

TARGETED SPATIAL TRANSCRIPTOMICS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 63/615,558, filed December 28, 2023, and U.S. Provisional Patent Application No. 63/738,312, filed December 23, 2024, each of which is incorporated herein by reference in their entirety.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] The Sequence Listing, which is a part of the present disclosure, is submitted concurrently with the specification as a text file. The name of the text file containing the Sequence Listing is "IP-2573_SeqListing.xml", which was created on December 26, 2024 and is 21,505 bytes in size. The subject matter of the Sequence Listing is incorporated herein in its entirety by reference.

BACKGROUND

[0003] The ability to determine the identity and location of target analytes in a biological sample is highly desirable. Obtaining sequence information from the 5' end and central regions of a target nucleic acid (*e.g.*, an RNA molecule), however, remains challenging.

SUMMARY

[0004] The emerging field of spatial transcriptomics is being driven by the development of new technologies that allow the mapping of single cell transcriptomes to their spatial locations in a tissue slice. One method for spatially mapping single-cell transcriptomes (called the *ex situ* approach) involves the use of a surface coated with barcoded oligonucleotides, where the spatial location of each barcode is known. The barcoded oligonucleotides are localized into individual features, where every oligonucleotide in the same feature carries the same spatial barcode. Different implementations of the surface include, for example and without limitation, a bead array, a spotted array, a clustered flow cell, and clustered particles arranged on a surface. These oligonucleotides also contain a capture nucleotide sequence (*e.g.*, an oligo(dT) capture nucleotide sequence) that binds a target nucleic acid (*e.g.*, polyA mRNA) and acts as a primer for reverse transcription. A tissue section is then placed on the surface and target nucleic acids (*e.g.*, polyA mRNA molecules) within the tissue diffuse to the features and are captured on the surface. In some embodiments, the captured target nucleic acid is RNA that is reverse transcribed into cDNA, linking the spatial barcode with the cDNA sequence. This is followed by library preparation, during which the appropriate sequencing adapter is added distal to the polyA sequence of a

target nucleic acid. During analysis of the sequencing data, the spatial barcode is used to map the physical location of the molecule from which the sequencing read is derived.

[0005] Current *ex situ* methods result in RNA-Seq libraries with a strong bias towards the 3' end of the transcript because the library preparation methods result in library fragments with adapters flanking the polyA tail (*e.g.*, when the target nucleic acid is mRNA) and nearby sequences. This can limit analysis to the 3' end of the transcript, which is useful for understanding expression levels but may not provide information about the rest of the target nucleic acid (*e.g.*, RNA) sequence, such as transcriptional start sites, splice junctions, or RNA variants. The present disclosure describes methods to generate spatially barcoded library fragments from the 5' and central regions of a target nucleic acid (*e.g.*, an RNA molecule). In various aspects, the present disclosure also provides a surface on which an immobilized library may be prepared, wherein the surface comprises two general types of oligonucleotides: (1) a capture oligonucleotide comprising: (i) a first adapter sequence that is immobilized on the surface and (ii) a capture nucleotide sequence that is configured to bind to target nucleic acids of a biological sample; and (2) a spatially barcoded oligonucleotide comprising: (i) a second adapter (Adp2) sequence that is immobilized on the surface, and (ii) a spatial barcode (SBC).

[0006] In some aspects, the disclosure provides a method of preparing an immobilized library of target nucleic acids of a biological sample, comprising: (a) providing a surface comprising: (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, and a template switching oligonucleotide (TSO) sequence; (b) contacting the biological sample with the surface, the contacting resulting in hybridization of the target nucleic acids of the biological sample to the capture nucleotide sequences of the plurality of capture oligonucleotides to form hybridized capture oligonucleotides; (c) extending the capture nucleotide sequence of the hybridized capture oligonucleotides to form first complementary strands of the target nucleic acids, wherein the extending comprises addition of a plurality of non-templated nucleotides to the end of the first complementary strands; (d) hybridizing a plurality of template switching oligonucleotides to the plurality of non-templated nucleotides of the first complementary strands such that each of the plurality of template switching oligonucleotides that is hybridized to the plurality of non-templated nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal

to the surface; (e) extending the plurality of non-templated nucleotides on the first complementary strands using the template switching oligonucleotides as template, thereby generating a complementary template-switching oligonucleotide (TSO') sequence on the first complementary strands; (f) hybridizing the complementary template-switching oligonucleotide (TSO') sequence on one or more of the first complementary strands to the template switching oligonucleotide (TSO) sequence of one or more of the plurality of spatially barcoded oligonucleotides, and extending the template-switching oligonucleotide binding site on the one or more of the first complementary strands using the one or more of the plurality of spatially barcoded oligonucleotides as template, thereby generating a spatially barcoded first strand cDNA comprising a sequence complementary to the spatial barcode (SBC') and a sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby preparing the immobilized library of target nucleic acids. In some embodiments, methods of the disclosure further comprise: (g) extending an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby generating one or more second complementary strands. In some embodiments, methods of the disclosure further comprise: (g) extending an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby generating one or more second complementary strands; and (h) amplifying the one or more first complementary strands and/or the one or more second complementary strands, thereby generating a plurality of hybridized first complementary strands and second complementary strands, or portions thereof. In some embodiments, methods of the disclosure further comprise: (g) extending an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby generating one or more second complementary strands; (h) amplifying the one or more first complementary strands and/or the one or more second complementary strands, thereby generating a plurality of hybridized first complementary strands and second complementary strands, or portions thereof; and (i) performing tagmentation on the plurality of hybridized first complementary strands and second complementary strands, or portions thereof, to prepare a plurality of tagged fragments. In further embodiments, the one or more second complementary strands are amplified in step (h) using a plurality of first primers that hybridize to the Adp2 sequence or the Adp2' sequence and a plurality of second primers that hybridize to the Adp1 sequence or the Adp1' sequence. In further embodiments, the first complementary strands and/or the second complementary strands are amplified in step (h) using a plurality of first primers that hybridize to the Adp2 sequence or the Adp2' sequence and a plurality of random primers comprising a random sequence. In some embodiments, methods of the disclosure further

comprise removing the one or more second complementary strands from the surface after step (g) and prior to step (h). In some embodiments, methods of the disclosure further comprise removing the one or more first complementary strands and the one or more second complementary strands from the surface after step (g) and prior to step (h). In various embodiments, one or more of the plurality of spatially barcoded oligonucleotides comprises a single molecule identifier (SMI). In some embodiments, the SMI is a unique molecular identifier (UMI). In various embodiments, extending the template-switching oligonucleotide binding site on the one or more of the first complementary strands using the one or more of the plurality of spatially barcoded oligonucleotides as template in step (f) generates a sequence complementary to the SMI (SMI'). In some embodiments, methods of the disclosure further comprise removing the target nucleic acids from the surface after step (f). In some embodiments, removing the target nucleic acids from the surface comprises denaturing the target nucleic acids. In some embodiments, removing the target nucleic acids from the surface comprises digesting the target nucleic acids. In some embodiments, methods of the disclosure further comprise removing the biological sample from the surface after step (c). In some embodiments, methods of the disclosure further comprise removing the biological sample from the surface after step (e). In various embodiments, each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence. In some embodiments, the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences. In further embodiments, the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof. In some embodiments, the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence. In further embodiments, the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence. In some embodiments, the universal capture sequence comprises one or more universal bases (*e.g.*, inosine, 5-Nitroindole). In various embodiments, the target nucleic acids are mRNA, genomic DNA (gDNA), rRNA, tRNA, or a combination thereof. In some embodiments, the target nucleic acids are rRNA, mRNA, or a combination thereof. In further embodiments, the target nucleic acids are gDNA, mRNA, or a combination thereof. In some embodiments, the extending of the capture nucleotide sequence in step (c) is carried out using a reverse transcriptase. In various embodiments, the target nucleic acids are polyadenylated prior to hybridization of the target nucleic acids to the capture nucleotide sequences. In some embodiments, the target nucleic acids are polyadenylated using a poly(A) polymerase. In further embodiments, the target nucleic acids are polyadenylated using chemical ligation or enzymatic ligation. In some embodiments, the one or more second complementary strands are amplified in step (h) by strand invasion of the one or

more spatially barcoded oligonucleotides in the presence of recombinase. In some embodiments, prior to step (c) a capping step is performed to add a cap to the target nucleic acids. In further embodiments, the cap is a m7G cap. In still further embodiments, the capping step is performed by a capping enzyme. In yet further embodiments, the capping enzyme is a Faustovirus capping enzyme (FCE). In some embodiments, the first adapter (Adp1) sequence is immobilized on the surface through a cleavage site. In some embodiments, the first adapter (Adp1) sequence is immobilized on the surface through a cleavage site. In further embodiments, the cleavage site is an enzymatic cleavage site. In still further embodiments, the enzymatic cleavage site comprises a restriction enzyme site, a uracil, an 8-oxoguanine, or a combination thereof. In some embodiments, the cleavage site is a chemical cleavage site. In various embodiments, the cleavage site is cleaved after step (f). In some embodiments, the cleavage site is cleaved after step (g). In some embodiments, the one or more second complementary strands is removed by heat or alkaline denaturation. In various embodiments, methods of the disclosure further comprise sequencing the plurality of tagged fragments to determine a sequence of the plurality of tagged fragments. In some embodiments, methods of the disclosure further comprise correlating the sequence of the plurality of tagged fragments to a position of the target nucleic acids in the biological sample.

[0007] In some aspects, the disclosure provides a method of preparing an immobilized library of target nucleic acids of a biological sample, comprising: (a) providing a surface comprising: (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, and a mosaic end (ME) transposase recognition sequence; (b) contacting the biological sample with the surface, the contacting resulting in hybridization of the target nucleic acids of the biological sample to the capture nucleotide sequences of the plurality of capture oligonucleotides to form hybridized capture oligonucleotides; (c) extending the capture nucleotide sequence of the hybridized capture oligonucleotides to form first complementary strands of the target nucleic acids, wherein the extending comprises addition of a plurality of non-templated nucleotides to the end of the first complementary strands; (d) hybridizing a plurality of template switching oligonucleotides (TSOs) to the plurality of non-templated nucleotides of the first complementary strands such that each of the plurality of template switching oligonucleotides (TSOs) that is hybridized to the plurality of non-templated

nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal to the surface; (e) extending the plurality of non-templated nucleotides on the first complementary strands using the template switching oligonucleotides (TSOs) as template, thereby generating a complementary template-switching oligonucleotide sequence (TSO') on the first complementary strands; (f) removing the target nucleic acids from the surface; (g) hybridizing an additional template-switching oligonucleotide (TSO) primer to the complementary template switching oligonucleotide sequence of one or more of the first complementary strands to generate a hybridized TSO primer, and extending the hybridized TSO primer to generate one or more second complementary strands, thereby generating a plurality of clusters comprising hybridized first complementary strands and second complementary strands; (h) hybridizing a plurality of oligonucleotides comprising a sequence complementary to the ME sequence (ME') to the ME transposase recognition sequence of one or more of the plurality of spatially barcoded oligonucleotides; (i) performing tagmentation on the plurality of clusters comprising hybridized first complementary strands and second complementary strands to prepare a plurality of tagged fragments, (j) adding an enzyme having 5' to 3' exonuclease activity; (k) hybridizing a plurality of oligonucleotides comprising a sequence complementary to the ME sequence (ME') and a sequence complementary to a third adapter (Adp3') sequence to the ME transposase recognition sequence of one or more of the plurality of spatially barcoded oligonucleotides and performing gap-fill ligation, thereby attaching the ME' and Adp3' sequences to the 3' end of one or more of the first complementary strands and the 3' end of one or more of the second complementary strands; (l) subjecting the surface to denaturing conditions to form one or more single-stranded first complementary strands and one or more single-stranded second complementary strands; hybridizing a plurality of primers comprising the third sequencing adapter (Adp3) sequence to the Adp3' sequence attached to the 3' ends of one or more of the single-stranded first complementary strands and one or more of the single-stranded second complementary strands; and extending the primers to form a plurality of clusters comprising Adp3'-tagged immobilized strands hybridized to Adp3-tagged non-immobilized strands, thereby preparing the immobilized library of target nucleic acids. In some aspects, the disclosure provides a method of preparing an immobilized library of target nucleic acids of a biological sample, comprising: (a) providing a surface comprising: (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, and a mosaic end (ME)

transposase recognition sequence; (b) contacting the biological sample with the surface, the contacting resulting in hybridization of the target nucleic acids of the biological sample to the capture nucleotide sequences of the plurality of capture oligonucleotides to form hybridized capture oligonucleotides; (c) extending the capture nucleotide sequence of the hybridized capture oligonucleotides to form first complementary strands of the target nucleic acids, wherein the extending comprises addition of a plurality of non-templated nucleotides to the end of the first complementary strands; (d) hybridizing a plurality of template switching oligonucleotides (TSOs) to the plurality of non-templated nucleotides of the first complementary strands such that each of the plurality of template switching oligonucleotides (TSOs) that is hybridized to the plurality of non-templated nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal to the surface; (e) extending the plurality of non-templated nucleotides on the first complementary strands using the template switching oligonucleotides (TSOs) as template, thereby generating a complementary template-switching oligonucleotide sequence (TSO') on the first complementary strands; (f) removing the target nucleic acids from the surface; (g) hybridizing an additional template-switching oligonucleotide (TSO) primer to the complementary template switching oligonucleotide sequence of one or more of the first complementary strands to generate a hybridized TSO primer, and extending the hybridized TSO primer to generate one or more second complementary strands, thereby generating a plurality of clusters comprising hybridized first complementary strands and second complementary strands; (h) hybridizing a plurality of oligonucleotides comprising a sequence complementary to the ME sequence (ME') to the ME transposase recognition sequence of one or more of the plurality of spatially barcoded oligonucleotides; (i) performing tagmentation on the plurality of clusters comprising hybridized first complementary strands and second complementary strands to prepare a plurality of tagged fragments, (j) adding an enzyme having 5' to 3' exonuclease activity; (k) hybridizing a plurality of oligonucleotides comprising a sequence complementary to the ME sequence (ME') and a sequence complementary to a third adapter (Adp3') sequence to the ME transposase recognition sequence of one or more of the plurality of spatially barcoded oligonucleotides and performing gap-fill ligation, thereby attaching the ME' and Adp3' sequences to the 3' end of one or more of the first complementary strands and the 3' end of one or more of the second complementary strands, thereby preparing the immobilized library of target nucleic acids. In some embodiments, methods of the disclosure further comprise subjecting the surface to denaturing conditions to form one or more single-stranded first complementary strands and one or more single-stranded second complementary strands; hybridizing a plurality of primers comprising the third sequencing adapter (Adp3) sequence to the Adp3' sequence attached to the 3' ends of one or more of the single-stranded first complementary strands

