

CLAIMS

1. A method of analyzing a biological sample, comprising:

(a) contacting a biological sample on a substrate with one or more nucleic acid probes that directly or indirectly hybridize to a first target nucleic acid or a complement or an amplification product thereof in the biological sample,

wherein the substrate comprises a plurality of capture agents directly or indirectly immobilized thereon, wherein a capture agent of the plurality of capture agents comprises (i) a capture domain capable of capturing a nucleic acid and (ii) a spatial barcode corresponding to the position of the capture agent on the substrate;

(b) detecting the one or more nucleic acid probes at a spatial location of the biological sample;

(c) providing conditions to allow the capture agents to directly or indirectly capture a second target nucleic acid or a complement or an amplification product thereof; and

(d) generating a spatially labeled polynucleotide comprising (i) a sequence of the second target nucleic acid or complement thereof and (ii) a sequence of the spatial barcode or complement thereof.

2. A method of analyzing a biological sample, comprising:

(a) contacting a biological sample on a first substrate with one or more nucleic acid probes that directly or indirectly hybridize to a first target nucleic acid or a complement or an amplification product thereof in the biological sample;

(b) detecting the one or more nucleic acid probes at a spatial location of the biological sample;

(c) providing conditions to allow a plurality of capture agents to directly or indirectly capture a second target nucleic acid or a complement thereof or an amplification product thereof, wherein a capture agent of the plurality of capture agents comprises:

(i) a capture domain capable of capturing the second target nucleic acid, and

(ii) a spatial barcode; and

(d) generating a spatially labeled polynucleotide comprising (i) a sequence of the second target nucleic acid or complement thereof and (ii) a sequence of the spatial barcode or complement thereof.

3. The method of claim 2, wherein the plurality of capture agents are joined directly or indirectly to the first substrate or to a second substrate.

4. The method of claim 2 or 3, wherein the spatial barcode corresponds to the position of the capture agent on the first substrate or on a second substrate.

5. The method of any of claims 2-4, further comprising before step (c), providing a second substrate comprising the plurality of capture agents to the biological sample.

6. A method of analyzing a target nucleic acid in a biological sample, comprising:
(a) contacting a biological sample on a first substrate with one or more nucleic acid probes that directly or indirectly hybridize to a first target nucleic acid or a complement or an amplification product thereof in the biological sample;

(b) detecting the one or more nucleic acid probes at a spatial location of the biological sample;

(c) providing conditions to allow a plurality of capture agents to directly or indirectly capture a second target nucleic acid or a complement thereof or an amplification product thereof, wherein the plurality of capture agents are joined directly or indirectly to the first substrate or to a second substrate, and wherein a capture agent of the plurality of capture agents comprises:

(i) a capture domain capable of capturing a nucleic acid, and

(ii) a spatial barcode corresponding to the position of the capture agent on the first substrate or on the second substrate; and

(d) generating a spatially labeled polynucleotide comprising (i) a sequence of the second target nucleic acid or complement thereof and (ii) a sequence of the spatial barcode or complement thereof.

7. The method of any of claims 1-6, wherein the first target nucleic acid and the second target nucleic acid comprise the same nucleic acid sequence.

8. The method of any of claims 1-7, wherein the first target nucleic acid and the second target nucleic acid comprise different nucleic acid sequences.

9. The method of any of claims 1-8, wherein the first target nucleic acid and the second target nucleic acid are the same molecule or different molecules.

10. The method of any of claims 1-9, wherein the second target nucleic acid is or comprises the first target nucleic acid, a sequence thereof, a complement thereof, a hybridization product thereof, a ligation product thereof, an extension product thereof, a replication product thereof, a transcription/reverse transcription product thereof, and/or an amplification product thereof.

11. The method of any of claims 1-10, wherein the second target nucleic acid is or comprises at least one of the one or more nucleic acid probes, a complement of the nucleic acid probe(s), a hybridization product of the nucleic acid probe(s), a ligation product of the nucleic acid probe(s), an extension product of the nucleic acid probe(s), a replication product of the nucleic acid probe(s), a transcription/reverse transcription product of the nucleic acid probe(s), and/or an amplification product of the nucleic acid probe(s).

