

cells or the cell's position relative to the tissue microenvironment) can affect, e.g., the cell's morphology, differentiation, fate, viability, proliferation, behavior, and signaling and cross-talk with other cells in the tissue.

Spatial heterogeneity has been previously studied using techniques that only provide data for a small handful of analytes in the contact of an intact tissue or a portion of a tissue, or provide a lot of analyte data for single cells, but fail to provide information regarding the position of the single cell in a parent biological sample (e.g., tissue sample).

Arrays that capture spatial information of analytes within a biological sample can be prepared in various ways. Arrays can be designed with pluralities of capture probes. Capture probes can be designed to capture a substantial population of analytes (e.g., mRNA) or to capture specific (e.g., gene specific) analytes. The methods described herein provide for ways of preparing arrays with capture probes and capturing analytes.

SUMMARY

Provided herein are methods of generating a spatial array including: (a) providing an array including a plurality of oligonucleotides, where the 3' end of an oligonucleotide of the plurality of oligonucleotides is attached to a substrate, (b) providing a plurality of primers, where a primer of the plurality of primers is substantially complementary to a portion of the oligonucleotide, (c) extending the primer using the oligonucleotide as a template, thereby generating a first oligonucleotide with a free 3' end, (d) extending the first oligonucleotide to produce a 3' overhang, (e) providing a splint oligonucleotide that hybridizes to the 3' end of the first oligonucleotide, and (f) ligating a second oligonucleotide to the 3' end of the first oligonucleotide, where the second oligonucleotide includes a sequence that is substantially complementary to a portion of the splint oligonucleotide, and where the second oligonucleotide includes a capture domain, thereby generating a spatial array.

In some examples of methods of generating a spatial array, the oligonucleotide of the plurality of oligonucleotides includes a spatial barcode. In some examples, the oligonucleotide of the plurality of oligonucleotides includes a first constant sequence. In some examples of methods of generating a spatial array, the oligonucleotide of the plurality of oligonucleotides further includes a second constant sequence. In some examples of methods of generating a spatial array, the oligonucleotide of the plurality of oligonucleotides has a length of between 20 and 60 nucleotides.

In some examples of methods of generating a spatial array, the second oligonucleotide further includes a constant sequence, and where the constant sequence is

substantially complementary to a portion of the splint oligonucleotide. In some examples of methods of generating a spatial array, the second oligonucleotide further includes a unique molecular identifier. In some examples of methods of generating a spatial array, the second oligonucleotide further includes a spatial barcode.

In some examples of methods of generating a spatial array, the capture domain includes a poly-T sequence. In some examples of methods of generating a spatial array, the capture domain includes a gene-specific sequence.

In some examples of methods of generating a spatial array, the 3' end overhang of step (d) is produced by an enzyme. In some examples of methods of generating a spatial array, the enzyme is a template-independent polymerase. In some examples of methods of generating a spatial array, the template-independent polymerase is terminal deoxynucleotidyl transferase (TdT) or poly(A) polymerase.

Provided herein are methods for determining the location of an analyte in a biological sample including (a) contacting the biological sample with a spatial array produced by any of the variety of methods herein (b) permeabilizing the biological sample to allow the analyte in the biological sample to specifically bind with a capture domain on the spatial array, and (c) identifying the analyte specifically bound to the capture domain, thereby determining the spatial location of the analyte in the biological sample.

In some examples of methods for determining the location of an analyte in a biological sample, a second analyte in the biological sample interacts with a second capture domain on the spatial array, and step (c) includes identifying the second analyte specifically bound to the second capture domain, thereby determining the spatial location of the analyte in the second biological sample.

Provided herein are methods for preparing a spatial array, including (a) contacting an array including one or more pluralities of first oligonucleotides with a hydrogel including one or more pluralities of second oligonucleotides, where a first oligonucleotide of the one or more pluralities of first oligonucleotides includes a capture domain, a second oligonucleotide of the one or more pluralities of second oligonucleotides includes a capture domain, or both, (b) attaching the first oligonucleotide to the second oligonucleotide to generate a capture probe including a capture domain, (c) reducing the size of the hydrogel including the capture probe by removing water to form a shrunken hydrogel including the capture probe, thereby generating the spatial array.

In some examples of methods for preparing a spatial array, the one or more pluralities of second oligonucleotides are immobilized in the hydrogel. In some examples

of methods for preparing a spatial array, the immobilized second oligonucleotides are partitioned such that a first population of the immobilized second oligonucleotides is partitioned away from another population of immobilized second oligonucleotides.

In some examples of methods for preparing a spatial array, the immobilized second oligonucleotides are partitioned into a plurality of second oligonucleotide partitions such that different populations of the immobilized second oligonucleotides are partitioned away from each other. In some examples of methods for preparing a spatial array, a first immobilized second oligonucleotide of a partitioned population of immobilized second oligonucleotides is different from a second immobilized second oligonucleotide of another partitioned population of immobilized second oligonucleotides. In some examples of methods for preparing a spatial array, the first immobilized second oligonucleotide, the second immobilized second oligonucleotide, or both include a spatial barcode.

In some examples of methods for preparing a spatial array, the first immobilized second oligonucleotide of a partitioned population of immobilized second oligonucleotides includes a spatial barcode that is different from a spatial barcode of the second immobilized second oligonucleotide of another partitioned population of immobilized second oligonucleotides.

In some examples of methods for preparing a spatial array, one or more second oligonucleotide partitions include a population of immobilized capture probes including a spatial barcode different from a spatial barcode of the population of immobilized capture probes of another second oligonucleotide partition.

In some examples of methods for preparing a spatial array, the one or more pluralities of first oligonucleotides are partitioned. In some examples of methods for preparing a spatial array, the one or more pluralities of first oligonucleotides are partitioned into a first oligonucleotide partitions. In some examples of methods for preparing a spatial array, one or more first oligonucleotide partitions include a population of first oligonucleotides that is different from first oligonucleotides in another first oligonucleotide partition. In some examples of methods for preparing a spatial array, a first oligonucleotide of a population of first oligonucleotides in one or more first oligonucleotide partitions includes a spatial barcode. In some examples of methods for preparing a spatial array, the first oligonucleotide of a population of first oligonucleotides in one or more first oligonucleotide partitions includes a spatial barcode that is different from the spatial barcode of a first oligonucleotide of a population of first oligonucleotides in another first oligonucleotide partition. In some examples of methods for preparing a spatial array, a

spatial barcode of the first oligonucleotide of a population of first oligonucleotides in one or more first oligonucleotide partitions is different from a spatial barcode of a second oligonucleotide of a population of second oligonucleotides in one or more second oligonucleotide partitions. In some examples of methods for preparing a spatial array, a first oligonucleotide partition includes a first population of first oligonucleotides including a spatial barcode that is different from a spatial barcode of a second population of first oligonucleotides in another first oligonucleotide partition.