and one or more of the single-stranded second complementary strands; and extending the primers to form a plurality of clusters comprising Adp3'-tagged immobilized strands hybridized to Adp3-tagged non-immobilized strands. In some embodiments, methods of the disclosure further comprise the step of dehybridizing the Adp3-tagged non-immobilized strands and amplifying the dehybridized Adp3-tagged non-immobilized strands using a plurality of first primers comprising the Adp2 sequence or the Adp2' sequence and a plurality of second primers comprising the Adp3 sequence or the Adp3' sequence. In some embodiments, one or more of the plurality of spatially barcoded oligonucleotides comprises a single molecule identifier (SMI). In further embodiments, the SMI is a unique molecular identifier (UMI). In some embodiments, removing the target nucleic acids from the surface comprises denaturing the target nucleic acids. In some embodiments, removing the target nucleic acids from the surface comprises digesting the target nucleic acids. In various embodiments, methods of the disclosure further comprise removing the biological sample from the surface after step (c). In some embodiments, methods of the disclosure further comprise removing the biological sample from the surface after step (e). In some embodiments, each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence. In further embodiments, the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences. In still further embodiments, the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof. In various embodiments, the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence. In further embodiments, the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence. In further embodiments, the target nucleic acids are mRNA, gDNA, rRNA, tRNA, or a combination thereof. In still further embodiments, the target nucleic acids are RNA, mRNA, or a combination thereof. In some embodiments, the extending of the capture nucleotide sequence in step (c) is carried out using a reverse transcriptase. In some embodiments, the target nucleic acids are polyadenylated prior to hybridization of the target nucleic acids to the capture nucleotide sequences. In various embodiments, the target nucleic acids are polyadenylated using a poly(A) polymerase. In some embodiments, the target nucleic acids are polyadenylated using chemical ligation or enzymatic ligation. In some embodiments, methods of the disclosure further comprise sequencing the plurality of tagged fragments to determine a sequence of the plurality of tagged fragments. In some embodiments, methods of the disclosure further comprise correlating the sequence of the plurality of tagged fragments to a position of the target nucleic acids in the biological sample.

[0008] In further aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) optionally releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) releasing the plurality of first surface oligonucleotides from the surface; (i) cleaving the cleavage site of the cluster of second surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of second surface oligonucleotides, thereby preparing the spatial array. In some aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a

second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) releasing the plurality of first surface oligonucleotides from the surface; (i) cleaving the cleavage site of the cluster of second surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of second surface oligonucleotides, thereby preparing the spatial array. In some embodiments, steps (f) and (g) are repeated 0, 1, 2, 3, 4, 5, or more times. In some embodiments, a method of the disclosure further comprises: (j) providing a second plurality of template oligonucleotides, wherein one or more of the second plurality of template oligonucleotides comprises, from 5' to 3': the second adapter (Adp2) sequence, a second spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to and capable of hybridizing to an Adp1 sequence; (k) hybridizing the Adp1' sequence of one or more of the second plurality of template oligonucleotides to a second Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (l) extending one or more of the second Adp1 sequence using the one or more of the second plurality of template oligonucleotides as a template to generate a plurality of third surface oligonucleotides each third surface oligonucleotide comprising: the second Adp1 sequence that is immobilized on the surface, the sequence that is complementary to the cleavage site, the sequence that is complementary to the TSO sequence, a sequence that is complementary to the second

spatial barcode, and the Adp2' sequence that is complementary to the Adp2 sequence; (m) releasing the one or more of the second plurality of template oligonucleotides from the plurality of third surface oligonucleotides; (n) hybridizing the Adp2' sequence of one or more of the plurality of third surface oligonucleotides to a second Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (o) extending the second Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of fourth surface oligonucleotides comprising: the second Adp2 sequence immobilized on the surface, the second spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to the first adapter (Adp1) sequence; (p) releasing the plurality of third surface oligonucleotides from the surface; (q) cleaving the cleavage site of the cluster of fourth surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of fourth surface oligonucleotides. In some embodiments, a method of the disclosure further comprises: (r) providing a third plurality of template oligonucleotides, wherein one or more of the third plurality of template oligonucleotides comprises, from 5' to 3': the second adapter (Adp2) sequence, a third spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to and capable of hybridizing to an Adp1 sequence; (s) hybridizing the Adp1' sequence of one or more of the third plurality of template oligonucleotides to a third Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (t) extending one or more of the third Adp1 sequence using the one or more of the third plurality template oligonucleotides as a template to generate a plurality of fifth surface oligonucleotides each fifth surface oligonucleotide comprising: the third Adp1 sequence that is immobilized on the surface, the sequence that is complementary to the cleavage site, the sequence that is complementary to the TSO sequence, a sequence that is complementary to the third spatial barcode, and the Adp2' sequence that is complementary to the Adp2 sequence; (u) releasing the one or more of the third plurality of template oligonucleotides from the plurality of fifth surface oligonucleotides; (v) hybridizing the Adp2' sequence of one or more of the plurality of fifth surface oligonucleotides to a third Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (w) extending the third Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of sixth surface oligonucleotides comprising: the third Adp2 sequence immobilized on the surface, the third spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to the first adapter (Adp1) sequence; (x) releasing the plurality of fifth surface oligonucleotides from the surface; (y) cleaving the cleavage site of the cluster of sixth surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of sixth surface

oligonucleotides. In some embodiments, a method of the disclosure further comprises immobilizing a plurality of capture oligonucleotides on the surface, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample. In further embodiments, the surface of (a) further comprises a plurality of capture oligonucleotides on the surface, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample. In still further embodiments, the plurality of capture oligonucleotides is immobilized on the surface through a spacer. In some embodiments, each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence. In some embodiments, the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences. In further embodiments, the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof. In some embodiments, the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence. In further embodiments, the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence. In various embodiments, one or more of the first plurality of template oligonucleotides, one or more of the second plurality of template oligonucleotides, and/or one or more of the third plurality of template oligonucleotides comprises a sequencing primer domain. In some embodiments, the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

[0009] In some aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first unique dual index (UDI1) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, a sequence that is complementary to and capable of hybridizing to a capture nucleotide sequence, a second UDI (UDI2) sequence, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first

adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, the UDI1 sequence, the capture nucleotide sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, the UDI2 sequence, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) optionally releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the UDI1 sequence, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, the sequence that is complementary to the capture nucleotide sequence, the UDI2 sequence, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) cleaving the cleavage site of the cluster of second surface oligonucleotides, thereby preparing the spatial array. In some aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first unique dual index (UDI1) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, a sequence that is complementary to and capable of hybridizing to a capture nucleotide sequence, a second UDI (UDI2) sequence, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, the UDI1 sequence, the capture nucleotide sequence, a sequence that is complementary to the cleavage site, a sequence

that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, the UDI2 sequence, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the UDI1 sequence, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, the sequence that is complementary to the capture nucleotide sequence, the UDI2 sequence, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) cleaving the cleavage site of the cluster of second surface oligonucleotides, thereby preparing the spatial array. In some embodiments, after cleavage of the cleavage site and optional dehybridization, the resulting prepared spatial array comprises (i) a plurality of first oligonucleotides, wherein each first oligonucleotide in the plurality comprises: the Adp2 sequence immobilized on the surface, the UDI1 sequence, the first spatial barcode, and the template switching oligonucleotide (TSO) sequence; and (ii) a plurality of second oligonucleotides, wherein each second oligonucleotide in the plurality comprises: the Adp1 sequence immobilized on the surface, the UDI2 sequence, and the capture nucleotide sequence. In some embodiments, one or more oligonucleotides in the plurality of first oligonucleotides comprises a sequencing primer sequence situated between the UDI1 sequence and the first spatial barcode. In some embodiments, steps (f) and (g) are repeated 0, 1, 2, 3, 4, 5, or more times. In some embodiments, one or more of the first plurality of template oligonucleotides comprises a sequencing primer domain. In further embodiments, the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

[0010] In further aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides immobilized on the surface and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the second adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) applying a library of template oligonucleotides to one or more regions of the surface, wherein the library comprises at least one first template oligonucleotide comprising, from 5' to 3': the Adp2 sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence

that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of the at least one first template oligonucleotide to the Adp1 sequence of at least one first adapter oligonucleotide and extending the hybridized Adp1 sequence to prepare at least one first double-stranded nucleic acid, wherein each first double-stranded nucleic acid comprises a first extended Adp1 strand immobilized on the surface and a first template strand, wherein the first extended Adp1 strand comprises, from 5' to 3': the Adp1 sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence, and wherein the first template strand comprises, from 5' to 3': the Adp2 sequence, the first spatial barcode, the TSO sequence, the cleavage site, and the Adp1' sequence; (d) hybridizing the Adp1 sequence of at least one first adapter oligonucleotide to the Adp1' sequence of at least one first template strand and extending the hybridized Adp1 sequence to prepare at least one additional first extended Adp1 strand immobilized on the surface; (e) hybridizing the Adp2 sequence of at least one second adapter oligonucleotide to the Adp2' sequence of at least one first extended Adp1 strand immobilized on the surface and extending the hybridized Adp2 sequence to prepare at least one first extended Adp2 strand immobilized on the surface, wherein each first extended Adp2 strand comprises, from 5' to 3': the Adp2 sequence, the first spatial barcode, the TSO sequence, the cleavage site, and the Adp1' sequence; (f) repeating steps (d) and (e) to produce a first cluster comprising a plurality of first extended Adp1 strands immobilized on the surface and a plurality of first extended Adp2 strands immobilized on the surface; (g) releasing the first extended Adp1 strands in the first cluster from the surface; (h) cleaving the cleavage sites of the first extended Adp2 strands in the first cluster to release the cleavage sites and the Adp1' sequences from the surface, thereby preparing the spatial array. In some embodiments, one or more of the first plurality of template oligonucleotides comprises a sequencing primer domain. In further embodiments, the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **Figures 1A-1C** depict an exemplary spatial 5' RNA workflow. **Figure 1A** shows the steps of extending the capture nucleotide sequence using a reverse transcriptase (RT) with terminal transferase activity and the subsequent RNA removal by alkaline or heat denaturation, Mg⁺ based RNA fragmentation, or RNase digestion. **Figure 1B** shows the steps of hybridization of TSO' to TSO, polymerase extension by RT or another DNA polymerase, and subsequent heat or alkaline denaturation. **Figure 1C** shows the steps of addition of excess Adp2 oligonucleotides, second strand synthesis with a DNA polymerase,

heat or alkaline denaturation, and subsequent PCR amplification with Adp1 and Adp2.

Figure 1D depicts a variation of the spatial 5' RNA workflow comprising direct extension of the non-templated deoxycytosines, and shows the steps of extending the capture nucleotide sequence using a reverse transcriptase (RT) with terminal transferase activity, the subsequent RNA removal by alkaline or heat denaturation, Mg⁺ based RNA fragmentation, or RNase digestion, and then allowing hybridization of CCC-GGG and extension with a polymerase. **Figure 1E** depicts a variation using a splint ligation approach and shows the steps of extending the capture nucleotide sequence using a reverse transcriptase (RT) with terminal transferase activity, the subsequent RNA removal by alkaline or heat denaturation, Mg⁺ based RNA fragmentation, or RNase digestion, splinting the oligonucleotides with a hyb-GGG to increase ligation efficiency, and then ligating to SBC adapter at 5' phosphate (or click ligation). **Figure 1F** shows a variation that does not require template switching and shows the steps of extending the capture nucleotide sequence using a reverse transcriptase (RT) (terminal transferase activity not required), the subsequent RNA removal by alkaline or heat denaturation, Mg⁺ based RNA fragmentation, or RNase digestion, and then a randomer in the spatially barcoded oligonucleotide (*e.g.*, N6 or N9) finds a complementary sequence in the cDNA and extends the SBC adapter back through the template. Subsequent cleavage below Adp2 releases the adapterized library.

[0012] **Figure 2** shows exemplary surface oligonucleotide sequences. Capture oligonucleotide is depicted on the left, while the spatially barcoded oligonucleotide is shown on the right. Spacer: moiety that provides sufficient distance from the surface to enable the reverse transcriptase (RT) to access the polyT capture sequence. Adp1: ME'V2B15' 5'–CTGTCTCTTATACACATCTCCGAGCCCACGAGAC-3' (SEQ ID NO: 1) :: Adp2: P7 5'–CAAGCAGAAGACGGCATACGAGAT-3' (SEQ ID NO: 2) :: SBC: Random spatial barcode (*e.g.*, 30N); same for all oligonucleotides within a feature :: SBS491: 5'–TCGCGAGTTAATGCAACGATCGTCGAAATTCGC-3' (SEQ ID NO: 3) :: UMI: Random UMI; unique sequence on every oligonucleotide :: SBS3: 5'–ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' (SEQ ID NO: 4). In some embodiments, a spacer is present between the surface and P7.

[0013] **Figure 3** depicts an optional 5' capping step.

[0014] **Figure 4A** shows exemplary cleavage options to remove cDNA. Cleavage site options include, but are not limited to, uracil (USER enzyme mix), 8-oxo-G (FPG, OGG1 enzymes), restriction enzyme site, and chemical cleavage (CCL). In some embodiments, Option 2 is advantageous because double stranded cleavage may be more efficient than single strand cleavage. **Figure 4B** depicts an embodiment comprising a double cleavage from the bridge.

[0015] **Figure 5** depicts an exemplary method of converting cDNA into a sequencing library using fragmentation. Fragmentation of full-length cDNA is generally needed if sequencing on a short-read sequencing machine. In this Figure, the Adp2 sequence was set to P7.

[0016] **Figures 6A-6B** depict an exemplary method of adding a template-switching oligonucleotide (TSO) sequence using ligation.

[0017] **Figures 7A-7H** depict an exemplary workflow for barcoding fragments within a target nucleic acid transcript. Note that in **Figure 7G**, the leftmost oligonucleotide will also form a P5-Adp1 product that can dehybridize into the solution (not shown) but should not amplify in PCR. **Figure 7H** depicts an exemplary workflow of non-surface fragmentation.

