, wherein the nucleic acid sequence is comprised in a concatemer nucleic acid molecule comprising tandem repeats of the nucleic acid sequence.

10. The method of claim 1, wherein the nucleic acid sequence further comprises at least one universal adapter sequence.

11~~9~~. The method of claim 1~~8~~, wherein the fluorescently-labeled nucleotide conjugate further comprises a core attachment moiety linking the plurality of the ~~second~~ nucleotide moieties to the core.

12. The method of claim 1, wherein the nucleotide moiety is selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

13. The method of claim 1, further comprising bringing the nucleic acid sequence into contact with a plurality of fluorescently-labeled nucleotide conjugates comprising the fluorescently-labeled nucleotide conjugate and an additional fluorescently-labeled nucleotide conjugate, wherein the nucleotide moiety of the fluorescently-labeled nucleotide conjugate is different than an additional nucleotide moiety of the additional fluorescently-labeled nucleotide conjugate.

14. The method of claim 13, wherein the additional nucleotide moiety is selected from the group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

~~10~~15. The method of claim 1, wherein the ~~second~~ nucleotide moiety comprises between about 3 and about 10 phosphate groups.

~~11~~16. The method of claim 1, wherein the ~~second~~ nucleotide ~~is a nucleotide triphosphate comprising~~of the nucleic acid sequence comprises a removable chain terminating moiety.

17. The method of claim 16, wherein the removable chain terminating moiety has a blocking moiety at the 3' sugar position of the nucleotide configured to prevent incorporation of the nucleotide moiety into the nucleic acid sequence.

~~12. The method of claim 1, further comprising bringing the primed nucleic acid sequence into contact with a polymerase under conditions sufficient to form the binding complex, wherein the binding complex further comprises the polymerase.~~

18~~13~~. The method of claim 1~~2~~, wherein the polymerizing enzyme is a DNA polymerase ~~lacks a detectable label~~.

19. The method of claim 1, wherein the polymerizing enzyme lacks a detectable label.

~~14. The method of claim 12, wherein the polymerase comprises a detectable label.~~

~~15. The method of claim 1, wherein the binding complex is immobilized to a support.~~

20~~16~~. The method of claim 1~~5~~, wherein the binding complex is immobilized to a ~~support comprises a~~ surface of a support, and wherein the surface comprises a hydrophilic coating layer coupled thereto.

21. The method of claim 20, wherein the image of the surface exhibits a contrast to noise ratio (CNR) that is greater than or equal to about 20 when the fluorescently-labeled nucleotide conjugate of the binding complex comprises a detectable label comprising Cyanine dye-3

(Cy3) and when the image of the surface is acquired using an inverted fluorescence microscope and a camera under non-signal saturating conditions while the surface is immersed in a buffer.

2217. The method of claim 2016, wherein the hydrophilic coating layer comprises a water contact angle of less than 50 degrees.

~~18. The method of claim 1, further comprising bringing the primed nucleic acid sequence into contact with a second fluorescently-labeled nucleotide conjugate under conditions sufficient to form a second binding complex comprising the next nucleotide of the primed nucleic acid molecule bound to a third nucleotide of the fluorescently-labeled nucleotide conjugate, wherein the third nucleotide is different than the second nucleotide.~~

~~19. The method of claim 1, wherein the primed nucleic acid sequence is comprised in a concatemer nucleic acid molecules comprising tandem repeats of the primed nucleic acid sequence.~~

23. The method of claim 20, wherein a plurality of the nucleic acid sequence is immobilized to the surface, and wherein a surface density of the plurality of the nucleic acid sequence comprises about 103 molecules/ millimeter $(mm)^2$ to about 5 x 10^5 molecules/mm^2.

2024. The method of claim 1, wherein the imaging reagent comprises a non-catalytic divalent cation that inhibits incorporation of the ~~second~~ nucleotide moiety of the fluorescently-labeled nucleotide conjugate.

25. The method of claim 24, wherein the non-catalytic divalent cation comprises strontium, barium, scandium, titanium, calcium, vanadium, chromium, iron, cobalt, nickel, copper, zinc, gallium, germanium, arsenic, selenium, rhodium, europium, tin or terbium ions.

26. The method of claim 1, wherein the nucleic acid sequence comprises a template nucleic acid sequence for a sequencing reaction and at least one universal adaptor sequence.