12. The method of any of claims 1-11, wherein the first target nucleic acid and/or the second target nucleic acid comprise an RNA sequence, optionally wherein the first target nucleic acid and/or the second target nucleic acid are an mRNA molecule.

13. The method of any of claims 1-12, wherein the biological sample is reversibly cross-linked prior to or during step (a).

14. The method of claim 13, wherein the conditions provided in step (c) comprises de-crosslinking the reversibly cross-linked biological sample.

15. The method of any of claims 1-14, wherein the conditions provided in step (c) comprises releasing the second target nucleic acid or the complement thereof or the amplification product thereof from the biological sample.

16. The method of any of claims 1-15, wherein the conditions provided in step (c) comprises contacting the second target nucleic acid or the complement thereof or the amplification product thereof in the biological sample with the capture agents of the substrate (or the first substrate) or the second substrate.

17. The method of any of claims 1-14, wherein the conditions provided in step (c) comprises releasing the plurality of capture agents from the substrate (or the first substrate) or

from the second substrate, and/or delivering or driving the released plurality of capture agents towards and/or into or onto the biological sample.

18. The method of any of claims 1-17, wherein the plurality of capture agents comprise a capture agent releasably coupled to an analyte capture agent.

19. The method of any of claims 1-18, wherein step (b) is performed on a biological sample that is processed or cleared.

20. The method of any of claim 1-19, wherein the biological sample comprises a tissue sample.

21. The method of claim 20, wherein the tissue sample is a tissue slice between about 1 μm and about 50 μm in thickness, optionally wherein the tissue slice is between about 5 μm and about 35 μm in thickness.

22. The method of claim 20 or 21, wherein the tissue sample is embedded in a hydrogel.

23. The method of any of claims 1-22, wherein the first target nucleic acid, the complement thereof, and/or the amplification product thereof are reversibly cross-linked to a matrix, *e.g.*, a hydrogel.

24. The method of any of claims 1-23, wherein at least one of the one or more nucleic acid probes comprise a barcode sequence indicative of a sequence or a complementary sequence thereof in the first target nucleic acid.

25. The method of any of claims 1-24, wherein the detecting the one or more nucleic acid probes comprises imaging the biological sample.

26. The method of claim 24 or 25, wherein in step (b), a sequence of the barcode sequence or a complementary sequence thereof or an amplified sequence thereof is determined.

27. The method of any of claims 1-26, wherein step (b) comprises *in situ* sequencing and/or sequential hybridization of a plurality of probes.

28. The method of any of claims 1-27, wherein the one or more nucleic acid probes comprise a primary probe that directly hybridizes to the first target nucleic acid or the complement or the amplification product thereof.

29. The method of claim 28, wherein the first target nucleic acid is an mRNA, the complement is a cDNA, and/or the amplification product is a rolling circle amplification (RCA) product.

30. The method of claim 28 or 29, wherein the primary probe comprises a padlock probe, a circular probe, or a circularized probe.

31. The method of any of claims 28-30, wherein the primary probe comprises one or more barcode sequences which optionally correspond to a sequence of the first target nucleic acid.

32. The method of any of claims 28-31, wherein step (b) comprises contacting the biological sample with one or more detectably labelled probes capable of directly or indirectly hybridizing to the primary probe (or a complement or amplification product thereof), optionally wherein the one or more detectably labelled probes hybridize to one or more barcode sequences of the primary probe (or a complement or amplification product thereof).

33. The method of any of claims 28-32, wherein step (b) comprises contacting the biological sample with one or more secondary probes capable of directly or indirectly hybridizing to the primary probe (or a complement or amplification product thereof), optionally wherein the one or more secondary probes hybridize to one or more barcode sequences of the primary probe (or a complement or amplification product thereof).

34. The method of claim 33, wherein step (b) further comprises contacting the biological sample with one or more detectably labelled probes capable of directly or indirectly hybridizing to the one or more secondary probes (or a complement or amplification product thereof), optionally wherein the one or more detectably labelled probes hybridize to one or more barcode sequences of the one or more secondary probes (or a complement or amplification product thereof).