[0018] **Figures 8A-8D** depict an alternative spatial 5' RNA workflow.

[0019] **Figure 9** depicts strategies for preventing extension from SBC oligonucleotides.

[0020] **Figure 10** shows both the capture oligonucleotide and the address oligonucleotide (spatially barcoded oligonucleotide). See also Figure 2.

[0021] **Figures 11A-11E** depict further exemplary workflows for establishing oligonucleotide clusters on a surface. The "X" shown in certain oligonucleotides in Figure 11A represents optional cleavage locations. At the point in the workflow that is depicted on the left side of Figure 11B, the clusters of second surface oligonucleotides are established on the surface, wherein each cluster comprises a unique spatial barcode. Figure 11B also depicts an embodiment in which the capture oligonucleotides are directly immobilized on the surface. Figure 11C depicts an embodiment in which the capture oligonucleotides are present from the start of the spatial array preparation (*e.g.*, the capture oligonucleotides may be immobilized to the surface along with the adapter sequences and would be present during the hybridization of the template oligonucleotides to an adapter sequence). Figure 11D depicts an embodiment in which single molecule identifiers (SMIs) are added to the clusters of second surface oligonucleotides. In some embodiments, a SMI is a unique molecular identifier (UMI). Figure 11E depicts an embodiment in which unique dual indexes (UDIs) are added to the spatially barcoded oligonucleotides.

[0022] **Figure 12** depicts simultaneous extension of the first complementary strand and the spatially barcoded strand.

[0023] **Figure 13** shows spatial heatmaps from both TSO- (13A and 13B) and LIG- (13C and 13D) LPs for two highly expressed transcripts (*Kap*, 13A and 13C; *Umod*, 13B and 13D) in mouse kidney. Figures 13A, 13B and 13C, 13D are from two different tissue slices, respectively. Spatial images are derived from UMIs mapped to specific locations (XY co-

ordinates) on the surface. The heatmap scale shows the relative abundance of UMIs per unit area ($10 \times 10 \mu\text{m}^2$).

DETAILED DESCRIPTION

[0024] The present disclosure is generally directed to methods of preparing a library of target nucleic acids of a biological sample. The methods provided herein advantageously generate spatially barcoded library fragments from the 5' and central regions of a target nucleic acid (*e.g.*, an RNA molecule).

TERMS

[0025] As used in this specification and the enumerated paragraphs herein, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0026] "About" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20-25 percent (%), for example, within 20 percent, 10 percent, 5 percent, 4 percent, 3 percent, 2 percent, or 1 percent of the stated value or range of values.

[0027] As used herein, the term "adapter" refers generally to any linear nucleic acid molecule that can be added (*e.g.*, through synthesis or ligation) to an oligonucleotide of the disclosure. In some embodiments, an adapter is a primer. In some embodiments, adapters are copied onto the library molecules using templated polymerase synthesis (*e.g.*, second strand cDNA synthesis as described herein). In some embodiments, adapters are ligated to a first complementary strand of the disclosure. In some embodiments, an adapter comprises two oligonucleotides that are double-stranded at one portion and single-stranded at another portion, forming an adapter with an overhang. In some embodiments, an adapter comprises a B15 nucleotide sequence. In some embodiments, the adapter that comprises a B15 nucleotide sequence is referred to herein as Adp1. In some embodiments, the adapter that comprises a P5 nucleotide sequence is referred to herein as Adp1. In some embodiments, an adapter comprises a P7 nucleotide sequence. In some embodiments, the adapter that comprises a P7 nucleotide sequence is referred to herein as Adp2. In some embodiments, an adapter comprises a sequence that is complementary to a primer. In further embodiments, an adapter comprises a sequence that is complementary to a P5 primer or a P5' primer. In some embodiments, an adapter comprises a sequence complementary to a P7 primer or a P7' primer. In some embodiments, an adapter comprises a sequence complementary to a B15 primer or a B15' primer.

[0028] The terms "P5", "P7", "B15", "P5'" (P5 prime), "P7'" (P7 prime), "B15'" (B15 prime), "P15", and "P17" may be used when referring to examples of oligonucleotide sequences of the disclosure, *e.g.*, clustering primers, and/or oligonucleotide sequences that are complementary to primers. In various embodiments, an adapter of the disclosure comprises a "P5", "P7", "B15", "P5'" (P5 prime), "P7'" (P7 prime), "B15'" (B15 prime), "P15", or "P17" nucleotide sequence, or a variant thereof. The terms "P5'" (P5 prime), "P7'" (P7 prime), and "B15'" (B15 prime) refer to the complement of P5, P7, and B15, respectively. It will be understood that any suitable primer can be used in the methods presented herein, and that the use of P5, P5', P7, P7', P15, P17, B15, and B15' are exemplary embodiments only. Uses of primers such as P5, P5', P7, P7', P15, P17, B15, and B15' or their complements on flow cells are known in the art, as exemplified by the disclosures of WO 2019/222264, WO 2007/010251, WO 2006/064199, WO 2005/065814, WO 2015/106941, WO 1998/044151, and WO 2000/018957, each of which is incorporated herein by reference in its entirety. For example, any suitable forward amplification primer, whether immobilized or in solution, can be useful in the methods presented herein for hybridization to a complementary sequence and amplification of a sequence. Similarly, any suitable reverse amplification primer, whether immobilized or in solution, can be useful in the methods presented herein for hybridization to a complementary sequence and amplification of a sequence. One of skill in the art will understand how to design and use primer sequences that are suitable for capture and/or amplification of nucleic acids as presented herein. In some embodiments, P5 comprises or consists of the polynucleotide sequence 5'-AAT GAT ACG GCG ACC ACC GA-3' (SEQ ID NO: 5), or a variant thereof. In some embodiments, P5 comprises or consists of the polynucleotide sequence 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-3' (SEQ ID NO: 6), or a variant thereof. In some embodiments, P7 comprises or consists of the polynucleotide sequence 5'-CAA GCA GAA GAC GGC ATA CG-3' (SEQ ID NO. 7), or a variant thereof. In some embodiments, P7 comprises or consists of the polynucleotide sequence 5' CAA GCA GAA GAC GGC ATA CGA GAT 3' (SEQ ID NO. 8), or a variant thereof. In some embodiments, P5' comprises or consists of the polynucleotide sequence 5' TCG GTG GTC GCC GTA TCA TT 3' (SEQ ID NO: 9), or a variant thereof. In some embodiments, P5' comprises or consists of the polynucleotide sequence 5' GTG TAG ATC TCG GTG GTC GCC GTA TCA TT 3' (SEQ ID NO: 10), or a variant thereof. In some embodiments, P7' comprises the polynucleotide sequence 5' CGT ATG CCG TCT TCT GCT TG 3' (SEQ ID NO: 11), or a variant thereof. In some embodiments, P7' comprises or consists of the polynucleotide sequence 5' ATC TCG TAT GCC GTC TTC TGC TTG 3' (SEQ ID NO. 12), or a variant thereof. In some embodiments, B15 comprises or consists of the polynucleotide sequence 5' GTCTCGTGGGCTCGG 3' (SEQ ID NO: 13), or a variant thereof. In some embodiments, B15' comprises or consists of the polynucleotide sequence

5' CCGAGCCCCACGAGAC 3' (SEQ ID NO: 14), or a variant thereof. In some embodiments, P15 comprises or consists of the polynucleotide sequence 5' TTTTSTAATG ATACGGCGAC CACCGAGANC TACAC 3' (SEQ ID NO: 15), or a variant thereof. In some embodiments, P17 comprises or consists of the polynucleotide sequence 5' TTTTSTNNNC AAGCAGAAGA CGGCATACGA GAT 3' (SEQ ID NO: 16), or a variant thereof. In some embodiments, a primer useful in the methods of the disclosure comprises or consists of a Mosaic End (ME) sequence, a ME' sequence, or a variant thereof. In some embodiments, the ME sequence comprises or consists of the polynucleotide sequence: 5'- AGATGTGTATAAGAGACAG - 3' (SEQ ID NO: 17), or a variant thereof. In various embodiments, the ME' sequence comprises or consists of the polynucleotide sequence: 5'-CTG TCT CTT ATA CAC ATC T - 3' (SEQ ID NO: 18), or a variant thereof. The term "variant" as used herein with reference to any of the sequences recited herein refers to a variant nucleic acid that is substantially identical, *i.e.*, has only some nucleotide sequence variations, for example to the non-variant sequence. In some embodiments, a variant has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% overall nucleotide sequence identity to the non-variant nucleic acid sequence. It will be understood that reference to P5 and P7 herein could refer to different primer sequences. Any suitable primer sequence combinations are encompassed by the present disclosure.

[0029] The term "bead" refers to a small body made of a rigid or semi-rigid material. The body can have a shape characterized, for example, as a sphere, oval, microsphere, or other recognized particle shape whether having regular or irregular dimensions. Example materials that are useful for beads include, without limitation, glass; plastic such as acrylic, polystyrene or a copolymer of styrene and another material, polypropylene, polyethylene, polybutylene, polyurethane or polytetrafluoroethylene (TEFLON®, from Chemours); polysaccharides or cross-linked polysaccharides such as agarose or Sepharose; nylon; nitrocellulose; resin; silica or silica-based materials including silicon and modified silicon; carbon-fiber, metal; inorganic glass; optical fiber bundle, or a variety of other polymers. Example beads include, without limitation, controlled pore glass beads, paramagnetic beads, thoria sol, Sepharose beads, nanocrystals and others known in the art as described, for example, in Microsphere Detection Guide from Bangs Laboratories, Fishers Ind. Beads may also be coated with a polymer that has a functional group that can attach to an oligonucleotide.

[0030] As used herein, "surface" can refer to a part of a substrate or support structure that is accessible to contact with reagents, beads, or analytes. The surface can be substantially flat or planar. Alternatively, the surface can be rounded or contoured. Example contours that

can be included on a surface are wells (*e.g.*, microwells or nanowells), depressions, pillars, ridges, channels or the like. Example materials that can be used as a substrate or support structure include glass such as modified or functionalized glass; plastic such as acrylic, polystyrene or a copolymer of styrene and another material, polypropylene, polyethylene, polybutylene, polyurethane or TEFLON; polysaccharides or cross-linked polysaccharides such as agarose or Sepharose; nylon; nitrocellulose; resin; silica or silica-based materials including silicon and modified silicon, carbon-fibre; metal; inorganic glass; optical fibre bundle, or a variety of other polymers. A single material or mixture of several different materials can form a surface useful in certain examples. In some examples, a surface comprises wells (*e.g.*, microwells or nanowells). In some aspects, the surface comprises an array of wells (*e.g.*, microwells or nanowells) on glass, silicon, plastic or other suitable solid supports with patterned, covalently-linked gel such as poly(N-(5-azidoacetamidylpentyl)acrylamide-coacrylamide) (PAZAM, see, for example, U.S. Pat. App. Pub. No. 2014/0079923 A1, which is incorporated herein by reference). In some embodiments, each nanowell comprises a unique oligonucleotide (*e.g.*, an oligonucleotide with a unique spatial barcode). In some examples, a support structure can include one or more layers. Non-limiting examples of a surface include a bead array, a spotted array, clustered particles arranged on a surface of a chip, a film, a multi-well plate, and a flow cell.

[0031] Exemplary flow cells include but are not limited to those used in a nucleic acid sequencing apparatus such as flow cells for the Genome Analyzer®, MiSeq®, NextSeq® or HiSeq® platforms commercialized by Illumina, Inc. (San Diego, Calif.); or for the SOLiD™ or Ion Torrent™ sequencing platform commercialized by Life Technologies (Carlsbad, Calif.). Exemplary flow cells and methods for their manufacture and use are also described, for example, in WO 2014/142841 A1; U.S. Pat. App. Pub. No. 2010/0111768 A1 and U.S. Pat. No. 8,951,781, each of which is incorporated herein by reference.

[0032] A bead array comprising oligonucleotide barcodes can also be used in a sequencing procedure, such as a sequencing-by-synthesis (SBS) technique. Briefly, SBS can be initiated by contacting the barcodes with one or more labeled nucleotides, DNA polymerase, etc. Those features where a primer is extended using the sequences comprising the barcode as a template will incorporate a labeled nucleotide that can be detected. Optionally, the labeled nucleotides can further include a reversible termination property that terminates further primer extension once a nucleotide has been added to a primer. For example, a nucleotide analog having a reversible terminator moiety can be added to a primer such that subsequent extension cannot occur until a deblocking agent is delivered to remove the moiety. Thus, for embodiments that use reversible termination, a deblocking reagent can be delivered to the flow cell (before or after detection occurs).

Washes can be carried out between the various delivery steps. The cycle can then be repeated *n* times to extend the primer by *n* nucleotides, thereby detecting a sequence of length *n*. Exemplary SBS procedures, fluidic systems and detection platforms that can be readily adapted for use with an array produced by the methods of the present disclosure are described, for example, in Bentley et al., Nature 456:53-59 (2008), WO 04/018497; WO 91/06678; WO 07/123744; U.S. Pat. Nos. 7,057,026; 7,329,492; 7,211,414; 7,315,019 or 7,405,281, and US Pat. App. Pub. No. 2008/0108082 A1, each of which is incorporated herein by reference.