27. The method of claim 1, wherein the imaging reagent further comprises 1-methyl-4-(1-methylpyridin-1-ium-4-yl)pyridin-1-ium;dichloride, Tris-HCl EDTA, NaCl, a non-ionic detergent, sucrose, strontium acetate or glycerol, or any combination thereof.

28. The method of claim 1, wherein said persistence time is more than 1 second.

~~21. A formulation for reducing photo-bleaching of a biological entity during imaging, the formulation comprising: at least one solvent, a pH buffering agent, a chelating agent, at least one monovalent cation, a non-catalytic divalent cation, a detergent and ascorbic acid.~~

29. The method of claim 1, further comprising identifying the nucleotide by analyzing the image obtained in (d).

~~22. The formulation of claim 21, wherein the pH buffering agent comprises Tris-HCl.~~

~~23. The formulation of claim 22, wherein the pH of the Tris-HCL is about 8.8.~~

~~24. The formulation of claim 21, wherein the chelating agent comprises EDTA.~~

~~25. The formulation of claim 21, wherein the monovalent cation comprises NaCl, KCl, (NH4)2SO4 or potassium glutamate.~~

~~26. The formulation of claim 21, wherein the non-catalytic divalent cation comprises strontium, barium, scandium, titanium, calcium, vanadium, chromium, iron, cobalt, nickel, copper, zinc, gallium, germanium, arsenic, selenium, rhodium, europium, tin or terbium ions, or a combination thereof.~~

~~27. The formulation of claim 21, wherein the formulation lacks a catalytic divalent cation which comprises magnesium or manganese, or a combination thereof.~~

~~28. The formulation of claim 21, wherein the detergent comprises Triton X-100.~~

~~29. The formulation of claim 21, wherein the formulation further comprises a sugar.~~

~~30. The formulation of claim 21, wherein the formulation further comprises a viscosity agent comprising glycerol.~~

~~31. The formulation of claim 21, wherein the formulation further comprises 1,3,5,7 cyclo-octatetraene (COT).~~

~~32. The formulation of claim 31, wherein the COT has a concentration of about 2 micromolar (mM).~~

~~33. The formulation of claim 21, wherein the ascorbic acid comprises sodium ascorbate.~~

~~34. The formulation of any one of claims 21-33, wherein the concentration of the ascorbic acid is at least 10 mM.~~

~~35. The formulation of any one of claims 21-33, wherein the concentration of the ascorbic acid is at least 20 mM~~

~~36. The formulation of any one of claims 21-33, wherein the concentration of the ascorbic acid is between about 10 m M and about 100 mM.~~

~~37. The formulation of any one of claims 21-33, wherein the concentration of the ascorbic acid is about 50 mM.~~

~~38. The formulation of claim 21, wherein the formulation further comprises Trolox.~~

39. The formulation of claim 37, wherein the concentration of the Trolox is about 2 mM.

40. The formulation of claim 21, wherein the formulation further comprises 3-nitrobenzoic acid (NBA).

41. The formulation of claim 21, wherein the formulation further comprises cysteamine.

42. A formulation comprising (i) at least one solvent, (ii) a pH buffering agent, (iii) a chelating agent, (iv) at least one monovalent cation, (v) a non-catalytic divalent cation, (vi) a detergent, (vii) a plurality of multivalent molecules and (viii) a sequencing polymerase enzyme, wherein each of the plurality of multivalent molecules comprises (1) a core, and (2) a plurality of nucleotides and a plurality of detectable moieties coupled to the core, and wherein the sequencing polymerase enzyme comprises an amino acid sequence that is at least 80% identical to any of SEQ ID NOS: 2-5.

43. The formulation of claim 42, wherein each of the plurality of multivalent molecules further comprises a linker that couples the plurality of nucleotides to the core, and wherein the linker comprises an aliphatic chain having 2-6 subunits or an oligo ethylene glycol chain having 2-6 subunits.

44. The formulation of claim 42, wherein the core is spheroidal.

45. The formulation of claim 41, wherein the plurality of nucleotides are of the same type of nucleotide selected from a group consisting of dATP, dGTP, dCTP, dTTP and dUTP.

46. The formulation of claim 41, wherein the plurality of the multivalent molecules comprises two or more different types of multivalent molecules, wherein each of the two or more different types of the multivalent molecules comprise a different plurality of nucleotides.

