

poly(A) mRNA capture, targeted RNA capture as described herein is less affected by RNA degradation associated with FFPE fixation compared to methods dependent on oligo-dT capture and reverse transcription of mRNA. Further targeted RNA capture as described herein allows for sensitive measurement of specific genes of interest that otherwise might be missed with a whole transcriptomic approach. Targeted RNA capture can be used to capture a defined set of RNA molecules of interest, or it can be used at a whole transcriptome level, or anything in between. When combined with the spatial methods disclosed herein, the location and abundance of the RNA targets can be determined.

In one example, this disclosure features a method for determining a location of an analyte in a biological sample including: (a) providing the biological sample on an array including a plurality of capture probes, where a capture probe of the plurality includes: (i) a spatial barcode and (ii) a capture domain; (b) contacting a first probe and a second probe with the biological sample, where the first probe and the second probe each include one or more sequences that are substantially complementary to sequences of the analyte, and where the second probe includes a capture probe capture domain; (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 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. In some instances, the methods include determining the abundance and location of the analyte in the biological sample. In some instances, the methods include determining the abundance of an analyte at a location in the biological sample.

In one aspect, the present disclosure refers to 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 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 sequences that are substantially complementary to sequences of the analyte, and wherein the second probe comprises a capture probe capture domain that is complementary to all or a portion of the capture domain; (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 ligation product from the analyte; (f) hybridizing the ligation product to the capture domain; and (g) determining (i) all or part of the sequence of the ligation product bound to the capture domain, or a complement thereof, and (ii) all or part of the sequence of the spatial barcode, or a complement thereof, and using the determined sequences of (i) and (ii) to determine the location of the analyte in the biological sample.

In some examples, the first probe and the second probe are substantially complementary to adjacent sequences of the analyte. In some examples, the first probe and the second probe hybridize to sequences that are not adjacent to each other on the analyte.

In some examples, the method further includes hybridizing a third probe to the first probe and the second probe, where the third probe includes 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. In some examples, hybridizing the first probe and the second probe to the analyte is performed at a first temperature. In some examples, hybridizing the third probe to the first probe and the

second probe is performed at a second temperature. In some examples, the first temperature is a higher temperature than the second temperature. In some examples, 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. In some examples, 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. In some examples, 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.

In some examples, the first probe includes a sequence that is substantially complementary to a first target sequence of the analyte. In some examples, the second probe further includes: (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 (iv) a capture probe capture domain that is capable of binding to a capture domain of a capture probe. In some examples, the first target sequence of the analyte is directly adjacent to the second target sequence of the analyte. In some examples, the second target sequence is not directly adjacent to the third target sequence on the analyte. In some examples, 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.

In some examples, the second probe includes a sequence that is substantially complementary to a third target sequence of the analyte. In some examples, the first probe includes: (i) a first sequence that is substantially complementary to a first target sequence of the analyte; (ii) a linker sequence; and (iii) second sequence that is substantially complementary to second target sequence of the analyte. In some examples, the second target sequence is directly adjacent to the third target sequence. In some examples, the first target sequence is not directly adjacent to the second target sequence on the analyte. In some examples, 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.

In some examples, the linker sequence includes a total of about 1 nucleotide to about 100 nucleotides. In some examples, the linker further includes a barcode sequence that serves as a proxy for identifying the analyte.

In some examples, the first probe includes at least two ribonucleic acid bases at the 3' end, and where the second probe includes a phosphorylated nucleotide at the 5' end.

In some examples, generating a ligation product includes 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, where the enzymatic ligation utilizes a ligase. In some examples, the ligase is one or more of a T4 RNA ligase (Rnl2), a splintR ligase, a single stranded DNA  
5 ligase, or a T4 DNA ligase.

In some examples, the second probe includes a pre-adenylated phosphate group at its 5' end, and where the first probe includes at least two ribonucleic acid bases at the 3' end. In some examples, the step of generating a ligation product includes ligating a 3' end of the first probe to the 5' end of the second probe using a ligase that does not require adenosine  
10 triphosphate for ligase activity. In some examples, 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.

In some examples, the first probe further includes a functional sequence, where the  
15 functional sequence is a primer sequence.