[0033] As described herein, in various aspects the disclosure provides a surface comprising a plurality of spatially barcoded oligonucleotides. In general, a spatially barcoded oligonucleotide comprises an adapter sequence (*e.g.*, P7) and a spatial barcode. A spatially barcoded oligonucleotide of the disclosure can comprise additional elements, including but not limited to a single molecule identifier (SMI) (*e.g.*, a unique molecular identifier (UMI)), an index sequence, a template-switching oligonucleotide (TSO) sequence, a homopolymeric nucleotide sequence (*e.g.*, "GGG" (see, *e.g.*, Figure 1D)), a random nucleotide sequence (*e.g.*, N9 as seen in Figure 1F), a sequencing primer binding site (*e.g.*, SBS12), or a combination thereof. In some aspects, a spatially barcoded oligonucleotide comprises an adapter sequence that is immobilized on the surface (*e.g.*, P7 as depicted in, *e.g.*, Figure 1A), a spatial barcode, and a template switching oligonucleotide (TSO) sequence. As used herein, the term "barcode" is intended to mean a series of nucleotides in an oligonucleotide that can be used to identify the oligonucleotide, a spatial address on a surface (*i.e.*, a "spatial barcode"), a characteristic of the oligonucleotide, or a manipulation that has been carried out on the oligonucleotide. The barcode can be a naturally occurring nucleotide sequence or a nucleotide sequence that does not occur naturally in the organism from which the barcoded nucleic acid was obtained. A barcode sequence can be unique to a single nucleic acid species in a population, or a barcode sequence can be shared by several different nucleic acid species in a population. For example, each spatially barcoded oligonucleotide in a plurality on a surface for spatial capture of nucleic acids in a biological sample, *e.g.*, a permeabilized biological sample (*e.g.*, tissue sample), a cell suspension, can include different barcode sequences from all other spatially barcoded oligonucleotides in the population. Alternatively, each spatially barcoded oligonucleotide in a plurality can include different barcode sequences from some or most other spatially barcoded oligonucleotides in a plurality. For example, each spatially barcoded oligonucleotide in a plurality can have a barcode that is present for several different spatially barcoded oligonucleotides in the plurality even though the spatially barcoded oligonucleotides with the common barcode differ from each other at other sequence regions along their length. In various embodiments, one

or more barcode sequences that are used with a biological tissue are not present in the genome, transcriptome or other nucleic acids of the biological specimen. For example, barcode sequences can have less than 80%, 70%, 60%, 50% or 40% sequence identity to the nucleic acid sequences in a particular biological tissue.

[0034] As used herein, the term "array" refers to a population of sites that can be differentiated from each other according to relative location. Different molecules that are at different sites of an array can be differentiated from each other according to the locations of the sites in the array. An individual site of an array can include one or more molecules of a particular type. For example, a site can include a single nucleic acid molecule having a particular sequence or a site can include several nucleic acid molecules having the same sequence (and/or complementary sequence, thereof). The sites of an array can be different features located on the same substrate. Exemplary features include without limitation, beads (or other particles) in or on a substrate, droplets, wells in a substrate, projections from a substrate, ridges on a substrate or channels in a substrate. The sites of an array can be separate substrates each bearing a different molecule. Different molecules attached to separate substrates can be identified according to the locations of the substrates on a surface to which the substrates are associated or according to the locations of the substrates in a liquid or gel. Exemplary arrays in which separate substrates are located on a surface include, without limitation, those having beads in wells.

[0035] As used herein, a "biological sample" may include one or more biological or chemical substances, such as nucleic acids, oligonucleotides, proteins, cells, tissues, organisms, and/or biologically active chemical compound(s), such as analogs or mimetics of the aforementioned species. As used herein, the term "tissue" is intended to mean an aggregation of cells, and, optionally, intercellular matter. Typically the cells in a tissue are not free floating in solution and instead are attached to each other to form a multicellular structure. Exemplary tissue types include muscle, nerve, epidermal and connective tissues. In some instances, the biological sample may include whole blood, lymphatic fluid, serum, plasma, sweat, tear, saliva, sputum, cerebrospinal fluid, amniotic fluid, seminal fluid, vaginal excretion, serous fluid, synovial fluid, pericardial fluid, peritoneal fluid, pleural fluid, transudates, exudates, cystic fluid, bile, urine, gastric fluid, intestinal fluid, fecal samples, liquids containing single or multiple cells, liquids containing organelles, fluidized tissues, fluidized organisms, viruses including viral pathogens, liquids containing multi-celled organisms, biological swabs and biological washes. In further examples, the sample can be derived from an organ, including for example, an organ of the musculoskeletal system such as muscle, bone, tendon or ligament; an organ of the digestive system such as salivary gland, pharynx, esophagus, stomach, small intestine, large intestine, liver, gallbladder or

pancreas; an organ of the respiratory system such as larynx, trachea, bronchi, lungs or diaphragm; an organ of the urinary system such as kidney, ureter, bladder or urethra; a reproductive organ such as ovary, fallopian tube, uterus, vagina, placenta, testicle, epididymis, vas deferens, seminal vesicle, prostate, penis or scrotum; an organ of the endocrine system such as pituitary gland, pineal gland, thyroid gland, parathyroid gland, or adrenal gland; an organ of the circulatory system such as heart, artery, vein or capillary; an organ of the lymphatic system such as lymphatic vessel, lymph node, bone marrow, thymus or spleen; an organ of the central nervous system such as brain, brainstem, cerebellum, spinal cord, cranial nerve, or spinal nerve; a sensory organ such as eye, ear, nose, or tongue; or an organ of the integument such as skin, subcutaneous tissue or mammary gland. In various embodiments, the tissue can be derived from a multicellular organism. In some embodiments, a tissue section can be contacted with a surface, for example, by laying the tissue on the surface. The tissue can be freshly excised from an organism, or it may have been previously preserved for example by freezing (*e.g.*, fresh frozen tissue), embedding in a material such as paraffin (*e.g.*, formalin fixed paraffin embedded (FFPE) samples), formalin fixation, infiltration, dehydration or the like. Optionally, a tissue section can be attached to a surface, for example, using techniques and compositions described in, for example, U.S. Patent No. 11,390,912, incorporated by reference herein in its entirety. In some embodiments, a tissue can be permeabilized and the cells of the tissue lysed when the tissue is in contact with a surface. Any of a variety of treatments can be used such as those set forth above in regard to lysing cells. Target proteins and/or nucleic acids that are released from a tissue that is permeabilized can be captured by capture oligonucleotides on the surface. Thus, in various embodiments, the biological sample is a tissue sample. The thickness of a tissue sample or other biological sample that is contacted with a surface in a method set forth herein can be any suitable thickness desired. In representative embodiments, the thickness will be at least 0.1 μm , 0.25 μm , 0.5 μm , 0.75 μm , 1 μm , 5 μm , 10 μm , 50 μm , 100 μm or thicker. Alternatively or additionally, the thickness of a biological sample that is contacted with a surface will be no more than 100 μm , 50 μm , 10 μm , 5 μm , 1 μm , 0.5 μm , 0.25 μm , 0.1 μm or thinner.

[0036] As used herein, a "capture oligonucleotide" is generally an oligonucleotide comprising a nucleotide sequence capable of hybridizing or otherwise associating with an analyte (*e.g.*, target nucleic acid). In various embodiments, a capture oligonucleotide comprises an adapter sequence and a capture nucleotide sequence that is configured to bind to target nucleic acids of a biological sample. In some embodiments, a capture oligonucleotide comprises an adapter comprising a clustering primer sequence (*e.g.*, a B15 sequence) and a capture nucleotide sequence (*e.g.*, a poly T sequence), and is between

about 30 bases to about 100 bases in length, or between about 30 bases to about 90 bases, or between about 30 bases and 80 bases, or between about 30 bases and 70 bases, or between about 30 bases and 60 bases, or between about 30 bases and 55 bases, or between about 30 bases and 50 bases in length. In further embodiments, a capture oligonucleotide comprises an adapter comprising a clustering primer sequence (*e.g.*, a P7 sequence), and is about 15 bases, 20 bases, 25 bases, 30 bases, 35 bases, 40 bases, 45 bases, 50 bases, 55 bases, 60 bases, 65 bases, 70 bases, 75 bases, 80 bases, 85 bases, 90 bases, 95 bases, or 100 bases in length. The capture nucleotide sequence capable of hybridizing or otherwise associating with an analyte is, for example and without limitation, a universal sequence (*e.g.*, a poly T sequence, a random nucleotide sequence, or a semi-random nucleotide sequence), or a target-specific (*e.g.*, a gene-specific) sequence. In various embodiments, a capture nucleotide sequence (*e.g.*, a poly T nucleotide sequence or a random nucleotide sequence) is, is about, or is at least about 2, 5, 8, 10, 12, 15, 18, 20, 22, 25, 28, 30, 32, 35, 38, 40, or more bases in length. Alternatively or additionally, a capture nucleotide sequence can include less than or equal to about 40, 38, 35, 32, 30, 28, 25, 22, 20, 18, 15, 12, 10, 8, 5, or 2 bases. In some embodiments, a surface comprises a plurality of capture oligonucleotides immobilized thereon, wherein one or more of the plurality of capture oligonucleotides comprises, from 5' to 3': (i) an adapter sequence (*e.g.*, Adp1 as shown in Figure 1A); and (ii) a capture nucleotide sequence. In some embodiments, beads are packed onto a solid support (*e.g.*, a planar support or flow cell), wherein the beads comprise a plurality of capture oligonucleotides immobilized thereon, wherein one or more of the plurality of capture oligonucleotides comprises, from 5' to 3': (i) an adapter sequence (*e.g.*, Adp1 as shown in Figure 1A); and (ii) a capture nucleotide sequence.

[0037] In some embodiments, the disclosure contemplates that a capture oligonucleotide comprises a "polyTVN" sequence, which is a poly T sequence followed by a V (any base but a T) and an N. The polyTVN sequence is used, in some embodiments, to bias reverse transcription to the base of the poly A tail on the mRNA molecule.

[0038] As used herein, a "splint oligonucleotide" is an oligonucleotide that improves the ligation efficiency of oligonucleotides. By way of example, when ligating two oligonucleotides together, the splint oligonucleotide hybridizes to an end of each oligonucleotide and the ends are subsequently ligated. See, for example, Figure 1E.

[0039] As used herein, the term "different", when used in reference to nucleic acids, means that the nucleic acids have nucleotide sequences that are not the same as each other. Two or more nucleic acids can have nucleotide sequences that are different along their entire length. Alternatively, two or more nucleic acids can have nucleotide sequences

that are different along a substantial portion of their length. For example, two or more nucleic acids can have target nucleotide sequence portions that are different for the two or more molecules while also having a universal sequence portion that is the same on the two or more molecules. The term can be similarly applied to proteins which are distinguishable as different from each other based on amino acid sequence differences.

[0040] As used herein, the term “clustering primer sequence” refers to a nucleotide sequence in solution and/or immobilized on a surface that is used for amplifying the template polynucleotides to create identical copies of the same templates (*i.e.*, clusters). Examples of clustering primer sequences may include but are not limited to P5 primer, P5' primer, P7 primer, P7' primer, P15 primer, P15' primer, P17' primer, P17' primer, B15 primer, and B15' primer as described herein. Clustering primers, their sequences, and their uses are also described, *e.g.*, in WO2019222264, incorporated by reference herein in its entirety.

[0041] By “complementary” is meant that a first oligonucleotide sequence can form a double-stranded structure by matching base-pairs with a second oligonucleotide sequence or portion thereof. In various embodiments, “complementary” oligonucleotides are 100% complementary to each other, while in other embodiments, a first oligonucleotide sequence is at least (meaning greater than or equal to) about 95% complementary to a second oligonucleotide sequence over the length of the first oligonucleotide, at least about 90%, at least about 85%, at least about 80%, at least about 75%, at least about 70%, at least about 65%, at least about 60%, at least about 55%, or at least about 50% complementary to the second oligonucleotide over the length of the first oligonucleotide to the extent that the oligonucleotides are able to hybridize to each other under the conditions being utilized. The percent complementarity is determined over the length of the oligonucleotide. For example, given a first oligonucleotide in which 18 of 20 nucleotides of the first oligonucleotide are complementary to a 20-nucleotide region in a second oligonucleotide of 100 nucleotides total length, the oligonucleotides would be 90 percent complementary. In this example, the remaining noncomplementary nucleotides may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleotides.

[0042] As used herein, “hybridize” is intended to mean noncovalently associating a first oligonucleotide to a second oligonucleotide along the lengths of those polymers to form a double-stranded “duplex.” For instance, two DNA oligonucleotide strands may associate through complementary base pairing. The strength of the association between the first and second oligonucleotides increases with the complementarity between the sequences of nucleotides within those oligonucleotides. The strength of hybridization between oligonucleotides may be characterized by a temperature of melting (T_m) at which 50% of the

duplexes have oligonucleotide strands that disassociate from one another. Oligonucleotides that are “partially” hybridized to one another means that they have sequences that are complementary to one another, but such sequences are hybridized with one another along only a portion of their lengths to form a partial duplex. Oligonucleotides with an “inability” to hybridize include those that are physically separated from one another such that an insufficient number of their bases may contact one another in a manner so as to hybridize with one another.

[0043] As used herein the term “analyte” is intended to include any of a variety of target nucleic acids (*e.g.*, DNA, RNA or analogs thereof) that are to be detected, characterized, modified, synthesized, or the like. A target nucleic acid, in various embodiments, is a RNA molecule, such as a mRNA. In further embodiments, the target nucleic acids are mRNA, gDNA, rRNA, tRNA, or a combination thereof. In some embodiments, the target nucleic acids are RNA, mRNA, or a combination thereof. An array can include multiple different species from a library of analytes (*e.g.*, nucleic acids having different sequences from a library of nucleic acids).

[0044] As used herein, a “primer” is a nucleic acid molecule that can hybridize to a target sequence, such as an adapter attached to an oligonucleotide (*e.g.*, a first complementary strand as described herein). As one example, a clustering primer can serve as a starting point for template amplification and cluster generation. As another example, a synthesized nucleic acid (template) strand may include a site to which a primer (*e.g.*, a sequencing primer) can hybridize in order to prime synthesis of a new strand that is complementary to the synthesized nucleic acid strand. Any primer can include any combination of nucleotides or analogs thereof. In some examples, the primer is a single-stranded oligonucleotide or polynucleotide. The primer length can be any number of bases long and can include a variety of non-natural nucleotides. In various embodiments, the sequencing primer is a short strand, ranging from 10 to 60 bases, or from 20 to 40 bases.

[0045] As used herein, the term “single molecular identifier” or “SMI” refers to a molecular tag, either random, non-random, or semi-random, that may be attached to a nucleic acid. In various embodiments, a SMI is a unique molecular identifier (UMI). When incorporated into a nucleic acid, a SMI can be used to correct for subsequent amplification bias by directly counting single molecular identifiers (SMIs) that are sequenced after amplification. A SMI (*e.g.*, a UMI) can be attached to similar nucleic acids, *e.g.*, adapters, making each nucleic acid unique. SMIs (*e.g.*, UMIs) may also be used to uniquely tag individual molecules (*e.g.*, individual mRNA molecules) in a sample (*e.g.*, individual mRNA molecules in a tissue sample, cell sample, or sample library). In some embodiments, a UMI is a random nucleotide sequence (*e.g.*, N9).