47. The formulation of claim 41, wherein the sequencing polymerase comprises a mutation in the amino acid sequence comprising a substitution at one or more positions relative to SEQ ID NO: 1 comprising Leu416, Tyr417, Pro418, Ala493, Arg515, Ile529 and Asn567.

48. The formulation of claim 41, wherein the sequencing polymerase comprises the amino acid sequence of any one of SEQ ID NOS: 2-5.

49. A kit comprising one or more containers containing the formulation of any one of claims 21-48.

50. The kit of claim 42, wherein the kit further comprises instructions for reducing photo-damage to the biological entity while imaging the biological entity during a biochemical reaction in the presence of the formulation.

51. The kit of claim 43, wherein the biochemical reaction comprises a sequencing reaction.

52. The kit of claim 43, wherein the instructions comprise submerging the biological entity in the formulation during the biochemical reaction prior to imaging the biological entity.

53. A kit comprising:

a trap reagent comprising at least one solvent, a pH buffering agent, a chelating agent, at least one monovalent cation, a non-catalytic divalent cation, a detergent, a plurality of fluorescently-labeled nucleotide conjugates and a first sequencing polymerase enzyme;

a post-trap reagent comprising at least one solvent, a pH buffering agent, a chelating agent, at least one monovalent cation, a non-catalytic divalent cation, a detergent and a first sequencing polymerase enzyme;

an imaging reagent comprising at least one solvent, a pH buffering agent, a chelating agent, at least one monovalent cation, a non-catalytic divalent cation, a detergent and ascorbic acid; and

a stepping reagent comprising at least one solvent, at least one pH buffering agent, at least one monovalent cation, a catalytic divalent cation, a detergent, a second sequencing polymerase enzyme and a plurality of nucleotides, wherein each nucleotide of the plurality of nucleotides comprises a cleavable terminator moiety attached to a 3' sugar position.

54. The kit of claim 53, further comprising instructions for using the trap reagent, wherein the instructions comprise contacting a plurality of immobilized template nucleic acid molecules with (i) the trap reagent and (ii) a plurality of sequencing primers under conditions sufficient to form a plurality of immobilized fluorescently-labeled ternary complexes without incorporating the plurality of fluorescently-labeled nucleotide conjugates into the sequencing primer.

55. The kit of claim 54, further comprising instructions for using the post-trap reagent, wherein the instructions comprise contacting the plurality of immobilized fluorescently-labeled ternary complexes with a post-trap reagent under conditions sufficient for preserving the plurality of immobilized fluorescently-labeled ternary complexes without incorporation of the plurality of immobilized fluorescently-labeled ternary complexes into the sequencing primer.

56. The kit of claim 55, further comprising instructions for using the stepping reagent, wherein the instructions comprise contacting the plurality of immobilized template nucleic acid molecules with the stepping reagent under conditions sufficient to extend the immobilized template nucleic acid molecule.

57. The kit of claim 53 further comprising:

a first amplification reagent comprising at least one solvent, a pH buffering agent, at least one monovalent cation, ammonium ions, a plurality of nucleotides and an amplification polymerase enzyme; and

a second amplification reagent comprising at least one solvent, a pH buffering agent, at least one monovalent cation, ammonium ions and a plurality of nucleotides.

58. The kit of claim 57, further comprising instructions for using the first amplification reagent and the second amplification reagent, wherein the instructions comprise:

contacting the immobilized template nucleic acid molecules with a first amplification reagent under conditions that inhibit activity of the amplification polymerase enzyme; and

contacting the immobilized template nucleic acid molecules with a second amplification reagent under a condition suitable for reviving the activity of the amplification polymerase enzyme to perform a plurality of nucleic acid amplification reactions.

59. The kit of any one of claims 53 to 58, further comprising:

a wash-removal reagent comprising at least one solvent, a pH buffering agent, a chelating agent, a detergent and a chaotropic agent.

60. The kit of any one of claims 53 to 59, further comprising a nucleic acid hybridization reagent, comprising:

at least one solvent, a pH buffering agent, and at least one monovalent cation; and

a detergent, a reducing agent, a chaotropic agent, a chelating agent, an alcohol, a zwitterion, a sugar alcohol or a crowding agent, or a combination thereof.