

WHAT IS CLAIMED IS:

1. A method for determining a location of an analyte in a biological sample comprising:
 - (a) providing the biological sample on an array comprising a plurality of capture probes, wherein a capture probe of the plurality of capture probes comprises: (i) a spatial
5 barcode and (ii) a capture domain;
 - (b) contacting a first probe and a second probe with the biological sample, wherein the first probe and the second probe each comprise one or more sequences that are substantially complementary to sequences of the analyte, and wherein the second probe comprises a capture probe capture domain;
 - 10 (c) hybridizing the first probe and the second probe to the analyte;
 - (d) generating a ligation product by ligating the first probe and the second probe;
 - (e) releasing the ligated product from the analyte;
 - (f) hybridizing the ligation product to the capture domain; and
 - (g) determining (i) all or a part of the sequence of the ligation product bound to
15 the capture domain, or a complement thereof, and (ii) all or a part of the sequence of the spatial barcode, or a complement thereof, and using the determined sequence of (i) and (ii) to identify the location of the analyte in the biological sample.
2. The method of claim 1, wherein the first probe and the second probe are substantially
20 complementary to adjacent sequences of the analyte.
3. The method of claim 1, where the first probe and the second probe hybridize to sequences that are not adjacent to each other on the analyte.
- 25 4. The method of any one of the preceding claims, further comprising hybridizing a third probe to the first probe and the second probe, wherein the third probe comprises a first sequence that is substantially complementary to a portion of the first probe and a second sequence that is substantially complementary to a portion of the second probe.
- 30 5. The method of any one of the preceding claims, wherein hybridizing the first probe and the second probe to the analyte is performed at a first temperature.
6. The method of claim 4 or 5, wherein hybridizing the third probe to the first probe and the second probe is performed at a second temperature.

7. The method of claim 6, wherein the first temperature is a higher temperature than the second temperature.
- 5 8. The method of any one of claims 5-7, wherein the first temperature is from about 50°C to about 75°C, from about 55°C to about 70°C, or from about 60°C to about 65°C.
9. The method of any one of claims 6-8, wherein the second temperature is from about 15°C to about 35°C, from about 20°C to about 30°C, or from about 25°C to about 30°C.
- 10 10. The method of any one of claims 3-9, wherein the first probe is extended with a DNA polymerase, thereby filling in a gap between the first probe and the second probe and generating an extended first probe.
- 15 11. The method of any one of the preceding claims, wherein the first probe comprises a sequence that is substantially complementary to a first target sequence of the analyte.
12. The method of claim 11, wherein the second probe further comprises:
- 20 (i) a first sequence that is substantially complementary to a second target sequence of the analyte;
- (ii) a linker sequence;
- (iii) a second sequence that is substantially complementary to a third target sequence of the analyte; and
- 25 (iv) a capture probe capture domain that is capable of binding to a capture domain of a capture probe.
13. The method of claim 11 or 12, wherein the first target sequence of the analyte is directly adjacent to the second target sequence of the analyte.
- 30 14. The method of any one of claims 11-13, wherein the second target sequence is not directly adjacent to the third target sequence on the analyte.

15. The method of any one of claims 11-14, wherein the second target sequence and the third target sequence are (i) on different exons of the analyte or (ii) located within the same exon of the analyte but are not adjacent on the analyte.
- 5 16. The method of any one of claims 1-10, wherein the second probe comprises a sequence that is substantially complementary to a third target sequence of the analyte.
17. The method of claim 16, wherein the first probe comprises:
- 10 (i) a first sequence that is substantially complementary to a first target sequence of the analyte;
- (ii) a linker sequence; and
- (iii) a second sequence that is substantially complementary to second target sequence of the analyte.
- 15 18. The method of claim 16 or 17, wherein the second target sequence is directly adjacent to the third target sequence.
19. The method of any one of claims 16-18, wherein the first target sequence is not directly adjacent to the second target sequence on the analyte.
- 20 20. The method of any one of claims 16-19, wherein the first target sequence and second target sequence are (i) on different exons of the analyte or (ii) located within the same exon but are not directly adjacent on the analyte.
- 25 21. The method of any one of claims 11-20, wherein the linker sequence comprises a total of about 1 nucleotide to about 100 nucleotides.
22. The method of claim 21, wherein the linker further comprises a barcode sequence that serves as a proxy for identifying the analyte.
- 30 23. The method of any one of claims 1-22, wherein the first probe comprises at least two ribonucleic acid bases at the 3' end, and wherein the second probe comprises a phosphorylated nucleotide at the 5' end.