[0046] As used herein, the term “universal sequence” refers to a series of nucleotides that is common to two or more nucleic acid molecules even if the molecules also have regions of sequence that differ from each other. A universal sequence that is present in different members of a collection of molecules can allow capture of multiple different nucleic acids using a population of universal capture nucleic acids that are complementary to the universal sequence. Similarly, a universal sequence present in different members of a collection of molecules can allow the replication or amplification of multiple different nucleic acids using a population of universal primers that are complementary to the universal sequence. Thus, a universal capture nucleic acid or a universal primer includes a sequence that can hybridize specifically to a universal sequence. Target nucleic acid molecules may be modified to attach universal adapters, for example, at one or both ends of the different target sequences. Universal capture oligonucleotides are applicable for interrogating a plurality of different oligonucleotides without necessarily distinguishing the different species whereas target-specific capture sequences are applicable for distinguishing the different species. A non-limiting example of a universal sequence is a polyT nucleotide sequence.

[0047] As used herein, the term “plurality” is intended to mean a population of two or more members, which may all be the same or two or more members may be different. Pluralities may range in size from small, medium, large, to very large. The size of small plurality may range, for example, from a few members to tens of members. Medium sized pluralities may range, for example, from tens of members to about 100 members or hundreds of members. Large pluralities may range, for example, from about hundreds of members to about 1000 members, to thousands of members and up to tens of thousands of members. Very large pluralities may range, for example, from tens of thousands of members to about hundreds of thousands, a million, millions, tens of millions and up to or greater than hundreds of millions of members. Therefore, a plurality may range in size from two to well over one hundred million members as well as all sizes, as measured by the number of members, in between and greater than the above example ranges. Accordingly, the definition of the term is intended to include all integer values greater than two. An upper limit of a plurality may be set, for example, by the theoretical diversity of bead types in an array.

[0048] As used herein, the term “tagmentation,” “tagment,” or “tagmenting” refers to transforming a nucleic acid, *e.g.*, DNA, into adaptor-modified templates in solution ready for cluster formation and sequencing by the use of transposase mediated fragmentation and tagging. This process often involves the modification of the nucleic acid by a transposome complex comprising transposase enzyme complexed with adaptors comprising transposon end sequence (*e.g.*, a ME sequence as described herein). Tagmentation results in the simultaneous fragmentation of the nucleic acid and ligation of the adaptors to the 5' ends of

both strands of duplex fragments. Following a purification step to remove the transposase enzyme, additional sequences (*e.g.*, P5-i5-A14ME) are added to the ends of the adapted fragments by PCR. See, *e.g.*, Figure 5. For additional discussion of tagmentation, see also International Publication No. WO 2022/031955, which is incorporated herein by reference in its entirety. In general, tagmentation is used for nucleic acids that are beyond a size range that is optimal for a commercial sequencer (*e.g.*, an Illumina sequencer). In various embodiments, the optimal size range for sequencing is about 300-500 bases.

[0049] A "transposase" is an enzyme that is capable of forming a functional complex with a transposon end-containing composition (*e.g.*, transposons, transposon ends, transposon end compositions) and catalyzing insertion or transposition of the transposon end-containing composition into the double-stranded target nucleic acid with which it is incubated, for example, in an *in vitro* transposition reaction. A transposase as presented herein can also include integrases from retrotransposons and retroviruses. Transposases, transpososome, and transposome complexes are generally known to those of skill in the art, as exemplified by the disclosure of US Patent Publication No. 2010/0120098, the content of which is incorporated herein by reference in its entirety. In some embodiments, the transposase used in tagmentation methods of the disclosure is a Tn5 transposase. In some embodiments, the Tn5 transposase is a hyperactive Tn5 transposase, or an active mutant thereof. In some aspects, the Tn5 transposase is a Tn5 transposase as described in International Publication No. WO 2015/160895, which is incorporated herein by reference. It will be appreciated, however, that any transposition system that is capable of inserting a transposon end with sufficient efficiency to 5'-tag and fragment a target nucleic acid for its intended purpose can be used in the methods of the present disclosure.

[0050] A "transposome complex" comprises at least one transposase and a transposon recognition sequence. In some embodiments, the transposase binds to a transposon recognition sequence to form a functional complex that is capable of catalyzing a transposition reaction. In some embodiments, the transposon recognition sequence is a double-stranded transposon end sequence. The transposase binds to a transposase recognition site in a target nucleic acid and inserts the transposon recognition sequence into a target nucleic acid. In some such insertion events, one strand of the transposon recognition sequence (or end sequence) is transferred into the target nucleic acid, resulting in a cleavage event. Exemplary transposition procedures and systems that can be readily adapted for use with the transposases are described in International Publication No. WO 2022/031955, which is incorporated herein by reference in its entirety.

[0051] The term "transposon end" refers to a double-stranded nucleic acid DNA that exhibits only the nucleotide sequences (the "transposon end sequences") that are necessary

to form the complex with the transposase or integrase enzyme that is functional in an in vitro transposition reaction. In some embodiments, a transposon end is capable of forming a functional complex with the transposase in a transposition reaction. As non-limiting examples, transposon ends can include the 19-bp outer end ("OE") transposon end, inner end ("IE") transposon end, or "mosaic end" ("ME") transposon end recognized by a wild-type or mutant Tn5 transposase, or the R1 and R2 transposon end as set forth in the disclosure of U.S. Patent Publication No. 2010/0120098, the content of which is incorporated herein by reference in its entirety. Transposon ends can comprise any nucleic acid or nucleic acid analogue suitable for forming a functional complex with the transposase or integrase enzyme in an in vitro transposition reaction. For example, the transposon end can comprise DNA, RNA, modified bases, non-natural bases, modified backbone, and can comprise nicks in one or both strands. Although the term "DNA" is used throughout the present disclosure in connection with the composition of transposon ends, it should be understood that any suitable nucleic acid or nucleic acid analogue can be utilized in a transposon end.

[0052] As used herein, a "semi-random" nucleotide sequence comprises or consists of a partially pre-determined nucleotide sequence combined with a random nucleotide sequence.

OLIGONUCLEOTIDES

[0053] An oligonucleotide is a polymer comprised of nucleotides. Oligonucleotides of the disclosure may be of any length and include, in various embodiments, DNA oligonucleotides, RNA oligonucleotides, analogs thereof, or a combination thereof. In any aspects or embodiments described herein, an oligonucleotide is single-stranded, double-stranded, or partially double-stranded.

[0054] Nucleotides may include naturally occurring nucleotides and functional analogs thereof. Examples of functional analogs are those that are capable of hybridizing to a nucleic acid in a sequence specific fashion or capable of being used as a template for replication of a particular nucleotide sequence. Naturally occurring nucleotides generally have a backbone containing phosphodiester bonds. An analog structure can have an alternate backbone linkage including any of a variety known in the art. Naturally occurring nucleotides generally have a deoxyribose sugar (*e.g.*, found in DNA) or a ribose sugar (*e.g.*, found in RNA). An analog structure can have an alternate sugar moiety including any of a variety known in the art. Nucleotides can include native or non-native bases. A native DNA can include one or more of adenine, thymine, cytosine and/or guanine, and a native RNA can include one or more of adenine, uracil, cytosine and/or guanine. Any non-native base may be used, such as a locked nucleic acid (LNA) and a bridged nucleic acid (BNA). Example modified nucleotides include inosine, xanthine, hypoxanthine, isocytosine,

isoguanine, 2-aminopurine, 5-methylcytosine, 5-hydroxymethyl cytosine, 2-aminoadenine, 6-methyl adenine, 6-methyl guanine, 2-propyl guanine, 2-propyl adenine, 2-thiouracil, 2-thiothymine, 2-thiocytosine, 15-halouracil, 15-halocytosine, 5-propynyl uracil, 5-propynyl cytosine, 6-azo uracil, 6-azo cytosine, 6-azo thymine, 5-uracil, 4-thiouracil, 8-halo adenine or guanine, 8-amino adenine or guanine, 8-thiol adenine or guanine, 8-thioalkyl adenine or guanine, 8-hydroxyl adenine or guanine, 5-halo substituted uracil or cytosine, 7-methylguanine, 7-methyladenine, 8-azaguanine, 8-azaadenine, 7-deazaguanine, 7-deazaadenine, 3-deazaguanine, 3-deazaadenine or the like. As is known in the art, certain nucleotide analogues cannot become incorporated into a polynucleotide, for example, nucleotide analogues such as adenosine 5'-phosphosulfate. Nucleotides may include any suitable number of phosphates, *e.g.*, three, four, five, six, or more than six phosphates.

[0055] Oligonucleotides contemplated by the disclosure also include those having at least one modified internucleotide linkage. In some embodiments, the oligonucleotide is all or in part a peptide nucleic acid. Other modified internucleoside linkages include at least one phosphorothioate linkage. Still other modified oligonucleotides include those comprising one or more universal bases. "Universal base" refers to molecules capable of substituting for binding to any one of A, C, G, T and U in nucleic acids by forming hydrogen bonds without significant structure destabilization. Examples of universal bases include but are not limited to 5'-nitroindole-2'-deoxyribose, 3-nitropyrrole, inosine and hypoxanthine.

[0056] In various aspects, an oligonucleotide of the disclosure, or a modified form thereof, is generally about 5 nucleotides to about 150 nucleotides in length. In further embodiments, an oligonucleotide of the disclosure is about 5 to about 125 nucleotides in length, about 5 to about 100 nucleotides in length, about 5 to about 90 nucleotides in length, about 5 to about 50 nucleotides in length, about 5 to about 45 nucleotides in length, about 5 to about 40 nucleotides in length, about 5 to about 35 nucleotides in length, about 5 to about 30 nucleotides in length, about 5 to about 25 nucleotides in length, about 5 to about 20 nucleotides in length, about 5 to about 15 nucleotides in length, about 5 to about 10 nucleotides in length, about 10 to about 150 nucleotides in length, about 10 to about 125 nucleotides in length, about 10 to about 100 nucleotides in length, about 10 to about 90 about 10 to about 50 nucleotides in length, about 10 to about 45 nucleotides in length, about 10 to about 40 nucleotides in length, about 10 to about 35 nucleotides in length, about 10 to about 30 nucleotides in length, about 10 to about 25 nucleotides in length, about 10 to about 20 nucleotides in length, about 10 to about 15 nucleotides in length, and all oligonucleotides intermediate in length of the sizes specifically disclosed to the extent that the oligonucleotide is able to achieve the desired result. Accordingly, in various embodiments, an oligonucleotide of the disclosure is or is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,

18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150 or more nucleotides in length. In further embodiments, an oligonucleotide of the disclosure is less than 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, or more nucleotides in length. In various embodiments, the length of an oligonucleotide (such as a primer) of the disclosure is between about 5 base pairs (bp) and 40 bp, or between about 5 bp and 35 bp, or between about 5 bp and 30 bp, or between about 10 bp and 35 bp, or between about 10 bp and 30 bp, or between about 20 bp and 40 bp, or between about 20 bp and 35 bp, or between about 20 bp and 30 bp, or between about 9 and 20 bp or between about 5 and 15 bp, or between about 9 and 15 bp in length. In some embodiments, the length of an oligonucleotide (such as a primer) of the disclosure is about 10 bp, 13 bp, 15 bp, 20 bp, 25 bp, 30 bp, 35 bp, or 40 bp. As described herein, in various embodiments the oligonucleotide may be a P5 primer, a P5' primer, a P7 primer, or a P7' primer.

[0057] As used herein, the term "poly T" or "poly A," when used in reference to a nucleic acid sequence (*e.g.*, a capture nucleotide sequence), is intended to mean a series of two or more thiamine (T) or adenine (A) bases, respectively. A poly T or poly A can include at least about 2, 5, 8, 10, 12, 15, 18, 20, 22, 25, 28, 30, 32, 35, 38, 40, or more of the T or A bases, respectively. Alternatively or additionally, a poly T or poly A can include at most about 40, 38, 35, 32, 30, 28, 25, 22, 20, 18, 15, 12, 10, 8, 5, or 2 of the T or A bases, respectively. In some embodiments, the disclosure contemplates use of a "polyTVN" sequence, wherein "T" is a capture nucleotide sequence, "V" is adenine (A), cytosine (C), or guanine (G), and "N" is adenine (A), cytosine (C), guanine (G), or thymine (T). The polyTVN sequence is used, in some embodiments, to bias reverse transcription to the base of the poly A tail on the mRNA molecule.

[0058] Spacers. In some embodiments, one or more oligonucleotides (*e.g.*, a capture oligonucleotide, a spatially barcoded oligonucleotide) immobilized on a surface comprises a spacer. "Spacer" as used herein means a moiety that serves to increase distance of an oligonucleotide from the surface. In some embodiments, the spacer provides sufficient distance from the surface to enable a reverse transcriptase (RT) to access the capture nucleotide sequence (*e.g.*, a polyT capture nucleotide sequence) of the capture oligonucleotide. In some aspects, the spacer when present is an organic moiety. In some aspects, the spacer is a polymer, including but not limited to a water-soluble polymer, a nucleic acid, a polypeptide, an oligosaccharide, a carbohydrate, a lipid, or a combination thereof. In various embodiments, an oligonucleotide (*e.g.*, a capture oligonucleotide, a spatially barcoded oligonucleotide) comprises 1, 2, 3, 4, 5, or more spacer moieties. In some embodiments, the spacer is an oligonucleotide spacer. An oligonucleotide spacer may have any sequence that does not interfere with the desired function of the oligonucleotide. In various embodiments, the bases of the oligonucleotide spacer are all adenylic acids, all thymidylic acids, all cytidylic acids, all guanylic acids, all uridylic acids, or all some other modified base. In various embodiments, the length of the spacer is or is equivalent to at least about 2 nucleotides, at least about 3 nucleotides, at least about 4 nucleotides, at least about 5 nucleotides, 5-10 nucleotides, 10-20 nucleotides, nucleotides, 10-30 nucleotides, or greater than 30 nucleotides.