In some examples, the method further includes providing a capture probe capture domain blocking moiety that interacts with the capture probe capture domain. In some examples, the method further includes releasing the capture probe capture domain blocking moiety from the capture probe capture domain prior to step (f). In some examples, the capture  
20 probe capture domain includes a poly-adenylated (poly(A)) sequence or a complement thereof. In some examples, the capture probe capture domain blocking moiety includes a poly-uridine sequence, a poly-thymidine sequence, or both. In some examples, releasing the poly-uridine sequence from the poly(A) sequence includes denaturing the ligation product or contacting the ligation product with an endonuclease, exonuclease or ribonuclease. In some  
25 examples, the capture probe capture domain includes a sequence that is complementary to all or a portion of the capture domain of the capture probe. In some examples, the capture probe capture domain includes a degenerate sequence.

In some examples, the first probe and/or the second probe is a DNA probe.

In some examples, the third probe is a DNA probe.

In some examples, the capture probe capture domain blocking moiety is a DNA  
30 probe.

In some examples, the releasing step (f) includes removing the ligated probe from the analyte.

In some examples, 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, includes contacting the ligated probe with an endoribonuclease. In some examples, the endoribonuclease is one or more of RNase H, RNase A, RNase C, or RNase I. In some  
5 examples, the RNase H includes RNase H1, RNase H2, or RNase H1 and RNase H2.

In some examples, the biological sample is a tissue sample. In some examples, the tissue sample is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, a fresh or a frozen tissue sample. In some examples, the tissue sample is the FFPE tissue sample, and the tissue sample is decrosslinked. In some examples, the biological sample was previously  
10 stained. In some examples, the biological sample was previously stained using immunofluorescence or immunohistochemistry. In some examples, the biological sample was previously stained using hematoxylin and eosin.

In some examples, the method further includes contacting the biological sample with a permeabilization agent, where the permeabilization agent is selected from an organic  
15 solvent, a detergent, and an enzyme, or a combination thereof. In some examples, 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™. In some examples, the endopeptidase is pepsin or proteinase K.

20 In some examples, the method further includes, prior to step (a), fixing the biological sample. In some examples, the step of fixing the biological sample is performed using one or both of methanol and acetone.

In some examples, the analyte includes RNA. In some examples, the RNA is an mRNA.

25 In some examples, the determining step includes amplifying all or part of the ligation product specifically bound to the capture domain. In some examples, an amplifying product includes (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. In some examples, the determining step includes sequencing. In  
30 some examples, the sequencing step includes *in situ* sequencing, Sanger sequencing methods, next-generation sequencing methods, and nanopore sequencing.

In another example, this disclosure features a kit including: (a) a substrate including a plurality of capture probes including a spatial barcode and a capture domain; (b) a system including: a plurality of first probes and second probes, where a first probe and a second  
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probe each includes sequences that are substantially complementary to an analyte, and where the second probe includes a capture binding domain; and (c) instructions for performing the method of any one of the preceding claims.

In another example, this disclosure features a kit including: (a) an array including a plurality of capture probes; (b) a plurality of probes including a first probe and a second, where the first probe and the second probe are substantially complementary to adjacent sequences of an analyte, where the second probe includes (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 including a ribonuclease and a ligase; and (d) instructions for performing the method of any one of the preceding claims.

In another example, this disclosure features a kit including: (a) an array including a plurality of capture probes; (b) a plurality of probes including a first probe and a second probe, where the first probe and the second probe are substantially complementary to adjacent sequences of an analyte, where the first probe includes a linker sequence, where the second probe includes a capture probe capture domain that is capable of binding to a capture domain of the capture probe; (c) a plurality of enzymes including a ribonuclease and a ligase; and (d) instructions for performing the method of any one of the preceding claims.

In another aspect, the present disclosure refers to a kit comprising: (a) a spatial array comprising a plurality of capture probes, wherein a capture probe of the plurality of capture probes comprises a spatial barcode and a capture domain; (b) a first probe and a second probe, 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 one of the first probe or the second probe comprises a capture probe capture domain that can hybridize to the capture domain of the capture probe on the spatial array; (c) one or more reagents comprising a permeabilization agent; and (d) one or more enzymes selects from a DNA polymerase, an endonuclease, a ligase, and any combination thereof.

In some examples, the kit includes a second probe including a preadenylated phosphate group at its 5' end, and a ligase that does not require adenosine triphosphate for ligase activity.