In some examples of methods for preparing a spatial array, the reduction in the size of the hydrogel in step (c) is isometric. In some examples of methods for preparing a spatial array, the removal of water in step (c) includes a dehydrating solvent. In some examples of methods for preparing a spatial array, the solvent includes at least one of an alcohol, a ketone, an aldehyde, acetonitrile, or a combination thereof. In some examples of methods for preparing a spatial array, the solvent includes at least one of glycerol, ethanol, acetonitrile, acetone, 2-propanol, and a combination thereof. In some examples, the removal of water in step (c) includes the use of a salt. In some examples of methods for preparing a spatial array, the salt includes an inorganic salt. In some examples of methods for preparing a spatial array, the removal of water in step (c) includes an acid. In some examples of methods for preparing a spatial array, the removal of water in step (c) includes a dehydrating process.

In some examples of methods for preparing a spatial array, the shrunken hydrogel is about 2 to about 10 fold smaller in a linear dimension than the size of the hydrogel prior to step (c) pre-shrunk hydrogel. In some examples of methods for preparing a spatial array, the shrunken hydrogel is at least 2-fold smaller in a linear dimension than the pre-shrunk hydrogel. In some examples of methods for preparing a spatial array, the shrunken hydrogel is at least 4-fold smaller in a linear dimension than the pre-shrunk hydrogel. In some examples of methods for preparing a spatial array, the shrunken hydrogel is at least 8-fold smaller in a linear dimension than the pre-shrunk hydrogel.

In some examples of methods for preparing a spatial array, attaching includes a polymerase chain reaction. In some examples of methods for preparing a spatial array, the hydrogel includes a DNA polymerase, a plurality of primers, and a plurality of nucleotides. In some examples of methods for preparing a spatial array, members of a plurality of first oligonucleotides are cross-linked to the hydrogel. In some examples of methods for preparing a spatial array, the capture probe is amplified. In some examples of

methods for preparing a spatial array, the capture probe is amplified prior to the reduction of the size of the hydrogel.

In some examples of methods for preparing a spatial array, the hydrogel is prepared from hydrogel subunits including at least one of: acrylamide, bis-acrylamide, a poly(ethylene glycol), a polyaliphatic polyurethane, a polyether polyurethane, a polyester polyurethane, a polyethylene copolymer, a polyamide, a polyvinyl alcohol, polypropylene glycol, polytetramethylene oxide, polyvinyl pyrrolidone, polyacrylamide, poly(hydroxyethyl acrylate), poly(hydroxyethyl methacrylate), collagen, hyaluronic acid, chitosan, dextran, agarose, gelatin, alginate, a protein polymer, methylcellulose, and a combination thereof.

In some examples of methods for preparing a spatial array, the first oligonucleotide of the one or more pluralities of first oligonucleotides includes a spatial barcode. In some examples of methods for preparing a spatial array, the first oligonucleotide of the one or more pluralities of first oligonucleotides includes a unique molecular identifier. In some examples of methods for preparing a spatial array, the first oligonucleotide of the one or more pluralities of first oligonucleotides includes a spatial barcode and a capture domain.

Provided herein are methods for determining the location of an analyte in a biological sample, including (a) providing a spatial array of any of the variety of methods described herein, (b) contacting a biological sample with a spatial array of any one of the spatial arrays described herein under conditions that allow the analyte to specifically bind to a capture probe of the spatial array, and (c) determining the identity of the analyte specifically bound to the capture probe, thereby determining the location of the analyte in the biological sample.

In some examples of methods for determining the location of an analyte in a biological sample, the biological sample is a tissue sample. In some examples of methods for determining the location of an analyte in a biological sample, the tissue sample is a fresh-frozen tissue sample. In some examples of methods for determining the location of an analyte in a biological sample, the tissue sample includes a tumor cell. In some examples of methods for determining the location of an analyte in a biological sample, the tissue sample includes a tissue section.

In some examples, methods for determining the location of an analyte in a biological sample further includes migrating the analyte in the biological sample to the capture probe. In some examples of methods for determining the location of an analyte in a biological sample, migrating includes passive migration. In some examples of methods

for determining the location of an analyte in a biological sample, migrating includes active migration.

In some examples of methods for determining the location of an analyte in a biological sample, the analyte includes at least one of RNA, DNA, protein, lipid, peptide, metabolite, small molecule, and an analyte capture agent.

Provided herein are methods of generating an array including (a) providing a substrate including a plurality of features immobilized on the substrate, where a feature of the plurality of features includes a first oligonucleotide, and where the first oligonucleotide includes a first spatial barcode, (b) delivering a second oligonucleotide to the first oligonucleotide, where the second oligonucleotide includes a second spatial barcode and a capture domain, and (c) attaching the first oligonucleotide and the second oligonucleotide, thereby generating a capture probe including the first spatial barcode, the second spatial barcode, and the capture domain.

In some examples of methods of generating an array, the first oligonucleotide is associated with one of a number of different first spatial barcodes. In some examples of methods of generating an array, the second oligonucleotide is associated with one of a number of different second spatial barcodes.

In some examples of methods of generating an array, step (c) includes ligating the first oligonucleotide and the second oligonucleotide. In some examples of methods of generating an array, the ligating includes the use of a ligase. In some examples of methods of generating an array, the first oligonucleotide and the second oligonucleotide are attached to one another by a chemical coupling reaction.

In some examples of methods of generating an array, the first oligonucleotide includes a 3' end that is masked by a protective group. In some examples of methods of generating an array, the protective group is removed prior to delivering the second oligonucleotide to the first oligonucleotide. In some examples of methods of generating an array, the protective group is a photocleavable protective group. In some examples of methods of generating an array, the photocleavable protective group is removed by exposing the first oligonucleotide to light. In some examples of methods of generating an array, the photocleavable protective group coupled to the first oligonucleotide is protected by applying a photomask.