[0059] As used herein, the term "immobilized" refers to the state of two things being joined, fastened, adhered, attached, connected, or bound to each other. For example, an analyte, such as a nucleic acid, can be immobilized on a material, such as a bead, gel, or surface, by a covalent or non-covalent bond. A covalent bond is characterized by the sharing of pairs of electrons between atoms. A non-covalent bond is a chemical bond that does not involve the sharing of pairs of electrons and can include, for example, hydrogen bonds, ionic bonds, van der Waals forces, hydrophilic interactions and hydrophobic interactions. In various embodiments, covalent attachment can be used, but all that is required is that the oligonucleotides remain stationary or attached to a surface under conditions in which it is intended to use the surface, for example, in applications requiring nucleic acid capture, amplification, and/or sequencing. Oligonucleotides to be used as capture oligonucleotides can be immobilized such that a 3'-end is available for enzymatic extension and at least a portion of the sequence is capable of hybridizing to a complementary sequence.

[0060] Exemplary covalent linkages include, for example, those that result from the use of click chemistry techniques. Exemplary non-covalent linkages include, but are not limited to, non-specific interactions (*e.g.*, hydrogen bonding, ionic bonding, van der Waals interactions etc.) or specific interactions (*e.g.*, affinity interactions, receptor-ligand interactions, antibody-

epitope interactions, avidin-biotin interactions, streptavidin-biotin interactions, lectin-carbohydrate interactions, etc.). Exemplary linkages are set forth in U.S. Pat. Nos. 6,737,236; 7,259,258; 7,375,234 and 7,427,678; and US Pat. Pub. No. 2011/0059865 A1, each of which is incorporated herein by reference.

[0061] As used herein, the term "extend," when used in reference to a nucleic acid, is intended to mean addition of at least one nucleotide to the nucleic acid. In particular embodiments, one or more nucleotides can be added to the 3' end of a nucleic acid, for example, via polymerase catalysis (e.g. DNA polymerase, RNA polymerase, or reverse transcriptase (RT)). Exemplary reverse transcriptase enzymes of the present disclosure include, but are not limited to, Maxima H- (Thermo Fisher Scientific Inc.) and Superscript IV (Thermo Fisher Scientific Inc.). Chemical or enzymatic methods can be used to add one or more nucleotide to the 3' or 5' end of a nucleic acid. An extension reaction, in which nucleotides are added to the 3' end of an oligonucleotide (e.g., a primer) is performed in the presence of a polymerase, such as a DNA or RNA polymerase. In some embodiments, the polymerase is a non-thermostable isothermal strand displacement polymerase. Suitable non-thermostable strand displacement polymerases according to the present disclosure can be found, for example, through New England BioLabs, Inc. and include phi29, Bsu, Klenow, DNA Polymerase I (*E. coli*), and Terminator. In some embodiments, the extension reaction is carried out by recombinase polymerase amplification (RPA). RPA comprises three core enzymes – a recombinase, a single-stranded DNA binding protein (SSB) and a strand-displacing polymerase. As described in Daher *et al.* (Rana K Daher, Gale Stewart, Maurice Boissinot, Michel G Bergeron, Recombinase Polymerase Amplification for Diagnostic Applications, Clinical Chemistry, Volume 62, Issue 7, 1 July 2016). One or more oligonucleotides can be added to the 3' or 5' end of a nucleic acid, for example, via chemical or enzymatic (e.g., ligase catalysis) methods. A nucleic acid can be extended in a template directed manner, whereby the product of extension is complementary to a template nucleic acid that is hybridized to the nucleic acid that is extended.

METHODS

[0062] The present disclosure is generally directed to methods of generating spatial transcriptomic libraries. In various aspects, the disclosure provides methods for spatially capturing target nucleic acids of a tissue sample. Use of methods of the disclosure provides the ability to spatially preserve the location of target nucleic acids in a biological sample (e.g., a tissue sample).

[0063] In general, in some aspects the disclosure provides methods of generating an immobilized library of target nucleic acids comprising the following general steps: (1)

providing a surface comprising a plurality of capture oligonucleotides and a plurality of spatially barcoded oligonucleotides; (2) contacting the surface with a biological sample, the contacting resulting in hybridization of the target nucleic acids of the biological sample to the capture oligonucleotides; (3) generating spatially-barcoded first strand cDNA, thereby generating the immobilized library of target nucleic acids.

[0064] In further aspects, the disclosure provides methods of preparing a spatial array via establishing oligonucleotide clusters on a surface. In various embodiments, the methods generally comprise the following general steps: (1) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface; (2) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage domain, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (3) hybridizing one or more of the first plurality of template oligonucleotides to the plurality of first adapter oligonucleotides immobilized on the surface; (4) extending one or more of the first adapter oligonucleotides using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides; (5) releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (6) hybridizing one or more of the plurality of first surface oligonucleotides to the plurality of second adapter oligonucleotides immobilized on the surface; (7) extending the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides; (8) releasing the plurality of first surface oligonucleotides from the surface; (9) cleaving the cleavage domain of the cluster of second surface oligonucleotides to release the cleavage domain and the Adp1' sequence from the cluster of second surface oligonucleotides, thereby preparing the spatial array. See, for example, Figure 11A. In further embodiments, a method of the disclosure comprises the following general steps: (1) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface; (2) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage domain, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (3) hybridizing one or more of the first plurality of template oligonucleotides to the plurality of first adapter oligonucleotides immobilized on the surface; (4) extending one or more of the first adapter oligonucleotides using the one or more of the

first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides; (5) releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (6) hybridizing one or more of the plurality of first surface oligonucleotides to the plurality of second adapter oligonucleotides immobilized on the surface; (7) extending the plurality of second adapter oligonucleotides immobilized on the surface; (8) hybridizing one or more of the plurality of first and second surface oligonucleotides to adapter oligonucleotides immobilized on the surface; (9) extending the plurality of first and second oligonucleotides on the surface; (10) repeating steps (8)-(9) zero, one, two, three, four, five, or more times; (11) releasing the plurality of first surface oligonucleotides from the surface; (12) cleaving the cleavage domain of the cluster of second surface oligonucleotides to release the cleavage domain and the Adp1' sequence from the cluster of second surface oligonucleotides, thereby preparing the spatial array. In various embodiments, amplification of an oligonucleotide on a surface is accomplished via bridge amplification or Examp.

[0065] Surface. The present disclosure provides, in various aspects, methods of preparing an immobilized library of target nucleic acids of a biological sample comprising providing a surface (e.g., an Illumina flow cell (Illumina Inc., San Diego Calif.) comprising pluralities of oligonucleotides. The surface, in various embodiments, comprises (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, and a template switching oligonucleotide (TSO) sequence (see, e.g., Figure 1A). In some embodiments, the surface comprises (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface, a first spatial barcode, and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a second spatial barcode, and a template switching oligonucleotide (TSO) sequence. In some embodiments, the first spatial barcode is the same as the second spatial barcode. In some embodiments, the first spatial barcode is different than the second spatial barcode. In some embodiments, the spatially barcoded oligonucleotide does not comprise a template-switching oligonucleotide (TSO) sequence. In various embodiments, the adapter

sequence Adp1 comprises a "P5", "P7", "B15", "P5'" (P5 prime), "P7'" (P7 prime), "B15'" (B15 prime), "P15", or "P17" nucleotide sequence. In some embodiments, the adapter sequence Adp1 comprises P5-A14ME. In some embodiments, the adapter sequence Adp1 comprises ME'V2B15'. In various embodiments, the adapter sequence Adp2 comprises a "P5", "P7", "B15", "P5'" (P5 prime), "P7'" (P7 prime), "B15'" (B15 prime), "P15", or "P17" nucleotide sequence.

[0066] Aspects of the disclosure include those in which a plurality of capture oligonucleotides is immobilized on a surface. The capture oligonucleotides, in various embodiments, hybridize to target nucleic acids of a biological sample. In some embodiments, each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence. In further embodiments, the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences. In still further embodiments, the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof. In various embodiments, the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence. In further embodiments, the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence. Aspects of the disclosure also include those in which capture nucleotide sequences are extended following hybridization of the capture oligonucleotide to the target nucleic acid. In some embodiments, the extending of the capture nucleotide sequence is performed using a reverse transcriptase (see, *e.g.*, Figure 1A). In some embodiments, the extending of the capture nucleotide sequence is performed using a terminal deoxynucleotidyl transferase (TdT). In some embodiments, the extending of the capture nucleotide sequence using a terminal deoxynucleotidyl transferase (TdT) occurs in the presence of only one type of substrate (*e.g.*, the extending is performed in the presence of only riboguanosine). In some embodiments, the extending of the capture nucleotide sequence using a terminal deoxynucleotidyl transferase (TdT) occurs in the presence of a locked nucleic acid (LNA) riboguanosine. In some implementations of a method of the disclosure, the target nucleic acids are polyadenylated prior to hybridization of the target nucleic acids to the capture nucleotide sequences. In some embodiments, the target nucleic acids are polyadenylated using a poly(A) polymerase. In further embodiments, the target nucleic acids are polyadenylated using chemical ligation or enzymatic ligation.

[0067] In some aspects, an oligonucleotide of the disclosure is immobilized on a surface through a spacer as described herein. Thus, in some embodiments, one or more or all of the plurality of capture oligonucleotides comprises a spacer, through which the one or more

or all of the plurality of capture oligonucleotides is immobilized on the surface (see, *e.g.*, Figure 2). In some embodiments, one or more or all of the plurality of spatially barcoded oligonucleotides comprises a spacer, through which the one or more or all of the plurality of spatially barcoded oligonucleotides is immobilized on the surface. In some embodiments, one or more or all of the plurality of capture oligonucleotides and one or more or all of the plurality of spatially barcoded oligonucleotides comprises a spacer, through which the one or more or all of the plurality of capture oligonucleotides and the one or more or all of the plurality of spatially barcoded oligonucleotides is immobilized on the surface.

[0068] Contacting the surface with a biological sample. As described herein, a biological sample (*e.g.*, a tissue section) can be attached to a surface, for example, using techniques and compositions described in, for example, U.S. Patent No. 11,390,912, incorporated by reference herein in its entirety. In some embodiments, a tissue sample can be permeabilized and the cells of the tissue lysed when the tissue is in contact with a surface. Target nucleic acids that are released from a tissue that is permeabilized can be captured by capture oligonucleotides on the surface.

[0069] In some embodiments, the biological tissue is removed from the surface after generation of a complementary template-switching oligonucleotide (TSO') sequence on the first complementary strands. In some embodiments, the biological tissue is removed from the surface after formation of the first complementary strands.

[0070] Methods of the disclosure further provide, in various embodiments, that the biological sample is digested. The digestion of the biological sample can occur, in various embodiments, after generation of the first complementary strands. In some embodiments, digestion of the biological sample occurs after generation of the first complementary strands but prior to generation of second complementary strands. The disclosure also provides methods in which the target nucleic acids (*e.g.*, RNA) are removed from the surface. Removal of target nucleic acids from the surface can occur, in various embodiments, after generation of the first complementary strands. In some embodiments, removal of the target nucleic acids occurs after generation of the first complementary strands but prior to generation of second complementary strands. Removal of the target nucleic acids from the surface is achieved, in various embodiments, by changing a condition. In further embodiments, the condition is temperature, pH, formamide concentration, Mg⁺ based RNA fragmentation, RNase digestion, or a combination thereof.

[0071] Generating spatially-barcoded first strand cDNA. In some embodiments, following formation of a first complementary strand, a plurality of template switching oligonucleotides is hybridized to the plurality of non-templated nucleotides of the first

complementary strands such that each of the plurality of template switching oligonucleotides that is hybridized to the plurality of non-templated nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal to the surface (see, *e.g.*, Figure 1A). The plurality of non-templated nucleotides on the first complementary strands is then extended using the template switching oligonucleotides as template, thereby generating a complementary template-switching oligonucleotide (TSO') sequence on the first complementary strands (see, *e.g.*, Figure 1A). In various embodiments comprising a template-switching reaction, the disclosure contemplates that the template switching reaction occurs concurrently with the reverse transcription reaction that is used to generate the first complementary strands. For example, the plurality of template switching oligonucleotides may be added directly into the reverse transcription mix.

[0072] Next, the complementary template-switching oligonucleotide (TSO') sequence is hybridized on one or more of the first complementary strands to the template switching oligonucleotide (TSO) sequence of one or more of the plurality of spatially barcoded oligonucleotides, and the template-switching oligonucleotide binding site is extended on the one or more of the first complementary strands using the one or more of the plurality of spatially barcoded oligonucleotides as template, thereby generating a spatially barcoded first strand cDNA comprising a sequence complementary to the spatial barcode (SBC') and a sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands (see, *e.g.*, Figure 1B), thereby preparing the immobilized library of target nucleic acids. In some embodiments, after the first complementary strand comprising the complementary template-switching oligonucleotide (TSO') sequence is generated, the TSO' sequence of the first complementary strand is hybridized to the TSO sequence on the spatially barcoded strand, and both strands are simultaneously extended by, *e.g.*, a reverse transcriptase (RT) or a DNA polymerase (see, *e.g.*, Figure 12). In some embodiments, the template switching oligonucleotide (TSO) sequence of one or more or all of the plurality of spatially barcoded oligonucleotides is 3' blocked such that extension is blocked. In some implementations, an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands is extended, thereby generating one or more second complementary strands (see, *e.g.*, Figure 1C). In some embodiments, the one or more first complementary strands and/or the one or more second complementary strands are amplified, thereby generating a plurality of hybridized first complementary strands and second complementary strands, or portions thereof (see, *e.g.*, Figure 1C). In some aspects, non-extended capture oligonucleotides are removed from the surface by an exonuclease (*e.g.*, as depicted in Figures 8A-8B). In some embodiments that include template switching, any cDNAs that are

generated that do not contain non-templated nucleotides (*e.g.*, non-templated deoxycytosines (dCs)) are rescued by direct ligation of an oligonucleotide comprising the TSO sequence and a random sequence ('SeqX') (see, *e.g.*, Figure 8C).

[0073] In further embodiments, one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, an optional single molecule identifier (SMI), and a homopolymeric nucleotide sequence. Following formation of a first complementary strand via reverse transcription with a reverse transcriptase having terminal transferase activity, the non-templated nucleotides of the first complementary strand hybridize directly to the homopolymer nucleotide sequence of the one or more of the plurality of spatially barcoded oligonucleotides. Next, the homopolymeric nucleotide sequence on the one or more of the first complementary strands is extended using the one or more of the plurality of spatially barcoded oligonucleotides as template, thereby generating a spatially barcoded first strand cDNA comprising a sequence complementary to the spatial barcode (SBC') and a sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands. See, *e.g.*, Figure 1D.

