

CLAIMS

1. 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 bind to a first target nucleic acid or a complement or an amplification product thereof in the biological sample, wherein the one or more nucleic acid probes are in a buffer;
 - (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, or a ligation product generated by hybridizing a pair of probes to the second target nucleic acid and ligating the pair of probes using the second target nucleic acid as template,
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.
2. 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 bind to a first target nucleic acid or a complement or an amplification product thereof in the biological sample, wherein the one or more nucleic acid probes are in a buffer,
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, or a ligation product generated by hybridizing a pair of probes to the second target nucleic acid and ligating the pair of probes using the second target nucleic acid as template; 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. A method of analyzing a biological sample, comprising:

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

(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, or a ligation product generated by hybridizing a pair of probes to the second target nucleic acid and ligating the pair of probes using the second target nucleic acid as template,

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.

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

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

6. 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.

7. The method of any of claims 1-6, 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.

8. The method of any of claims 1-7, 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).

9. The method of any of claims 1-8, 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.

10. The method of any of claims 1-9, wherein the biological sample is reversibly cross-linked prior to or during step (a), optionally wherein the conditions provided in step (c) comprises de-crosslinking the reversibly cross-linked biological sample.

11. The method of any of claims 1-10, wherein the conditions provided in step (c) comprise releasing the second target nucleic acid or the complement thereof or the amplification product thereof from the biological sample.

12. The method of any of claims 1-11, wherein the conditions provided in step (c) comprise migrating the second target nucleic acid or the complement thereof or the amplification product thereof to the capture agents on the second substrate.

13. The method of any of claims 1-12, wherein the biological sample is a cell or tissue sample.

14. The method of any of claims 1-13, wherein the detecting the one or more nucleic acid probes in step (b) comprises imaging the biological sample.

15. The method of any of claims 1-14, wherein the detecting the one or more nucleic acid probes in step (b) comprises *in situ* sequencing and/or sequential hybridization of a plurality of probes.

16. The method of any of claims 1-15, 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, optionally wherein the primary probe comprises a circular or circularizable probe.

17. The method of claim 16, 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.

18. The method of any of claims 15-17, wherein the primary probe comprises one or more barcode sequences which correspond to a sequence of the first target nucleic acid.

19. The method of any of claims 1-18, comprising contacting the biological sample with two or more probes that hybridize to an RNA or a DNA molecule in the biological sample, and wherein the two or more probes are ligated using the RNA or DNA molecule as template to generate the second target nucleic acid.

20. The method of any of claims 1-18, wherein the second target nucleic acid is an mRNA molecule, and the capture agent comprises a capture probe.

21. The method of claim 20, 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.

22. The method of claim 21, wherein the free 3' end comprises an oligo dT, a random sequence, or a gene-specific sequence.

23. The method of claim 21 or 22, 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.

24. The method of any of claims 1-23, wherein the generated spatially labeled polynucleotide is a cDNA or amplification product thereof.

25. The method of any of claims 1-24, wherein the capture domain does not capture the first target nucleic acid, the complement thereof, or the amplification product thereof prior to step (c).

26. The method of any of claims 1-25, 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.

27. The method of any of claims 1-26, wherein the spatially labeled polynucleotide or a portion thereof is released from the first or second substrate for analysis.

28. The method of any of claims 1-27, wherein the method comprises determining at least a portion of the spatially labeled polynucleotide or a complement thereof.

29. The method of any of claims 1-28, 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 in the biological sample.

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

(a) contacting the biological sample with a first nucleic acid probe in a buffer and a second nucleic acid probe in a buffer;

(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 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 spatially labeled polynucleotide is removed from the first substrate or the second substrate to determine a sequence of the spatially labeled polynucleotide.

31. The method of claim 30, wherein the second nucleic acid probe comprises two or more probes that hybridize to an RNA or DNA molecule in the biological sample, wherein the two or more probes optionally comprise one or more barcode sequences.

32. The method of claim 31, 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 or a product thereof.

33. The method of claim 31 or 32, 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.

34. The method of claim 33, wherein the ligated second nucleic acid probe is captured by the capture agent, wherein the ligation is an RNA-templated reaction.

35. A method of analyzing a biological sample, comprising:
- (a) contacting the biological sample on a first substrate with a first nucleic acid probe in a buffer;
 - (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 a 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 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.