In some examples of methods of generating an array, a first feature is separated from other features of the plurality by one or more physical barriers, where the one or more

physical barriers form one or more chambers, and where the first oligonucleotide is delivered to a chamber of the one or more chambers.

In some examples of methods of generating an array, the one or more physical barriers include a hydrophobic surface. In some examples of methods of generating an array, the one or more physical barriers include one or more chamber walls having a tapered end in contact with the substrate. In some examples of methods of generating an array, the first oligonucleotide includes a unique molecular identifier.

Provided herein are methods of generating an array including (a) providing a substrate including two or more partitions, where a first partition of the two or more partitions includes a feature immobilized on the substrate, where the feature includes a first oligonucleotide, and where the first oligonucleotide includes a first spatial barcode, (b) delivering a second oligonucleotide to the first partition, where the second oligonucleotide includes a second spatial barcode and a capture domain, and (c) attaching the first oligonucleotide and the second oligonucleotide in the first partition, thereby generating a capture probe including the first spatial barcode, the second spatial barcode, and the capture domain in the first partition.

In some examples, methods of generating an array further include (d) delivering a third oligonucleotide to a second partition of the two or more partitions, where the third oligonucleotide includes a third spatial barcode and a second capture domain, where the second partition includes a second feature, where the second feature includes a fourth oligonucleotide including the first spatial barcode, and (e) attaching the third oligonucleotide and the fourth oligonucleotide, thereby generating a second capture probe including the first spatial barcode, the third spatial barcode, and the second capture domain in the second partition.

In some examples of methods of generating an array, the two or more partitions are separated by one or more physical barriers. In some examples of methods of generating an array, the one or more physical barriers are printed on the substrate. In some examples of methods of generating an array, the one or more physical barriers form one or more chambers including one or more chamber walls. In some examples of methods of generating an array, the one or more chamber walls include a hydrophobic surface. In some examples, the one or more chamber walls include a tapered end in contact with the substrate. In some examples of methods of generating an array, the second oligonucleotide is delivered using a microfluidic device.

Provided herein are methods for enriching a population of capture probes including (a) providing a plurality of capture probes where: (i) members of the plurality of capture probes include a plurality of different spatial barcodes, and (ii) a target capture probe of the population of capture probes to be enriched includes a target spatial barcode, and (b) enriching a population of target capture probes including the target spatial barcode from the plurality of capture probes.

Provided herein are methods for determining the location of an analyte in a biological sample including (a) providing a substrate including a plurality of capture probes, where a subset of the plurality of capture probes is attached at a location on the substrate, and where two or more capture probes of the subset of capture probes includes a first common spatial barcode, (b) contacting the biological sample with the substrate such that a capture probe of the subset of capture probes specifically binds to the analyte from the biological sample, where the analyte that is specifically bound to the capture probe includes the first common spatial barcode or a complement thereof, (c) identifying a region of interest within the biological sample, where the region of interest includes the analyte specifically bound to the capture probe, and (d) enriching the analyte specifically bound to the capture probe to determine the location of the analyte in the biological sample.

In some examples of methods for determining the location of an analyte in a biological sample, the substrate further includes a second subset of capture probes attached at a second location on the substrate, where two or more capture probes of the second subset of capture probes include a second common spatial barcode that is different from the first common spatial barcode.

In some examples of methods for determining the location of an analyte in a biological sample, the substrate further includes a third subset of capture probes attached at a third position on the substrate, where two or more capture probes of the third subset of capture probes includes a third common spatial barcode that is different from the first common spatial barcode and the second common spatial barcode. In some examples of methods for determining the location of an analyte in a biological sample, the capture probe includes a capture domain.

In some examples of methods for determining the location of an analyte in a biological sample, the analyte includes at least one of RNA, DNA, and a analyte capture agent. In some examples of methods for determining the location of an analyte in a biological sample, the analyte capture agent includes an antigen binding molecule. In some examples of methods for determining the location of an analyte in a biological sample,

the analyte capture agent includes a region configured to couple or specifically bind to the capture domain of the capture probe.

In some examples of methods for determining the location of an analyte in a biological sample, the region of interest is identified by performing shallow sequencing of at least a subset of a plurality of analytes specifically bound to capture probes, and identifying the first common spatial barcode associated with the region of interest. In some examples of methods for determining the location of an analyte in a biological sample, the region of interest is identified by at least one of immunostaining, immunohistochemistry, microscopy, visual inspection, and combinations thereof.

In some examples of methods for determining the location of an analyte in a biological sample, step (d) includes performing a nucleic acid extension reaction using (i) the analyte specifically bound to the capture probe and (ii) a primer including a sequence specific for the first common spatial barcode or a complement thereof to generate an enriched plurality of the analyte specifically bound to the capture probe(s) including the first common barcode or a complement thereof.

In some examples of methods for determining the location of an analyte in a biological sample, the analyte is amplified by performing a polymerase chain reaction (PCR).

In some examples, methods for determining the location of an analyte in a biological sample further include enriching a population of capture probes present in a subregion of interest (a) where the subregion of interest is located within the region of interest, where the subregion of interest includes a sub-plurality of capture probes, and where: (i) two or more capture probes of the sub-plurality of capture probes include a first common sub-spatial barcode, and (ii) the first common sub-spatial barcode sequence is associated with the subregion of interest, and (b) enriching a population of capture probe including the first common sub-spatial barcode.

In some examples, methods for determining the location of an analyte in a biological sample further include enriching a population of capture probes present in a sub-subregion of interest (a) where the sub-subregion of interest is located within the subregion of interest, where the sub-subregion of interest includes a sub-sub-plurality of capture probes, and where: (i) two or more capture probes of the sub-sub plurality of capture probes include a first common sub-sub spatial barcode, and (ii) the first common sub-sub spatial barcode is associated with the sub-sub region of interest, and (b) enriching the population of capture probes including the first sub-sub spatial barcode.

In some examples of methods for determining the location of an analyte in a biological sample, the first common spatial barcode is associated with a single cell of the biological sample.

In some examples of methods for determining the location of an analyte in a biological sample, the analyte includes an mRNA. In some examples of methods for determining the location of an analyte in a biological sample, the analyte includes a protein. In some examples of methods for determining the location of an analyte in a biological sample, the analyte includes an antibody. In some examples of methods for determining the location of an analyte in a biological sample, the analyte includes DNA. In some examples of methods for determining the location of an analyte in a biological sample, the DNA encodes a T-cell receptor. In some examples of methods for determining the location of an analyte in a biological sample, the DNA includes one or more regions of chromatin in the biological sample.