[0074] In some implementations, one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, an optional single molecule identifier (SMI), and a 'hyb' nucleotide sequence, wherein the 'hyb' sequence is, in various embodiments, a TSO nucleotide sequence. Following formation of a first complementary strand via reverse transcription with a reverse transcriptase having terminal transferase activity and removal of the target nucleic acids (*e.g.*, RNA), the non-templated nucleotides of the first complementary strand are ligated directly to the 'hyb' nucleotide sequence of the one or more of the plurality of spatially barcoded oligonucleotides using a splint oligonucleotide comprising a 'hyb'-GGG' nucleotide sequence. See, *e.g.*, Figure 1E. Next, the spatially barcoded first strand cDNA comprising a sequence complementary to the spatial barcode (SBC') and a sequence complementary to the second adapter (Adp2') sequence may be cleaved from the surface. See, *e.g.*, Figure 1E.

[0075] In some embodiments, one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, an optional single molecule identifier (SMI), and a random nucleotide sequence (*e.g.*, N6 or N9 as seen in Figure 1F). Following formation of a first complementary strand via reverse transcription (here, it is not required that the reverse transcriptase possesses terminal transferase activity), the target nucleic acids (*e.g.*, RNA) are removed from the surface. Next, the random nucleotide sequence of the spatially

barcoded oligonucleotides hybridizes to a complementary nucleotide sequence in the first complementary strand and extends the spatially barcoded oligonucleotides back through the template. Finally, cleavage below the Adp2 sequence releases the adapterized library from the surface.

[0076] In some embodiments, a capture oligonucleotide is immobilized on the surface via a cleavage site. In various embodiments, the cleavage site is uracil (cleaved by USER enzyme mix), 8-oxo-G (cleaved by FPG), a restriction enzyme site, or through chemical cleavage. In some embodiments, following generation of a spatially barcoded first strand cDNA, the spatially barcoded first strand cDNA is cleaved before second strand synthesis. In further embodiments, following generation of a spatially barcoded first strand cDNA, the spatially barcoded first strand cDNA is cleaved after second strand synthesis. See, *e.g.*, Figure 4A.

[0077] The disclosure also provided methods of preparing an immobilized library utilizing full length target nucleic acids (*e.g.*, RNA transcripts). If the RNA transcripts are full-length, then in some embodiments tagmentation is used to simultaneously fragment the cDNA and add the other sequencing adapter closer to the TSO end of the fragment. See, *e.g.*, Figure 5.

[0078] In some embodiments, a TSO sequence is added to a capture oligonucleotide via ligation. See, *e.g.*, Figure 6. After permeabilization of the biological sample (*e.g.*, tissue sample), reverse transcription is performed using a reverse transcriptase with or without terminal transferase activity. The target nucleic acid (*e.g.*, mRNA) is then removed from the surface (Figure 6A). Oligonucleotides capable of hybridizing to the TSO sequence (*i.e.*, TSO' oligonucleotides as shown in Figure 6B) on the spatially barcoded oligonucleotides are then hybridized to the surface to block extension from the 3' ends of the spatially barcoded oligonucleotides (Figure 6B). Then, the TSO' oligonucleotide (where TSO' refers to a sequence that hybridizes to the TSO sequence) is ligated to the 3' ends of the cDNA molecules using annealed oligonucleotides with a 3' overhang comprising random bases ("NNN" as shown in Figure 6B). After ligation, the hybridized oligonucleotides are removed using alkaline or heat denaturation (Figure 6B). In some embodiments, the workflow then proceeds with the steps outlined in Figure 1B.

[0079] Methods of the disclosure also include those that enable spatial barcoding of fragments within a target nucleic acid (*e.g.*, a mRNA transcript). Here, the surface contains two types of oligonucleotides, capture oligonucleotides and spatially barcoded oligonucleotides, that comprise a transposon ME sequence. An oligonucleotide comprising a ME' sequence is then hybridized onto the surface oligonucleotides after the clusters have

been generated (see, *e.g.*, Figure 7B). After template switching, second strand cDNA is generated. Then transposase is added to the surface. The transposase enzyme binds to the ME sequences and tags the cDNA, covalently attaching the spatial barcode oligonucleotides. After tagmentation, Tn5 is removed with SDS and a 5'→3' exonuclease (*e.g.*, T7 exonuclease) is used to digest away the oligonucleotides that are not attached to the surface (see, *e.g.*, Figure 7C). Sequencing adapters are then hybridized and gap-fill ligation is performed to attach them to the 3' ends of the cDNA (see, *e.g.*, Figure 7E and Figure 7F). The strands are then denatured, and polymerase extension is used to generate a copy that is dehybridized from the surface, followed by PCR amplification of the final library (see, *e.g.*, Figure 7G). Figure 7H shows an embodiment of steps that may be performed after the bridged stage that is depicted in, *e.g.*, far right panel of Figure 1F. In the variation shown in Figure 7H, a randomizer in the spatially barcoded oligonucleotide (*e.g.*, N6 or N9) finds a complementary sequence in the cDNA and extends the SBC adapter back through the template to create a double-stranded molecule. Next, free transposomes are added and the double-stranded molecule is directly tagmented to add a second adapter sequence (*e.g.*, P5/P5'). This is followed by runoff or a gap-fill-ligation to complete the reaction. Next, all non-bound strands (*e.g.*, second strand cDNA) are washed out using, *e.g.*, NaOH, and subsequent cleavage below Adp2 and dehybridization of the second cDNA sequence releases the adapterized library.

[0080] In some aspects, a spatially barcoded oligonucleotide of the disclosure further comprises a random sequence or a sequence designed to have minimal complementarity to a target nucleic acid so as not to hybridize to the target nucleic acid (*e.g.*, mRNA) ("SeqX" as depicted in Figure 8A) at the terminus of the spatially barcoded oligonucleotide that is distal to the surface to which the spatially barcoded oligonucleotide is attached. The sequence designed to have minimal complementarity to a target nucleic acid so as not to hybridize to the target nucleic acid may be designed, *e.g.*, bioinformatically based on the target nucleic acid sequence. Following formation of a first complementary strand via reverse transcription with a reverse transcriptase possessing terminal transferase activity, a plurality of template switching oligonucleotides is hybridized to the plurality of non-templated nucleotides of the first complementary strands such that each of the plurality of template switching oligonucleotides that is hybridized to the plurality of non-templated nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal to the surface (see, *e.g.*, Figure 8A). The plurality of non-templated nucleotides on the first complementary strands is then extended using the template switching oligonucleotides as template, thereby generating a complementary template-switching oligonucleotide (TSO') sequence on the first complementary strands (see, *e.g.*, Figure 8A). Next, oligonucleotides

comprising a sequence that hybridizes to SeqX ("SeqX" as depicted in Figure 8A) are added, followed by exonuclease digestion of single stranded capture oligonucleotides (*i.e.*, capture oligonucleotides that did not hybridize to a target nucleic acid). The target nucleic acids (*e.g.*, RNA), template-switching oligonucleotides, and SeqX' oligonucleotides are then removed (see, *e.g.*, Figure 8B). Additional oligonucleotides comprising a sequence that hybridizes to SeqX ("SeqX" as depicted in Figure 8B) are added (*e.g.*, to block ligation).

[0081] Next, the complementary template-switching oligonucleotide (TSO') sequence is hybridized on one or more of the first complementary strands to the template switching oligonucleotide (TSO) sequence of one or more of the plurality of spatially barcoded oligonucleotides, and the template-switching oligonucleotide binding site is extended on the one or more of the first complementary strands using the one or more of the plurality of spatially barcoded oligonucleotides as template, thereby generating a spatially barcoded first strand cDNA comprising a sequence complementary to the spatial barcode (SBC') and a sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands (see, *e.g.*, Figure 1B), thereby preparing the immobilized library of target nucleic acids. In some embodiments, the template switching oligonucleotide (TSO) sequence of one or more or all of the plurality of spatially barcoded oligonucleotides is 3' blocked such that extension is blocked. In some implementations, an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands is extended, thereby generating one or more second complementary strands (see, *e.g.*, Figure 1C). In some embodiments, the one or more first complementary strands and/or the one or more second complementary strands are amplified, thereby generating a plurality of hybridized first complementary strands and second complementary strands, or portions thereof (see, *e.g.*, Figure 1C).

[0082] Following generation of spatially barcoded first strand cDNA, second complementary strands are generated. Generation of second complementary strands may be performed "on surface" or "off surface". See, *e.g.*, Figure 4A. In some embodiments, for on surface generation of second complementary strands the first complementary strands remain immobilized on the surface while second complementary strands are extended using the spatially barcoded first strand cDNA as template. In some embodiments, the second complementary strands are removed (eluted) from the surface, after which the second complementary strands are subjected to PCR for amplification. In some embodiments, for on surface generation of second complementary strands an Exclusion Amplification (ExAmp) mix comprising an adapter-index oligonucleotide is contacted with the spatially barcoded first strand cDNA on the surface, thereby generating second complementary strands via strand invasion and isothermal amplification. In various embodiments, the ExAmp mix further

comprises a recombinase, a single-strand DNA binding protein (*e.g.*, gp32 ssDNA binding protein), and a polymerase. As with any of the methods of generating second complementary strands described herein, generation of the second complementary strands may subsequently be followed by amplification of the second complementary strands (*e.g.*, by indexed PCR), during which a second clustering primer sequence (*e.g.*, P5) may be added to one or more of the second complementary strands. In some embodiments, the amplifying comprises index PCR during which a first primer (*e.g.*, Adp1') hybridizes to the first clustering primer sequence (*e.g.*, Adp1) and a second primer (*e.g.*, Adp2) hybridizes to the adapter nucleotide sequence (*e.g.* Adp2'). In various embodiments, an indexing sequence (*e.g.*, i5) is also added to one or more of the second complementary strands during amplification. In some embodiments, the second primer further comprises the indexing sequence. Addition of the second clustering primer sequence and optionally the indexing sequence to the one or more of the second complementary strands occurs, in various embodiments, off of the surface (*e.g.*, in solution). Amplification of the second complementary strands may subsequently be followed by sequencing. The sequencing of tagged fragments is, in various aspects, performed on a fresh substrate (*e.g.*, flow cell). The sequencing information may subsequently be correlated with a spatial location of the target nucleic acids in the biological sample.

[0083] In some embodiments, for off surface generation of second complementary strands, formation of the spatially barcoded first strand cDNA is followed by cleavage of the first complementary strands from the surface, and second complementary strand synthesis is performed off of the surface (*e.g.*, in solution). See, *e.g.*, Figure 4A. In some embodiments, the second complementary strands are then amplified (*e.g.*, via indexed PCR), during which a second clustering primer sequence (*e.g.*, P5) may be added to one or more of the second complementary strands. In various embodiments, an indexing sequence (*e.g.*, i5) is also added to one or more of the second complementary strands during amplification. Addition of the second clustering primer sequence and optionally the indexing sequence to the one or more of the second complementary strands occurs, in various embodiments, off of the surface (*e.g.*, in solution). The amplification of the second complementary strands may subsequently be followed by sequencing. The sequencing of tagged fragments is, in various aspects, performed on a fresh substrate (*e.g.*, flow cell). The sequencing information may subsequently be correlated with a spatial location of the target nucleic acids in the biological sample.

[0084] In various embodiments, a primer (*e.g.*, an oligonucleotide primer that is hybridized to the spatially barcoded first strand cDNA and then extended) used in a method of the disclosure is used at a concentration in the range of 0.1 μ M to 100 μ M, 1 μ M to 100 μ M or 3

μM to $75 \mu\text{M}$ or 5 to $50 \mu\text{M}$. In some embodiments, the primer is used at a concentration of $0.25 \mu\text{M}$, $0.5 \mu\text{M}$ or $1.1 \mu\text{M}$ or $2.2 \mu\text{M}$. In still further embodiments, the primer is used at a concentration of $1 \mu\text{M}$, $5 \mu\text{M}$, $10 \mu\text{M}$, $25 \mu\text{M}$ or $50 \mu\text{M}$. In various embodiments, such a primer is a P5 primer, a P5' primer, a P7 primer, or a P7' primer.

[0085] Generating oligonucleotide clusters on a surface. The disclosure provides methods for generating a surface comprising a plurality of capture oligonucleotides and a plurality of spatially barcoded oligonucleotides (see, *e.g.*, Figures 2 and 10). In various embodiments, the plurality of spatially barcoded oligonucleotides is present on the surface in clusters. In any of the aspects or embodiments of the disclosure, a spatially barcoded oligonucleotide comprises (i) a SMI (*e.g.*, a UMI) that is unique to each strand in a cluster; and (ii) a spatial barcode that is unique to each cluster. In some embodiments, a spatially barcoded oligonucleotide comprises a spatial barcode that is unique to each cluster but does not include a SMI. In various embodiments, the plurality of capture oligonucleotides and the plurality of spatially barcoded oligonucleotides are present on the surface in a ratio. In various embodiments, the ratio of capture oligonucleotides to spatially barcoded oligonucleotides on the surface is greater than 1:1. In other words, there is one capture oligonucleotide on the surface for every spatially barcoded oligonucleotide. In further embodiments, the ratio of capture oligonucleotides to spatially barcoded oligonucleotides on the surface is from about 1:1 to about 10:1, or from about 1:1 to about 9:1, or from about 1:1 to about 8:1, or from about 1:1 to about 7:1, or from about 1:1 to about 6:1, or from about 1:1 to about 5:1, or from about 1:1 to about 4:1, or from about 1:1 to about 3:1, or from about 1:1 to about 2:1. In still further embodiments, the ratio of capture oligonucleotides to spatially barcoded oligonucleotides on the surface is or is at least about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1. In still further embodiments, the ratio of spatially barcoded oligonucleotides to capture oligonucleotides on the surface is from about 1:1 to about 10:1, or from about 1:1 to about 9:1, or from about 1:1 to about 8:1, or from about 1:1 to about 7:1, or from about 1:1 to about 6:1, or from about 1:1 to about 5:1, or from about 1:1 to about 4:1, or from about 1:1 to about 3:1, or from about 1:1 to about 2:1. In further embodiments, the ratio of spatially barcoded oligonucleotides to capture oligonucleotides on the surface is or is at least about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1.