24. The method of any one of claims 1-23, wherein generating a ligation product comprises ligating (i) the first probe to the second probe or (ii) the extended first probe to the second probe using enzymatic ligation or chemical ligation, wherein the enzymatic ligation utilizes a ligase.
- 5
25. The method of claim 24, wherein the ligase is one or more of a T4 RNA ligase (Rnl2), a splintR ligase, a single stranded DNA ligase, or a T4 DNA ligase.
26. The method of any one of claims 1-22, wherein the second probe comprises a pre-adenylated phosphate group at its 5' end, and wherein the first probe comprises at least two ribonucleic acid bases at the 3' end.
- 10
27. The method of claim 26, wherein the step of generating a ligation product comprises ligating a 3' end of the first probe to the 5' end of the second probe using a ligase that does not require adenosine triphosphate for ligase activity.
- 15
28. The method of claim 27, wherein the ligase is selected from the group consisting of: thermostable 5' AppDNA/RNA Ligase, truncated T4 RNA Ligase 2, truncated T4 RNA Ligase 2 K227Q, truncated T4 RNA Ligase 2 KQ, Chlorella Virus PBCV-1 DNA Ligase, or any combination thereof.
- 20
29. The method of any one of claims 1-28, wherein the first probe further comprises a functional sequence, wherein the functional sequence is a primer sequence.
- 25
30. The method of any one of claims 1-29, further comprising providing a capture probe capture domain blocking moiety that interacts with the capture probe capture domain.
31. The method of claim 30, further comprising releasing the capture probe capture domain blocking moiety from the capture probe capture domain prior to step (f).
- 30
32. The method of any one claims 1-31, wherein the capture probe capture domain comprises a poly-adenylated (poly(A)) sequence or a complement thereof.

33. The method of claim 32, wherein the capture probe capture domain blocking moiety comprises a poly-uridine sequence, a poly-thymidine sequence, or both.

34. The method of claim 33, wherein releasing the poly-uridine sequence from the poly(A) sequence comprises denaturing the ligation product or contacting the ligation product with an endonuclease, exonuclease or ribonuclease.

35. The method of any one of claims 1-34, wherein the capture probe capture domain comprises a sequence that is complementary to all or a portion of the capture domain of the capture probe.

36. The method of any one of claims 1-35, wherein the capture probe capture domain comprises a degenerate sequence.

37. The method of any one of claims 1-36, wherein the first probe and/or the second probe is a DNA probe.

38. The method of any one of claims 4-37, wherein the third probe is a DNA probe.

39. The method of any one of claims 30-38, wherein the capture probe capture domain blocking moiety is a DNA probe.

40. The method of any one of claims 1-39, wherein the releasing step (f) comprises removing the ligated probe from the analyte.

41. The method of claim 40, wherein the releasing of (i) the ligation product from the analyte or (ii) the capture probe capture domain blocking moiety from the capture domain binding domain, comprises contacting the ligated probe with an endoribonuclease.