Provided herein are methods for generating a spatial array including (a) providing a substrate including a plurality of first oligonucleotides, where a first oligonucleotide of the plurality of first oligonucleotides is attached to a region of the substrate through its 3'-end or its 5'-end, (b) attaching a second oligonucleotide to an unattached end of the first oligonucleotide, where the second oligonucleotide includes a spatial barcode.

In some examples, methods for generating a spatial array further include after (b): (c) attaching a third oligonucleotide segment to an unattached end of the second oligonucleotide.

In some examples of methods for generating a spatial array, the first oligonucleotide is attached to the substrate through its 5'-end and the second oligonucleotide is generated using spatially-directed in situ synthesis on the 3'-end of the first oligonucleotide. In some examples of methods for generating a spatial array, the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is generated using spatially-directed in situ synthesis on the 5'-end of the first oligonucleotide.

In some examples of methods for generating a spatial array, phosphodiester and exocyclic amine moieties of the first oligonucleotide are protected.

In some examples of methods for generating a spatial array, the second oligonucleotide has a length selected from 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23

nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, or 30 nucleotides.

In some examples of methods for generating a spatial array, the in situ synthesis is light-directed synthesis.

In some examples of methods for generating a spatial array, the region of the substrate is less than about 2500 μm^2 , less than about 1600 μm^2 , less than about 400 μm^2 , less than about 100 μm^2 , less than about 50 μm^2 , less than about 25 μm^2 , less than about 15 μm^2 , less than about 10 μm^2 , or less than about 5 μm^2 .

In some examples of methods for generating a spatial array, two or more second oligonucleotides are attached to two or more first oligonucleotides, where a member of the two or more second oligonucleotides differs from another member of the two or more second oligonucleotides.

In some examples of methods for generating a spatial array, the first oligonucleotide includes a protected phosphodiester and exocyclic amine moieties.

In some examples of methods for generating a spatial array, the substrate further includes a substantially complementary oligonucleotide hybridized to the first oligonucleotide.

In some examples of methods for generating a spatial array, the first oligonucleotide has a length of at least about 2 nucleotides, 5 nucleotides, 8 nucleotides, 10 nucleotides, 15 nucleotides, 18 nucleotides, 20 nucleotides, 22 nucleotides, 25 nucleotides, 27 nucleotides, 30 nucleotides, 33 nucleotides, or 35 nucleotides. In some examples of methods for generating a spatial array, the first oligonucleotide has a length of about 2 to 45 nucleotides, 5 to 40 nucleotides, 15 to 40 nucleotides, 25 to 40 nucleotides, 25 to 35 nucleotides, or 27 to 33 nucleotides.

In some examples of methods for generating a spatial array, the first oligonucleotide is prepared by synthesis off of the substrate and the first oligonucleotide is subsequently attached to the substrate.

In some examples of methods for generating a spatial array, the phosphodiester and exocyclic amine moieties of the first oligonucleotide are protected prior to attachment of the first oligonucleotide to the substrate. In some examples of methods for generating a spatial array, the phosphodiester and exocyclic amine moieties of the first oligonucleotide are protected after attachment of the first oligonucleotide to the substrate.

In some examples of methods for generating a spatial array, the first oligonucleotide is prepared by in situ synthesis on the substrate. In some examples of

methods for generating a spatial array, the in situ synthesis is not light-directed synthesis. In some examples of methods for generating a spatial array, the in situ synthesis is not spatially-directed synthesis.

In some examples of methods for generating a spatial array, the first oligonucleotide includes a sequence including a cleavage site. In some examples of methods for generating a spatial array, the first oligonucleotide is attached to the substrate through a linker. In some examples of methods for generating a spatial array, a chemical group including: silane, amine, peptide, polyethylene glycol, biotin, desthiobiotin, streptavidin, thiol, acrydite, azide, digoxigenin, hexynyl, or a combination thereof. In some examples of methods for generating a spatial array, the linker is a branched linker or a dendrimeric linker.

In some examples of methods for generating a spatial array, the third oligonucleotide is attached to the second oligonucleotide by ligation.

In some examples of methods for generating a spatial array, any protected moieties of the first and second oligonucleotides are deprotected prior to ligating the third oligonucleotide to the second oligonucleotide.

In some examples of methods for generating a spatial array, the plurality of third oligonucleotides have a length of at least about 5 nucleotides, 7 nucleotides, 9 nucleotides, 13 nucleotides, 20 nucleotides, 25 nucleotides, 27 nucleotides, 30 nucleotides, 33 nucleotides, or 35 nucleotides. In some examples of methods for generating a spatial array, the plurality of third oligonucleotides have a length of about 5 to 40 nucleotides, 5 to 20 nucleotides, 5 to 10 nucleotides, 10 to 35 nucleotides, 15 to 30 nucleotides, 25 to 35 nucleotides, or 27 to 33 nucleotides.

In some examples of methods for generating a spatial array, the third oligonucleotide includes a capture domain of at least 6 nucleotides in length at the 3'-end. In some examples of methods for generating a spatial array, the capture domain includes a poly-T sequence. In some examples of methods for generating a spatial array, the third oligonucleotide includes a random sequence of 6 to 20 nucleotides in length.

Provided herein are compositions including a plurality of oligonucleotides attached to a plurality of regions on a substrate, where an oligonucleotide of the plurality of oligonucleotides includes: a first oligonucleotide of 2 to 50 nucleotides in length attached to the substrate through its 3'-end or its 5'-end, and a second oligonucleotide of 8 to 30 nucleotides in length that is attached to an unattached end of the first oligonucleotide, where the second oligonucleotide includes a spatial barcode.

Provided herein are compositions where the first oligonucleotide and the second oligonucleotide include protected phosphodiester and exocyclic amine moieties.

Provided herein are compositions where the first oligonucleotide is attached to the substrate through its 5'-end and the second oligonucleotide is attached to the 3'-end of the first oligonucleotide.

Provided herein are compositions where the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is attached to the 5'-end of the first oligonucleotide.

In some examples, compositions where the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is attached to the 5'-end of the first oligonucleotide further include a third oligonucleotide of 5 to 40 nucleotides in length that is attached to an unattached end of the second oligonucleotide, where the third oligonucleotide includes a capture domain.