35. The method of any of claims 31-34, comprising imaging the biological sample to sequence the one or more barcode sequences of the primary probe (or a complement or amplification product thereof), and/or the one or more barcode sequences of the one or more secondary probes (or a complement or amplification product thereof), optionally wherein the sequencing comprises sequencing by ligation or sequencing by hybridization.

36. The method of any of claims 24-35, further comprising generating an amplification product comprising a sequence of the barcode sequence or a complementary sequence thereof.

37. The method of claim 36, wherein the amplification product is generated by rolling circle amplification.

38. The method of claim 36 or 37, wherein the amplification product comprises one or more modified nucleotides.

39. The method of any of claims 27-38, wherein the *in situ* sequencing comprises sequencing by ligation.

40. The method of any of claims 27-39, wherein the *in situ* sequencing comprises sequencing by hybridization.

41. The method of any of claims 25-40, further comprising permeabilizing the biological sample on the substrate after the imaging.

42. The method of any of claims 1-41, wherein the second target nucleic acid is an mRNA molecule, and the capture agent comprises a capture probe.

43. The method of claim 42, wherein the capture probe comprises a free 3' end such that the capture probe functions as a reverse transcriptase (RT) primer using the second target nucleic acid as a template for primer extension.

44. The method of claim 43, wherein the free 3' end comprises an oligo dT, a random sequence, or a gene-specific sequence.

45. The method of claim 43 or 44, wherein the capture probe further comprises a universal domain which is 5' to the spatial barcode, wherein the universal domain comprises:

- (i) an amplification domain; and/or
- (ii) a cleavage domain for releasing the generated spatially labeled polynucleotide from the surface of the substrate.

46. The method of any of claims 1-45, wherein the generated spatially labeled polynucleotide is a cDNA or amplification product thereof.

47. The method of any of claims 1-46, wherein the capture domain does not capture the first target nucleic acid, the complement thereof, or the amplification product thereof prior to step (c).

48. The method of any of claims 1-46, wherein the capture domain does not capture the first target nucleic acid, the complement thereof, or the amplification product thereof prior to de-crosslinking the reversibly cross-linked biological sample.

49. The method of any of claims 1-48, wherein the capture agent captures the second target nucleic acid or complement thereof or amplification product thereof by hybridization, by ligation, or by hybridization followed by ligation, *e.g.*, by splinted ligation.

50. The method of any of claims 1-49, wherein the capture agent captures the one or more nucleic acid probes or complements thereof or amplification products thereof.

51. The method of any of claims 1-50, wherein the capture agent captures the one or more nucleic acid probes hybridized to the first target nucleic acid.

52. The method of claim 51, wherein the method comprises releasing the one or more nucleic acid probes hybridized to the first target nucleic acid prior to step (c).

53. The method of claim 51 or claim 52, wherein the spatially labeled polynucleotide comprises (i) a sequence of a nucleic acid probe of the one or more nucleic acid probes or a complement thereof and (ii) a sequence of the spatial barcode or complement thereof.

54. The method of any of claims 1-53, wherein the spatially labeled polynucleotide or a portion thereof is released (*e.g.*, from the first or second substrate) for analysis.

55. The method of any of claims 1-54, wherein the method comprises determining at least a portion of the spatially labeled polynucleotide or a complement thereof.

56. The method of 55, wherein the determining comprises sequencing by ligation, sequencing by hybridization, sequencing by synthesis, and/or sequencing by binding.

57. The method of any of claims 54-56, wherein the released spatially labeled polynucleotide is analyzed by direct sequencing or indirect sequencing, optionally with amplification prior to the sequencing.

58. The method of any of claims 1-57, wherein the method further comprises correlating the spatial barcode of the spatially labeled polynucleotide and the detected spatial location of the one or more nucleic acid probes.