42. The method of claim 41, wherein the endoribonuclease is one or more of RNase H, RNase A, RNase C, or RNase I.

43. The method of claim 42, wherein the RNase H comprises RNase H1, RNase H2, or RNase H1 and RNase H2.

44. The method of any one of claims 1-43, wherein the biological sample is a tissue sample.
- 5 45. The method of claim 44, wherein the tissue sample is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, a fresh or a frozen tissue sample.
46. The method of claim 44 or 45, wherein the tissue sample is the FFPE tissue sample, and the tissue sample is decrosslinked.
- 10 47. The method of any one of claims 1-46, wherein the biological sample was previously stained.
48. The method of claim 47, wherein the biological sample was previously stained using
- 15 immunofluorescence or immunohistochemistry.
49. The method of claim 48, wherein the biological sample was previously stained using hematoxylin and eosin.
- 20 50. The method of any one of claims 1-49, wherein the method further comprises contacting the biological sample with a permeabilization agent, wherein the permeabilization agent is selected from an organic solvent, a detergent, and an enzyme, or a combination thereof.
- 25 51. The method of claim 50, wherein the permeabilization agent is selected from the group consisting of: an endopeptidase, a protease sodium dodecyl sulfate (SDS), polyethylene glycol tert-octylphenyl ether, polysorbate 80, and polysorbate 20, N-lauroylsarcosine sodium salt solution, saponin, Triton X-100™, and Tween-20™.
- 30 52. The method of claim 51, wherein the endopeptidase is pepsin or proteinase K.
53. The method of any one of claims 1-52, wherein the method further comprises, prior to step (a), fixing the biological sample.

54. The method of claim 53, wherein the step of fixing the biological sample is performed using one or both of methanol and acetone.
55. The method of any one of claims 1-54, wherein the analyte comprises RNA.
56. The method of claim 55, wherein the RNA is an mRNA.
57. The method of any one of claims 1-56, wherein the determining step comprises amplifying all or part of the ligation product specifically bound to the capture domain.
58. The method of claim 57, wherein an amplifying product comprises (i) all or part of sequence of the ligation product specifically bound to the capture domain, or a complement thereof, and (ii) all or a part of the sequence of the spatial barcode, or a complement thereof.
59. The method of any one of claims 1-58, wherein the determining step comprises sequencing.
60. The method of claim 59, wherein the sequencing step comprises *in situ* sequencing, Sanger sequencing methods, next-generation sequencing methods, and nanopore sequencing.
61. A kit comprising:
- (a) a substrate comprising a plurality of capture probes comprising a spatial barcode and a capture domain;
 - (b) a system comprising: a plurality of first probes and second probes, wherein a first probe and a second probe each comprises sequences that are substantially complementary to an analyte, and wherein the second probe comprises a capture binding domain; and
 - (c) instructions for performing the method of any one of the preceding claims.
62. A kit comprising:
- (a) an array comprising a plurality of capture probes;
 - (b) a plurality of probes comprising a first probe and a second, wherein the first probe and the second probe are substantially complementary to adjacent sequences of an

analyte, wherein the second probe comprises (i) a capture probe capture domain that is capable of binding to a capture domain of the capture probe and (ii) a linker sequence;

- (c) a plurality of enzymes comprising a ribonuclease and a ligase; and
- (d) instructions for performing the method of any one of the preceding claims.

5

63. A kit comprising:

- (a) an array comprising a plurality of capture probes;
- (b) a plurality of probes comprising a first probe and a second probe, wherein the first probe and the second probe are substantially complementary to adjacent sequences of an
10 analyte, wherein the first probe includes a linker sequence, wherein the second probe comprises a capture probe capture domain that is capable of binding to a capture domain of the capture probe;
- (c) a plurality of enzymes comprising a ribonuclease and a ligase; and
- (d) instructions for performing the method of any one of the preceding claims.

15

64. The kit of any one of claims 61-63, wherein the kit comprises a second probe comprising a preadenylated phosphate group at its 5' end, and a ligase that does not require adenosine triphosphate for ligase activity.

- 20 65. A composition comprising a spatial array comprising capture probes, wherein the capture probes comprise a spatial barcode and a capture domain, a biological sample on the spatial array wherein the biological sample comprises a plurality of analytes of interest, a first probe oligonucleotide and a second probe oligonucleotide hybridized to an analyte and ligated together, wherein the first probe oligonucleotide and the second probe oligonucleotide
25 each comprise a sequence that is substantially complementary to adjacent sequences of the analyte and wherein one of the first probe or the second probe comprises a capture probe capture domain.

30 66. The composition of claim 65, wherein the composition further comprises an RNase H enzyme.

67. The composition of claim 66, wherein the composition further comprises a ligase.

68. The composition of claim 67, wherein the probe oligonucleotide that does not comprise a capture probe capture domain comprises a functional domain.