In some examples, compositions where the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is attached to the 5'-end of the first oligonucleotide, the second oligonucleotide has a length selected from 8 nucleotides, 9 nucleotides, 10 nucleotides, 11 nucleotides, 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 21 nucleotides, 22 nucleotides, 23 nucleotides, 24 nucleotides, 25 nucleotides, 26 nucleotides, 27 nucleotides, 28 nucleotides, 29 nucleotides, or 30 nucleotides.

In some examples, compositions where the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is attached to the 5'-end of the first oligonucleotide, the regions are less than about 2500 μm^2 , less than about 1600 μm^2 , less than about 400 μm^2 , less than about 100 μm^2 , less than about 50 μm^2 , less than about 25 μm^2 , less than about 15 μm^2 , less than about 10 μm^2 , less than about 5 μm^2 , or less than about 1 μm^2 .

In some examples, compositions where the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is attached to the 5'-end of the first oligonucleotide, the length of the oligonucleotide in each of the regions is at least 15 nucleotides, at least 20 nucleotides, at least 40 nucleotides, at least 50 nucleotides, or at least 60 nucleotides.

In some examples, compositions where the first oligonucleotide is attached to the substrate through its 3'-end and the second oligonucleotide is attached to the 5'-end of the

first oligonucleotide, each of the regions is from 15 to 100 nucleotides, 20 to 100 nucleotides, 40 to 100 nucleotides, from 50 to 90 nucleotides, or from 60 to 80 nucleotides.

Provided herein are methods of preparing a spatial array including (a) providing a substrate, (b) immobilizing a plurality of sets of primers on the substrate, where a first set of the plurality of sets of primers includes a first primer and a second primer, (c) providing a template oligonucleotide including a reverse complement of a spatial barcode domain, a reverse complement of a capture domain, a first hybridization domain capable of hybridizing to the first primer, and a second hybridization domain, where a reverse complement of the second hybridization domain is capable of hybridizing to the second primer, (d) hybridizing the template oligonucleotide to the first primer, (e) extending the first primer using the template oligonucleotide as a template to generate a capture probe including the first primer, the spatial barcode, and the capture domain, which capture probe is immobilized on the substrate, and (f) releasing the template oligonucleotide from the capture probe, thereby producing the spatial array.

In some examples, methods of preparing a spatial array further include amplifying the capture probe to create a cluster of one or more capture probes.

In some examples of methods of preparing a spatial array, amplifying includes (a) hybridizing the capture probe to the second primer, (b) extending the second primer using the capture probe as a template to generate a copy of the template oligonucleotide that is immobilized on the substrate, (c) hybridizing the copy of the template oligonucleotide to a first primer of a second set of the plurality of primers, and (d) extending the first primer of the second set of the plurality of primers using the copy of template oligonucleotide as a template to generate a copy of the capture probe that is immobilized on the substrate, where the cluster of capture probes includes the capture probe and the copy of the capture probe.

In some examples of methods of preparing a spatial array, the template oligonucleotide includes a reverse complement of a unique molecular identifier (UMI), and where the generated capture probe includes the UMI.

In some examples of methods of preparing a spatial array, the first primer is affixed to the substrate at a 5' end of the first primer. In some examples of methods of preparing a spatial array, the first primer includes a sequence that is complementary to the template oligonucleotide. In some examples of methods of preparing a spatial array, the first primer includes a poly (A) domain. In some examples of methods of preparing a spatial array, the first primer includes cleavage domain.

In some examples of methods of preparing a spatial array, the second primer is affixed to the substrate at a 5' end of the second primer. In some examples of methods of preparing a spatial array, the second primer includes a cleavage domain.

In some examples of methods of preparing a spatial array, the copy of the template oligonucleotide is released by cleaving the cleavage domain after the copy of the capture probe is generated. In some examples of methods of preparing a spatial array, the template oligonucleotide is attached to a bead.

In some examples of methods of preparing a spatial array, detaching the bead from the template oligonucleotide after the step of extending the first primer using the template oligonucleotide as a template to generate a capture probe including the first primer, the spatial barcode, and the capture domain, which capture probe is immobilized on the substrate.

In some examples of methods of preparing a spatial array, the bead is a gel bead. In some examples of methods of preparing a spatial array, the bead is 2 microns or less in diameter. In some examples of methods of preparing a spatial array, the bead is 1 micron or less in diameter. In some examples of methods of preparing a spatial array, the bead is removed prior to the step of extending the first primer. In some examples of methods of preparing a spatial array, the bead is removed after the step of extending the first primer.

In some examples of methods of preparing a spatial array, all or a portion of the sequence of the generated capture probe is determined. In some examples of methods of preparing a spatial array, all or a portion of the sequence of the generated capture probe is determined after the template oligonucleotide is released.

In some examples of methods of preparing a spatial array, all or a portion of the sequence of the generated capture probe is determined in situ. In some examples of methods of preparing a spatial array, all or a portion of the sequence of the generated capture probe is determined by in situ sequencing. In some examples of methods of preparing a spatial array, the in situ sequencing is performed via sequencing-by-synthesis (SBS), sequential fluorescence hybridization, sequencing by ligation, nucleic acid hybridization, or high-throughput digital sequencing techniques.

In some examples of methods of preparing a spatial array, the capture domain includes a sequence complementary to an analyte of interest. In some examples of methods of preparing a spatial array, the capture domain includes a poly(T) domain.

In some examples of methods of preparing a spatial array, the substrate includes a glass slide. In some examples of methods of preparing a spatial array, the glass slide is a treated glass slide.

Provided herein are methods for determining a location of an analyte in a biological sample including (a) preparing a spatial array using any of the variety of methods described herein, (b) decoding the one or more capture probes by determining all or a portion of the sequence of the spatial barcode of the one or more capture probes, thereby associating the spatial barcode of the one or more capture probe with a location on the spatial array, (c) contacting the biological sample to the spatial array under conditions where the analyte specifically binds to capture probe(s), (d) extending the one or more capture probes via a polymerization reaction using the analyte as a template to generate an extended one or more capture probes including the one or more capture probes and the reverse complement of the analyte, and (e) determining the sequence of the extended one or more capture probes, and associating the spatial barcode of the one or more capture probes with the analyte, thereby determining the location of the analyte in the biological sample.