In another example, this disclosure features a composition including a spatial array including capture probes, where the capture probes include a spatial barcode and a capture domain, a biological sample on the spatial array where the biological sample includes a plurality of analytes of interest, a first probe oligonucleotide and a second probe oligonucleotide hybridized to an analyte and ligated together, where the first probe oligonucleotide and the second probe oligonucleotide each include a sequence that is substantially complementary to adjacent sequences of the analyte and where one of the first probe or the second probe includes a capture probe capture domain.

In another aspect, the present disclosure refers to a composition comprising: (a) a spatial array comprising a plurality of capture probes, wherein a capture probe of the plurality of capture probes comprises a spatial barcode and a capture domain; (b) a biological sample on the spatial array wherein the biological sample comprises a plurality of analytes; and (c) a first probe and a second probe that are ligated together, 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 one of the first probe or the second probe comprises a capture probe capture domain that is hybridized to the capture domain of the capture probe on the spatial array.

In some examples, the composition further includes an RNase H enzyme. In some examples, the composition further includes a ligase. In some examples, the probe oligonucleotide that does not include a capture probe capture domain includes a functional domain.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent,

patent application, or item of information was specifically and individually indicated to be incorporated by reference. To the extent publications, patents, patent applications, and items of information incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

Where values are described in terms of ranges, it should be understood that the description includes the disclosure of all possible sub-ranges within such ranges, as well as specific numerical values that fall within such ranges irrespective of whether a specific numerical value or specific sub-range is expressly stated.

The term “each,” when used in reference to a collection of items, is intended to identify an individual item in the collection but does not necessarily refer to every item in the collection, unless expressly stated otherwise, or unless the context of the usage clearly indicates otherwise.

The singular form “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes one or more cells, comprising mixtures thereof. “A and/or B” is used herein to include all of the following alternatives: “A”, “B”, “A or B”, and “A and B”.

Various examples of the features of this disclosure are described herein. However, it should be understood that such examples are provided merely by way of example, and numerous variations, changes, and substitutions can occur to those skilled in the art without departing from the scope of this disclosure. It should also be understood that various alternatives to the specific examples described herein are also within the scope of this disclosure.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings illustrate certain embodiments of the features and advantages of this disclosure. These embodiments are not intended to limit the scope of the appended claims in any manner. Like reference symbols in the drawings indicate like elements.

**FIG. 1** shows an exemplary spatial analysis workflow.

**FIG. 2** is a schematic diagram showing an example of a barcoded capture probe, as described herein.

**FIG. 3** is a schematic illustrating a cleavable capture probe, wherein the cleaved capture probe can enter into a non-permeabilized cell and bind to target analytes within the sample.

**WHAT IS CLAIMED IS:**

1. A composition comprising:
  - (a) a spatial array comprising a plurality of capture probes, wherein a capture  
5 probe of the plurality of capture probes comprises a spatial barcode and a capture domain;
  - (b) a biological sample on the spatial array wherein the biological sample  
comprises a plurality of analytes; and
  - (c) a first probe and a second probe that are ligated together, wherein the first  
10 probe and the second probe each comprise one or more sequences that are substantially  
complementary to sequences of the analyte and wherein one of the first probe or the second  
probe comprises a capture probe capture domain that is hybridized to the capture domain of  
the capture probe on the spatial array.
2. The composition of claim 1, wherein the first probe or the second probe that does not  
15 comprise the capture probe capture domain comprises a functional domain.
3. The composition of claim 1 or 2, wherein the second probe comprises the capture  
probe capture domain.
- 20 4. The composition of any one of claims 1-3, wherein the first probe and the second  
probe are substantially complementary to adjacent sequences of the analyte.
5. The composition of any one of claims 1-4, wherein the first probe and the second  
probe are substantially complementary to sequences that are not adjacent to each other on the  
25 analyte, wherein one of the first probe or the second probe is extended with a DNA  
polymerase.
6. The composition of any one of claims 1-5, wherein the first probe and/or the second  
probe is a DNA probe.
- 30 7. The composition of any one of claims 1-6, further comprising 5000 or more ligated  
probe pairs, wherein a ligated probe pair of the 5000 or more ligated probe pairs comprises  
the first probe and the second probe that are ligated together, and wherein at least one of the  
ligated probe pairs is hybridized to the capture domain of a capture probe on the spatial array.