59. A method of analyzing a biological sample, comprising:

(a) contacting the biological sample with a first nucleic acid probe and a second nucleic acid probe, wherein the first and second nucleic acid probes are not immobilized on an artificial substrate;

(b) generating a rolling circle amplification (RCA) product *in situ* in the biological sample, the RCA product comprising a sequence of the first nucleic acid probe or complement thereof;

(c) detecting a signal (e.g., fluorescent signal) associated with the RCA product at a spatial location of the biological sample on a first substrate;

(d) providing conditions to allow a plurality of capture agents to directly or indirectly capture the second nucleic acid probe and/or a product thereof, wherein the plurality of capture agents are joined directly or indirectly to the first substrate or to a second substrate, and wherein a capture agent of the plurality of capture agents comprises:

(i) a capture domain capable of capturing a nucleic acid, and

(ii) a spatial barcode corresponding to the position of the capture agent on the first substrate or on the second substrate; and

(e) generating a spatially labeled polynucleotide comprising (i) a sequence of the second nucleic acid probe and/or product thereof and (ii) a sequence of the spatial barcode or complement thereof,

wherein the method does not comprise detecting the spatially labeled polynucleotide on the first substrate or on the second substrate.

60. The method of claim 59, wherein the first and second nucleic acid probes target the same or different analytes and/or bind to the same or different molecules in the biological sample.

61. The method of claim 59 or 60, wherein the method does not comprise generating an RCA product comprising a sequence or complement thereof of the second nucleic acid probe.

62. The method of any of claims 59-61, wherein the method does not comprise detecting a signal (e.g., fluorescent signal) associated with the second nucleic acid probe or a product thereof at a spatial location on the first substrate or on the second substrate.

63. The method of any of claims 59-62, further comprising:

(f) removing the spatially labeled polynucleotide from the first substrate or the second substrate, wherein a sequence of the spatially labeled polynucleotide is determined after the removing step.

64. The method of any of claims 59-63, wherein the first nucleic acid probe comprises a padlock probe which optionally comprises one or more barcode sequences.

65. The method of any of claims 59-64, wherein the second nucleic acid probe comprises two or more probes that hybridize to an RNA or DNA (e.g., cDNA) molecule in the biological sample, wherein the two or more probes optionally comprise one or more barcode sequences.

66. The method of claim 65, wherein the two or more probes hybridize to an mRNA molecule in the biological sample, or wherein the two or more probes hybridize to the first nucleic acid probe (e.g., a padlock probe) or a product thereof.

67. The method of claim 65 or 66, wherein the method further comprises ligating the two or more probes hybridized to the RNA or DNA molecule to generate a ligated second nucleic acid probe.

68. The method of claim 67, wherein the ligated second nucleic acid probe is captured by the capture agent, optionally wherein the ligation is an RNA-templated or DNA-templated reaction.

69. A method of analyzing a biological sample, comprising:

(a) contacting the biological sample on a first substrate with a first nucleic acid probe and a second nucleic acid probe, wherein the first and second nucleic acid probes are not immobilized on an artificial substrate;

(b) generating a rolling circle amplification (RCA) product *in situ* in the biological sample, the RCA product comprising a sequence of the first nucleic acid probe or complement thereof;

(c) providing conditions to allow a plurality of capture agents to directly or indirectly capture the second nucleic acid probe and/or a product thereof, wherein the plurality of capture agents are joined directly or indirectly to the first substrate or to a second substrate, and wherein a capture agent of the plurality of capture agents comprises:

(i) a capture domain capable of capturing a nucleic acid, and

(ii) a spatial barcode corresponding to the position of the capture agent on the first substrate or on the second substrate; and

(d) generating a spatially labeled polynucleotide comprising (i) a sequence of the second nucleic acid probe and/or product thereof and (ii) a sequence of the spatial barcode or complement thereof,

wherein a signal (e.g., fluorescent signal) associated with the RCA product is detected at a spatial location of the biological sample on the first substrate, and the spatially labeled polynucleotide is removed from the first substrate or the second substrate to determine a sequence of the spatially labeled polynucleotide.

70. The method of claim 69, further comprising:

(e) detecting the signal (e.g., fluorescent signal) associated with the RCA product at the spatial location of the biological sample on the first substrate.

71. The method of claim 69 or 70, further comprising:

(f) removing the spatially labeled polynucleotide from the first substrate or the second substrate to determine a sequence of the spatially labeled polynucleotide.

72. The method of claim 63 or claim 71, further comprising determining the sequence of the removed spatially labeled polynucleotide or a complement thereof using sequencing by ligation, sequencing by hybridization, sequencing by synthesis, and/or sequencing by binding.

73. The method of claim 67 or claim 68, wherein the ligation is an RNA-templated or DNA-templated reaction.