In some examples of methods for determining a location of an analyte in a biological sample, one or more capture probes includes a template oligonucleotide and a bead. In some examples, methods for determining a location of an analyte in a biological sample further include detaching the bead from the template oligonucleotide after the step of extending the one or more capture probes via a polymerization reaction using the analyte as a template to generate an extended one or more capture probes including the one or more capture probes and the reverse complement of the analyte.

In some examples of methods for determining a location of an analyte in a biological sample, the first primer and the second primer are at a ratio of between 1:1.5 and 1.5:1. In some examples of methods for determining a location of an analyte in a biological sample, the first primer and the second primer are less than 5 μm apart.

Provided herein are spatial arrays produced by any of the methods described herein.

Provided herein are spatial array including (a) a substrate, and (b) a plurality of sets of primers immobilized on the substrate, where a first set of the plurality of sets of primers includes a first primer and a second primer, and where the first primer includes a sequence that is complementary to a capture probe.

In some examples, the spatial array further includes a template oligonucleotide including a reverse complement of a unique molecular identifier (UMI), and where the capture probe includes the UMI.

In some examples of spatial arrays provided herein, the first primer is affixed to the substrate at a 5' end of the first primer. In some examples of spatial arrays provided herein, the first primer includes a sequence that is complementary to the template oligonucleotide. In some examples of spatial arrays provided herein, the first primer includes poly (A) domain. In some examples of spatial arrays provided herein, the first primer includes a cleavage domain.

In some examples of spatial arrays provided herein, the second primer is affixed to the substrate at a 5' end of the second primer. In some examples of spatial arrays provided herein, the second primer includes a cleavage domain.

In some examples of spatial arrays provided herein, the template oligonucleotide is attached to a bead. In some examples of spatial arrays provided herein, the bead is a gel bead. In some examples of spatial arrays provided herein, the bead is 2 microns or less in diameter. In some examples of spatial arrays provided herein, the bead is 1 micron or less in diameter.

In some examples of spatial arrays provided herein, the substrate includes a glass slide. In some examples of spatial arrays provided herein, the glass slide is a treated glass slide.

In some examples of spatial arrays provided herein, the first primer and the second primer are at a ratio of between 1:1.5 and 1.5:1. In some examples of spatial arrays provided herein, the first primer and the second primer are less than 5 μm apart.

In one aspect, the present disclosure refers to a method of generating a spatial array comprising:

- (a) providing a substrate comprising a plurality of acceptor oligonucleotides, wherein an acceptor oligonucleotide of the plurality of acceptor oligonucleotides comprises a spatial barcode and a first ligation handle, and wherein the acceptor oligonucleotide comprises a 5' end that is attached to the substrate;
- (b) providing a single-stranded splint oligonucleotide comprising a sequence substantially complementary to the first ligation handle and a sequence substantially complementary to a second ligation handle;
- (c) providing a plurality of donor oligonucleotides, wherein a donor oligonucleotide of the plurality of donor oligonucleotides comprises the second ligation handle and a capture domain;
- (d) hybridizing the single-stranded splint oligonucleotide to the first ligation handle and the second ligation handle; and
- (e) ligating the acceptor oligonucleotide to the donor oligonucleotide, thereby generating a spatial array.

Provided herein are methods of generating a spatial array including (a) providing a substrate including an arrayed plurality of acceptor oligonucleotides, where an acceptor oligonucleotide of the plurality of acceptor oligonucleotides includes a spatial barcode and a first ligation handle, and where the 5' end of the acceptor oligonucleotide is attached to the substrate, (b) providing a splint oligonucleotide including a sequence complementary to the first ligation handle and a sequence complementary to a donor oligonucleotide, and (c) ligating a donor oligonucleotide including a capture domain to the 3' end the acceptor oligonucleotide to generate a capture probe, thereby generating a spatial array.

In some examples of methods of generating a spatial array, the acceptor oligonucleotides further includes a cleavage domain, a unique molecular identifier, a functional sequence, or any combination thereof.

In some examples of methods of generating a spatial array, the sequence complementary to the capture domain of splint oligonucleotide is a poly(A) sequence. In some examples, the sequence complementary to the capture domain of the splint oligonucleotide is a gene-specific sequence. In some examples, the gene-specific sequence is a Penk-specific sequence. In some examples, the sequence complementary to the capture domain of the splint oligonucleotide is a random sequence. In some examples, the random sequence is a hexamer.

In some examples, methods of generating a spatial array further include contacting a biological sample to the spatial array after step (c). In some examples, methods of generating a spatial array further include permeabilizing the biological sample to allow the analyte in the biological sample to interact with the capture probe.

In some examples, methods of generating a spatial array further include migrating the analyte in the biological sample to the plurality of capture probes. In some examples of methods of generating a spatial array, the migrating includes passive migration. In some examples of methods of generating a spatial array, the migrating includes active migration.

Provided herein are methods of generating a spatial array including (a) providing a substrate including an arrayed plurality of acceptor oligonucleotides, where an acceptor oligonucleotide of the plurality of acceptor oligonucleotides includes a spatial barcode and a first ligation handle, and where the 5' end of the acceptor oligonucleotide is attached to the substrate, (b) providing a splint oligonucleotide including a sequence complementary to the first ligation handle and a sequence complementary to a second ligation handle, and (c) ligating a donor oligonucleotide including the second ligation handle and a capture domain to the 3' end the acceptor oligonucleotide to generate a capture probe, thereby generating a spatial array.

In some examples of methods of generating a spatial array, the acceptor oligonucleotides further includes a cleavage domain, a unique molecular identifier, a functional sequence, or any combination thereof.

In some examples of methods of generating a spatial array, the capture domain includes a poly(T) sequence. In some examples of methods of generating a spatial array, the capture domain includes a gene-specific sequence. In some examples of methods of

generating a spatial array, the gene-specific sequence is a Penk-specific sequence. In some examples of methods of generating a spatial array, the capture domain includes a random sequence. In some examples of methods of generating a spatial array, the random sequence is a hexamer.

In some examples, methods of generating a spatial array further include contacting a biological sample to the spatial array after step (c). In some examples, methods of generating a spatial array further include permeabilizing the biological sample to allow an analyte in the biological sample to interact with the capture probe.

In some examples, methods of generating a spatial array further include migrating the analyte in the biological sample to the capture probe. In some examples of methods of generating a spatial array, the migrating includes passive migration. In some examples of methods of generating a spatial array, the migrating includes active migration.