8. The composition of any one of claims 1-7, wherein prior to ligation to the second probe the first probe comprised a pre-adenylated phosphate group at its 5' end.
- 5 9. The composition of any one of claims 1-8, wherein the capture probe capture domain comprises a poly-adenylated sequence or a complement thereof.
10. The composition of any one of claims 1-9, further comprising a DNA polymerase.
- 10 11. The composition of any one of claims 1-10, further comprising an RNase H enzyme, wherein the RNase H enzyme comprises RNase H1, RNase H2, or both.
12. The composition of any one of claims 1-11, further comprising a permeabilization agent, wherein the permeabilization agent is proteinase K or pepsin.
- 15 13. The composition of any one of claims 1-12, further comprising a ligase, 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.
- 20 14. The composition of any one of claims 1-13, wherein the tissue sample is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, a fresh tissue sample, or a frozen tissue sample.
15. A kit comprising:
- 25 (a) a spatial array comprising a plurality of capture probes, wherein a capture probe of the plurality of capture probes comprises a spatial barcode and a capture domain;
- (b) a first probe and a second probe, 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 one of the first probe or the second probe comprises a capture probe
- 30 capture domain that can hybridize to the capture domain of the capture probe on the spatial array;
- (c) one or more reagents comprising a permeabilization agent; and
- (d) one or more enzymes selected from a DNA polymerase, an endonuclease, a ligase, and any combination thereof.

16. The kit of claim 15, wherein the permeabilization agent is proteinase K or pepsin.
17. The kit of claim 15 or 16, wherein the endonuclease is RNase H.
- 5 18. The kit of any one of claims 15-17, 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.
19. A method for determining a location of an analyte in a biological sample comprising:
- 10 (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 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 sequences that are substantially complementary to sequences of the analyte, and wherein the second probe comprises a
- 15 capture probe capture domain that is complementary to all or a portion of the capture domain;
- (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 ligation product from the analyte;
- (f) hybridizing the ligation product to the capture domain; and
- 20 (g) determining (i) all or part of the sequence of the ligation product bound to the capture domain, or a complement thereof, and (ii) all or part of the sequence of the spatial barcode, or a complement thereof, and using the determined sequences of (i) and (ii) to determine the location of the analyte in the biological sample.
- 25 20. The method of claim 19, wherein the first probe and the second probe are substantially complementary to adjacent sequences of the analyte.
21. The method of claim 19, where the first probe and the second probe hybridize to sequences that are not adjacent to each other on the analyte, and wherein the first probe is
- 30 extended with a DNA polymerase, thereby (i) filling in a gap between the first probe and the second probe and (ii) generating an extended first probe.
22. The method of any one of claims 19-21, further comprising (i) hybridizing the first probe and the second probe to the analyte at a temperature from about 50°C to about 75°C,

and (ii) hybridizing the first probe and the second probe to a third probe at a temperature from about 15°C to about 35°C.

23. The method of any one of claims 19-22, wherein the contacting the first probe and the second probe with the biological sample comprises contacting the biological sample with 5000 or more probe pairs, wherein a probe pair of the 5000 or more probe pairs comprises the first probe and the second probe.

24. The method of any one of claims 19-23, wherein the generating the ligation product comprises ligating the first probe to the second probe using enzymatic ligation or chemical ligation, wherein the enzymatic ligation utilizes a ligase, 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.

25. The method of any one of claims 19-24, wherein the first probe further comprises a primer sequence.

26. The method of any one of claims 19-25, wherein the first probe and/or the second probe is a DNA probe.

27. The method of any one of claims 19-26, wherein the releasing the ligation product from the analyte utilizes an endonuclease.

28. The method of claim 27, wherein the endonuclease is an RNase H enzyme.

29. The method of any one of claims 19-28, wherein the biological sample is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, a fresh tissue sample, or a frozen tissue sample.

30. The method of any one of claims 19-29, wherein the biological sample was previously stained using immunofluorescence, immunohistochemistry, or hematoxylin and eosin.

31. The method of any one of claims 19-30, further comprising contacting the biological sample with a permeabilization agent, wherein the permeabilization agent is selected from an organic solvent, a detergent, an enzyme, or a combination thereof.
- 5 32. The method of claim 31, wherein the permeabilization agent comprises proteinase K or pepsin.
33. The method of any one of claims 19-32, wherein the analyte comprises RNA, optionally wherein the RNA is an mRNA.
- 10 34. The method of any one of claims 19-33, wherein the determining step (g) comprises sequencing (i) all or a part of the sequence of the ligation product, or a complement thereof, and (ii) all or a part of the sequence of the spatial barcode, or a complement thereof.