Provided herein are methods of generating a capture probe bound to substrate including dispensing a second solution onto a portion of a surface of a substrate coated with a first solution, where after the dispensing, molecules within the first solution and the second solution do not substantially mix with each other, where the second solution includes a capture probe, and the dispensing results in attachment of the capture probe to the surface of the substrate.

In some examples, methods of generating a capture probe bound to substrate further include: curing the second solution, and removing the first solution from the substrate, where the cured second solution is retained on the surface of the substrate.

In some examples of methods of generating a capture probe bound to substrate, the surface of the substrate to which the capture probe is attached has an area that is smaller than a corresponding area generated by dispensing the second solution onto the surface of the substrate in the absence of the first solution. In some examples of methods of generating a capture probe bound to substrate, the surface of the substrate to which the capture probe is attached has an area that is about two-fold less than the corresponding area generated by dispensing the second solution onto the surface of the substrate in the absence of the first solution.

In some examples, methods of generating a capture probe bound to substrate further include curing the first and second solutions on the substrate, thus generating a matrix. In some examples of methods of generating a capture probe bound to substrate, the first and second solutions are cured by chemical means. In some examples of methods of

generating a capture probe bound to substrate, the first and second solutions are cured by photo-reactive means.

In some examples, methods of generating a capture probe bound to substrate further include shrinking the matrix, thus generating a shrunken matrix having an area to which the capture probe is attached, where the area in the shrunken matrix is reduced as compared to a corresponding area in the matrix.

In some examples, methods of generating a capture probe bound to substrate further include prior to the step of dispensing the second solution: curing the first solution to form a first solution matrix, where the first solution matrix can be reversibly expanded along one or more of its axes. In some examples of methods of generating a capture probe bound to substrate, the step of dispensing the second solution onto the portion of the substrate including dispensing the second solution onto a surface of the first solution matrix in an expanded state. In some examples, methods of generating a capture probe bound to substrate further includes, after the dispensing step: shrinking the first solution matrix along the one or more axes of the surface of the substrate.

In some examples of methods of generating a capture probe bound to substrate, the first solution has a viscosity that is about 0.1x-fold, 0.2x-fold, 0.3x-fold, 0.4x-fold, 0.5x-fold, 0.6x-fold, 0.7x-fold, 0.8x-fold, 0.9x-fold, 1.0x-fold, 1.1x-fold, 1.2x-fold, 1.3x-fold, 1.4-fold, 1.5x-fold, 1.6x-fold, 1.7x-fold, 1.8x-fold, 1.9x-fold, 2.0x-fold, 2.5x-fold, 3.0x-fold, 4.0x-fold, 5.0x fold, 6.0x-fold, 7.0x-fold, 8.0x fold, 9.0x-fold or 10x-fold greater than the viscosity of the second solution.

In some examples of methods of generating a capture probe bound to substrate, the first solution has a lower nucleic acid diffusivity as compared to the second solution.

In some examples of methods of generating a capture probe bound to substrate, the capture probe includes a spatial barcode. In some examples, the capture probe includes a unique molecular identifier. In some examples of methods of generating a capture probe bound to substrate, the capture probe includes a functional domain. In some examples of methods of generating a capture probe bound to substrate, the capture probe includes a cleavage domain, a spatial barcode, a unique molecular identifier, a functional domain, and a capture domain.

Provided herein are methods of generating an array of capture probes bound to a substrate including dispensing a droplet of a second solution onto a portion of a surface of a substrate coated with a first solution, where after the dispensing, molecules within the first solution and the droplet of the second solution do not substantially mix with

each other, where the second solution includes a capture probe, and the dispensing results in attachment of the capture probe to the surface of the substrate.

In some examples, methods of generating an array of capture probes bound to a substrate further include: curing the second solution, and removing the first solution from the substrate, where the cured second solution is retained on the surface of the substrate.

In some examples of methods of generating an array of capture probes bound to a substrate, the surface of the substrate to which the capture probe is attached has an area that is smaller than a corresponding area generated by dispensing the second solution onto the surface of the substrate in the absence of the first solution. In some examples of methods of generating an array of capture probes bound to a substrate, the surface of the substrate to which the capture probe is attached has an area that is about two-fold less than the corresponding area generated by dispensing the second solution onto the surface of the substrate in the absence of the first solution.

In some examples, methods of generating an array of capture probes bound to a substrate further include curing the first and second solutions on the substrate, thus generating a matrix. In some examples of methods of generating an array of capture probes bound to a substrate, the first solution and the second solutions are cured by chemical means. In some examples of methods of generating an array of capture probes bound to a substrate, the first and second solutions are cured by photo-reactive means.

In some examples, methods of generating an array of capture probes bound to a substrate further include shrinking the matrix, thus generating a shrunken matrix having an area to which the capture probe is attached, where the area in the shrunken matrix is reduced as compared to a corresponding area in the matrix.

In some examples, methods of generating an array of capture probes bound to a substrate further include, prior to the step of dispensing the second solution: curing the first solution to form a first solution matrix, where the first solution matrix can be reversibly expanded along one or more of its axes. In some examples of methods of generating an array of capture probes bound to a substrate, the step of dispensing the second solution onto the portion of the substrate including dispensing the second solution onto a surface of the first solution matrix in an expanded state.

In some examples, methods of generating an array of capture probes bound to a substrate further include after the dispensing step: shrinking the first solution matrix along the one or more axes of the surface of the substrate.

In some examples of methods of generating an array of capture probes bound to a substrate, the first solution has a viscosity that is about 0.1x-fold, 0.2x-fold, 0.3x-fold, 0.4x-fold, 0.5x-fold, 0.6x-fold, 0.7x-fold, 0.8x-fold, 0.9x-fold, 1.0x-fold, 1.1x-fold, 1.2x-fold, 1.3x-fold, 1.4-fold, 1.5x-fold, 1.6x-fold, 1.7x-fold, 1.8x-fold, 1.9x-fold, 2.0x-fold, 2.5x-fold, 3.0x-fold, 4.0x-fold, 5.0x fold, 6.0x-fold, 7.0x-fold, 8.0x fold, 9.0x-fold or 10x-fold greater than the viscosity of the second solution.

In some examples of methods of generating an array of capture probes bound to a substrate, the first solution has a lower nucleic acid diffusivity as compared to the second solution.

In some examples of methods of generating an array of capture probes bound to a substrate, the capture probe includes a spatial barcode. In some examples of methods of generating an array of capture probes bound to a substrate, the at least one of the capture probes includes a unique molecular identifier. In some examples of methods of generating an array of capture probes bound to a substrate, the capture probe includes a functional domain. In some examples of methods of generating an array of capture probes bound to a substrate, the capture probe includes a cleavage domain, a spatial barcode, a unique molecular identifier, a functional domain, and a capture domain.

Provided herein are methods for spatially profiling an analyte in a biological sample including (a) dispensing a droplet of a second solution onto a portion of a surface of a substrate coated with a the first solution, where after the dispensing, molecules within the first solution and the droplet of the second solution do not substantially mix with each other, where the second solution includes a capture probe, and the dispensing results in attachment of the capture probe to the surface of the substrate, (b) contacting the biological sample with the spatial array such that the analyte present in the biological sample is captured by the capture probes, thereby determining the spatial profile of the captured analyte.

In some examples of methods for spatially profiling an analyte in a biological sample, at least one of the capture probes includes a cleavage domain, a spatial barcode, a unique molecular identifier, a functional domain, and a capture domain.

All publications, patents, patent applications, and information available on the internet and 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.

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.

DESCRIPTION OF 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 shows an exemplary spatial analysis workflow.

FIG. 3 shows an exemplary spatial analysis workflow.

FIG. 4 shows an exemplary spatial analysis workflow.

FIG. 5 shows an exemplary spatial analysis workflow.

FIG. 6 is a schematic diagram showing an example of a barcoded capture probe, as described herein.

FIG. 7 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.

FIG. 8 is a schematic diagram of an exemplary multiplexed spatially-barcoded feature.

FIG. 9 is a schematic diagram of an exemplary analyte capture agent.

WHAT IS CLAIMED IS:

1. A method of generating a spatial array comprising:
 - (a) providing a substrate comprising a plurality of acceptor oligonucleotides,
5 wherein an acceptor oligonucleotide of the plurality of acceptor oligonucleotides comprises a spatial barcode and a first ligation handle, and wherein the acceptor oligonucleotide comprises a 5' end that is attached to the substrate;
 - (b) providing a single-stranded splint oligonucleotide comprising a sequence
substantially complementary to the first ligation handle and a sequence substantially
10 complementary to a second ligation handle;
 - (c) providing a plurality of donor oligonucleotides, wherein a donor
oligonucleotide of the plurality of donor oligonucleotides comprises the second ligation
handle and a capture domain;
 - (d) hybridizing the single-stranded splint oligonucleotide to the first ligation
15 handle and the second ligation handle; and
 - (e) ligating the acceptor oligonucleotide to the donor oligonucleotide, thereby
generating a spatial array.
2. The method of claim 1, wherein the donor oligonucleotide further comprises a
20 functional sequence, a donor spatial barcode, a unique molecular identifier, or a combination
thereof.
3. The method of claim 1 or 2, wherein the acceptor oligonucleotide further comprises a
cleavage domain, a unique molecular identifier, a functional sequence, or a combination
25 thereof.
4. The method of any one of claims 1 to 3, wherein the capture domain comprises a
poly(T) sequence.
- 30 5. The method of any one of claims 1 to 4, wherein the capture domain is a non-
homopolymeric sequence.

6. The method of any one of claims 1 to 5, wherein the method further comprises contacting a biological sample to the generated spatial array.
7. The method of claim 6, further comprising permeabilizing the biological sample after
5 contacting with the generated spatial array to allow an analyte in the biological sample to interact with the capture domain.
8. The method of claim 7, wherein the interacting comprises hybridization to the capture domain.
- 10 9. The method of claim 7, wherein the interacting comprises ligation to the capture domain.
10. The method of claim 7, wherein the interacting comprises hybridization and ligation
15 to the capture domain.
11. The method of any one of claims 7 to 10, wherein the method further comprises migrating the analyte in the biological sample to the capture probe.
- 20 12. The method of claim 11, wherein the migrating comprises passive migration.
13. The method of claim 11, wherein the migrating comprises active migration.
14. The method of any one of claims 6 to 13, further comprising imaging the biological
25 sample.
15. The method of any one of claims 6 to 14, further comprising staining the biological sample.
- 30 16. The method of claim 15, wherein the staining comprises hematoxylin and eosin (H&E) staining.
17. The method of any one of claims 1 to 16, wherein the ligation comprises enzymatic ligation or chemical ligation.

18. The method of claim 17, wherein the enzymatic ligation comprises a ligase selected from SplintR ligase, T4 DNA ligase, App ligase, and RNA ligase.
- 5 19. The method of any one of claims 1 to 18, wherein the single-stranded splint oligonucleotide comprises DNA, RNA, or a combination thereof.
20. The method of any one of claims 1 to 19, wherein the acceptor oligonucleotide comprises a 3' end comprising a protective group which is released prior to ligation to the
10 donor oligonucleotide.
21. The method of claim 20, wherein the protective group is a photocleavable protective group.
- 15 22. The method of claim 21, wherein the photocleavable protective group is released by exposing the acceptor oligonucleotide to light.
23. The method of claim 21 or 22, wherein, prior to releasing, the photocleavable protective group is protected by applying a photomask.
20
24. The method of any one of claims 1 to 23, wherein the substrate comprises two or more partitions and wherein a partition of the two or more partitions comprises a plurality of features.
- 25 25. The method of claim 24, wherein the plurality of features comprises a plurality of immobilized acceptor oligonucleotides.
26. The method of claim 25, wherein an immobilized acceptor oligonucleotide of the plurality of immobilized acceptor oligonucleotides within the partition comprises a spatial
30 barcode that is different from immobilized acceptor oligonucleotides in other partitions.
27. The method of claim 26, wherein the plurality of donor oligonucleotides is delivered to the partition, wherein the donor oligonucleotide of the plurality of donor oligonucleotides further comprises a donor spatial barcode, wherein the donor spatial barcode comprises a

different sequence compared to the spatial barcode of the immobilized acceptor oligonucleotides within the partition.

28. The method of claim 26 or 27, wherein the two or more partitions are separated by
5 physical barriers.
29. The method of claim 28, wherein the physical barriers comprise walls or hydrophobic regions on the substrate.
- 10 30. The method of any one of claims 27 to 29, wherein the plurality of donor oligonucleotides is delivered using a microfluidic device.