[0086] In some aspects, the disclosure provides a method of preparing a spatial array, comprising (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence (Figure 11A); (b) providing a first plurality of template oligonucleotides,

wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence (Figure 11A, top left); (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) optionally releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) releasing the plurality of first surface oligonucleotides from the surface; (i) cleaving the cleavage site of the cluster of second surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of second surface oligonucleotides, thereby preparing the spatial array. In some aspects, the disclosure provides a method of preparing a spatial array, comprising (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence (Figure 11A); (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence (Figure 11A, top left); (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first

Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) releasing the plurality of first surface oligonucleotides from the surface; (i) cleaving the cleavage site of the cluster of second surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of second surface oligonucleotides, thereby preparing the spatial array. In various embodiments, steps (f) and (g) are repeated 0, 1, 2, 3, 4, 5, or more times. In various embodiments, the plurality of first surface oligonucleotides comprise one or more cleavage sites that facilitate release of the plurality of first surface oligonucleotides from the surface. In various embodiments, each of the plurality of first surface oligonucleotides comprises about 1 to about 3 cleavage sites. In further embodiments, each of the plurality of first surface oligonucleotides comprises about 1, about 2, or about 3 cleavage sites. In various embodiments, and without limitation, the cleavage site is uracil (cleaved by USER enzyme mix), 8-oxo-G (cleaved by FPG), a restriction enzyme site, or through chemical cleavage. In some embodiments, a method of the disclosure further comprises (j) providing a second plurality of template oligonucleotides, wherein one or more of the second plurality of template oligonucleotides comprises, from 5' to 3': the second adapter (Adp2) sequence, a second spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to and capable of hybridizing to an Adp1 sequence; (k) hybridizing the Adp1' sequence of one or more of the second plurality of template oligonucleotides to a second Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (l) extending one or more of the second Adp1 sequence using the one or more of the second plurality of template oligonucleotides as a template to generate a plurality of third surface oligonucleotides each third surface oligonucleotide comprising: the second Adp1 sequence that is immobilized on the surface, the sequence that is complementary to the cleavage site,

the sequence that is complementary to the TSO sequence, a sequence that is complementary to the second spatial barcode, and the Adp2' sequence that is complementary to the Adp2 sequence; (m) releasing the one or more of the second plurality of template oligonucleotides from the plurality of third surface oligonucleotides; (n) hybridizing the Adp2' sequence of one or more of the plurality of third surface oligonucleotides to a second Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (o) extending the second Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of fourth surface oligonucleotides comprising: the second Adp2 sequence immobilized on the surface, the second spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to the first adapter (Adp1) sequence; (p) releasing the plurality of third surface oligonucleotides from the surface; (q) cleaving the cleavage site of the cluster of fourth surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of fourth surface oligonucleotides. In further embodiments, a method of the disclosure further comprises (r) providing a third plurality of template oligonucleotides, wherein one or more of the third plurality of template oligonucleotides comprises, from 5' to 3': the second adapter (Adp2) sequence, a third spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to and capable of hybridizing to an Adp1 sequence; (s) hybridizing the Adp1' sequence of one or more of the third plurality of template oligonucleotides to a third Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (t) extending one or more of the third Adp1 sequence using the one or more of the third plurality of template oligonucleotides as a template to generate a plurality of fifth surface oligonucleotides each fifth surface oligonucleotide comprising: the third Adp1 sequence that is immobilized on the surface, the sequence that is complementary to the cleavage site, the sequence that is complementary to the TSO sequence, a sequence that is complementary to the third spatial barcode, and the Adp2' sequence that is complementary to the Adp2 sequence; (u) releasing the one or more of the third plurality of template oligonucleotides from the plurality of fifth surface oligonucleotides; (v) hybridizing the Adp2' sequence of one or more of the plurality of fifth surface oligonucleotides to a third Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (w) extending the third Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of sixth surface oligonucleotides comprising: the third Adp2 sequence immobilized on the surface, the third spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to the first adapter (Adp1) sequence; (x) releasing the plurality of fifth surface oligonucleotides from the surface; (y)

cleaving the cleavage site of the cluster of sixth surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of sixth surface oligonucleotides. See, *e.g.*, Figures 11A and 11B. In various embodiments, one or more of the first plurality of template oligonucleotides, one or more of the second plurality of template oligonucleotides, and/or one or more of the third plurality of template oligonucleotides comprises a sequencing primer domain. In further embodiments, the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence. See, *e.g.*, Figure 11A.

[0087] Next, and in some embodiments, a plurality of capture oligonucleotides is immobilized on the surface, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample. The disclosure also contemplates, in various embodiments, that a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample, is immobilized on the surface at the same time that the plurality of first adapter oligonucleotides and the plurality of second adapter oligonucleotides are immobilized on the surface. See, *e.g.*, Figure 11C. In further embodiments, a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample, is immobilized on the surface after the spatial barcode(s) are decoded. In various embodiments, the first adapter (Adp1) sequence may comprise any primer binding site. In some embodiments, the plurality of capture oligonucleotides is immobilized on the surface through a spacer. In further embodiments, each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence. In some embodiments, the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences. In further embodiments, the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof. In still further embodiments, the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence. In various embodiments, the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence. At this point in a method of the disclosure (depicted, *e.g.*, on the right side of Figure 11B), the disclosure provides further embodiments in which SMIs (*e.g.*, UMIs) are added to the spatially barcoded oligonucleotides. An example of such an embodiment is depicted in Figure 11D. Figure

11D shows a variation of the method in which the first plurality of template oligonucleotides is modified relative to the template oligonucleotide shown in Figure 11A. Here, the modified template oligonucleotide comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to a first adapter sequence immobilized on a surface (*i.e.*, in various embodiments the modified template oligonucleotide does not comprise a TSO). The method proceeds as depicted in Figures 11A and 11B and described hereinabove, ultimately resulting in clusters of spatially barcoded oligonucleotides and capture oligonucleotides immobilized on the surface (as depicted in Figure 11D, bottom left). Next, an 'extension oligonucleotide' comprising a 'hyb' sequence, a SMI and a TSO sequence is ligated (*e.g.*, via hybridization of a splint oligonucleotide to both the spatially barcoded oligonucleotide and the 'hyb' sequence of the extension oligonucleotide, followed by ligation) to a spatially barcoded oligonucleotide, as shown in Figure 11D. In some embodiments, the modified template oligonucleotide comprises a sequencing primer domain. In further embodiments, the sequencing primer domain is situated between the first spatial barcode and the cleavage site. In some embodiments, addition of a SMI is accomplished by hybridizing a template oligonucleotide comprising: a nucleotide sequence complementary to the SMI and a nucleotide sequence complementary to the TSO (*e.g.*, SMI'-TSO') to the spatially barcoded oligonucleotide and then copying over the reverse complement of the UMI and TSO sequences using a polymerase.

[0088] The disclosure also contemplates aspects in which unique dual index (UDI) pairs are incorporated into surface oligonucleotides (*e.g.*, capture oligonucleotides and spatially barcoded oligonucleotides). UDIs are also described in International Publication No. WO/2019/090251, which is incorporated herein by reference in its entirety. In various embodiments, UDIs comprise a first unique dual index (UDI1) sequence and a second unique dual index (UDI2) sequence. For example, Figure 11E depicts a method of generating a surface with capture oligonucleotides and spatially barcoded oligonucleotides in which a single template oligonucleotide (shown at top of Figure 11E) comprising Adp1/Adp2 (*e.g.*, P5/P7) ends is added to the surface and bridge-amplified. Next, the single template oligonucleotide is cleaved (*e.g.*, restriction digested) and then dehybridized, thereby resulting in the capture oligonucleotides and spatially barcoded oligonucleotides being generated on the surface. Accordingly, in some aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter

oligonucleotides comprises a second adapter (Adp2) sequence; (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first unique dual index (UDI1) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, a sequence that is complementary to and capable of hybridizing to a capture nucleotide sequence, a second UDI (UDI2) sequence, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, the UDI1 sequence, the capture nucleotide sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, the UDI2 sequence, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) optionally releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the UDI1 sequence, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, the sequence that is complementary to the capture nucleotide sequence, the UDI2 sequence, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) cleaving the cleavage site of the cluster of second surface oligonucleotides, thereby preparing the spatial array. See, *e.g.*, Figure 11E. In further aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first unique dual index (UDI1) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, a sequence that is

complementary to and capable of hybridizing to a capture nucleotide sequence, a second UDI (UDI2) sequence, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface; (d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, the UDI1 sequence, the capture nucleotide sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, the UDI2 sequence, and an Adp2' sequence that is complementary to the Adp2 sequence; (e) releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides; (f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface; (g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the UDI1 sequence, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, the sequence that is complementary to the capture nucleotide sequence, the UDI2 sequence, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence; (h) cleaving the cleavage site of the cluster of second surface oligonucleotides, thereby preparing the spatial array. See, e.g., Figure 11E. In some variations, and as depicted in Figure 11E, a restriction enzyme cleaves the cleavage site on both the first and second surface oligonucleotides that are hybridized together. The non-covalently bound 3' ends of the molecules are dehybridized and washed away, leaving only the 5' ends. In various embodiments, steps (f) and (g) are repeated 0, 1, 2, 3, 4, 5, or more times. In some embodiments, one or more of the first plurality of template oligonucleotides comprises a sequencing primer domain. In further embodiments, the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

[0089] In additional aspects, the disclosure provides a method of preparing a spatial array, comprising: (a) providing a surface comprising a plurality of first adapter oligonucleotides immobilized on the surface and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the second

adapter oligonucleotides comprises a second adapter (Adp2) sequence; (b) applying a library of template oligonucleotides to one or more regions of the surface, wherein the library comprises at least one first template oligonucleotide comprising, from 5' to 3': the Adp2 sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence; (c) hybridizing the Adp1' sequence of the at least one first template oligonucleotide to the Adp1 sequence of at least one first adapter oligonucleotide and extending the hybridized Adp1 sequence to prepare at least one first double-stranded nucleic acid, wherein each first double-stranded nucleic acid comprises a first extended Adp1 strand immobilized on the surface and a first template strand, wherein the first extended Adp1 strand comprises, from 5' to 3': the Adp1 sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence, and wherein the first template strand comprises, from 5' to 3': the Adp2 sequence, the first spatial barcode, the TSO sequence, the cleavage site, and the Adp1' sequence; (d) hybridizing the Adp1 sequence of at least one first adapter oligonucleotide to the Adp1' sequence of at least one first template strand and extending the hybridized Adp1 sequence to prepare at least one additional first extended Adp1 strand immobilized on the surface; (e) hybridizing the Adp2 sequence of at least one second adapter oligonucleotide to the Adp2' sequence of at least one first extended Adp1 strand immobilized on the surface and extending the hybridized Adp2 sequence to prepare at least one first extended Adp2 strand immobilized on the surface, wherein each first extended Adp2 strand comprises, from 5' to 3': the Adp2 sequence, the first spatial barcode, the TSO sequence, the cleavage site, and the Adp1' sequence; (f) repeating steps (d) and (e) to produce a first cluster comprising a plurality of first extended Adp1 strands immobilized on the surface and a plurality of first extended Adp2 strands immobilized on the surface; (g) releasing the first extended Adp1 strands in the first cluster from the surface; (h) cleaving the cleavage sites of the first extended Adp2 strands in the first cluster to release the cleavage sites and the Adp1' sequences from the surface, thereby preparing the spatial array. In some embodiments, one or more of the first plurality of template oligonucleotides comprises a sequencing primer domain. In further embodiments, the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

[0090] To further illustrate the present disclosure, examples are given herein. It is to be understood that these examples are provided for illustrative purposes and are not to be construed as limiting the scope of the present disclosure.

EXAMPLES**EXAMPLE 1**

[0091] Methods of preparing an immobilized library of target nucleic acids of a biological sample (*e.g.*, tissue sample) are provided herein. The methods of the disclosure advantageously generate spatially barcoded library fragments from the 5' and central regions of a target nucleic acid (*e.g.*, an RNA molecule).

[0092] A schematic workflow of a method of the disclosure is shown in Figure 1. Figure 1 shows a library prep workflow for generating sequencing libraries from the 5' ends of polyA-tailed RNA transcripts (fragmented or full-length). The surface shown in Figure 1A contains two types of oligonucleotides, capture oligonucleotides and spatially barcoded oligonucleotides comprising a template-switching oligonucleotide (TSO) sequence. This TSO sequence can be any sequence, such as the Illumina SBS3 sequence. On a flow cell, the spatially barcoded oligonucleotides can be generated by Bridge Amplification. The capture oligonucleotides may also be directly grafted onto the flow cell and will be present within and between the clusters. Potential oligonucleotide sequences of capture oligonucleotides and spatially barcoded oligonucleotides for use in various embodiments are shown in Figure 2.

[0093] After tissue permeabilization, RNA diffuses down to the surface and hybridizes to the capture oligonucleotides through its polyA tail. Reverse transcription (RT) is performed using an RT enzyme with terminal transferase activity (*e.g.*, Maxima H- or Superscript IV) and in the presence of a TSO. The TSO can then hybridize to the untemplated nucleotides at the end of the cDNA molecule, and the RT enzyme will add the complement of the TSO sequence to the end of the cDNA molecule (Figure 1A).

[0094] Template switching is more efficient when the 5' ends of the RNA are capped. Thus, an m7G capping step may be included prior to reverse transcription to artificially cap the RNA fragments using capping enzymes such as the Faustovirus Capping Enzyme (New England Biolabs) (Figure 3). The presence of the m7G cap improves the efficiency of template switching.

[0095] After reverse transcription, the RNA is denatured or digested away, and the complementary TSO sequence on the cDNA will hybridize to the TSO sequence on the spatially barcoded oligonucleotides. Polymerase extension can then copy the spatially barcoded oligonucleotide sequence onto the cDNA strand, to the region corresponding to the 5' end of the RNA molecule (Figures 1A-1B).

[0096] Library preparation can then be completed. Tissue permeabilization often results in fragmented RNA. For example, a common tissue permeabilization enzyme, pepsin, is active in a low pH solution, and RNA fragments at low pH. With fragmented RNA, library preparation can be completed by generating second-strand cDNA using the Adp2 sequence as a primer, followed by denaturation of the second-strand and PCR amplification (Figure 1C). Alternatively, a cleavage site can be included in the capture oligonucleotides to enable cleavage of the cDNA, with or without second strand synthesis (Figure 4).

[0097] If the RNA transcripts are full-length, tagmentation can be used to simultaneously fragment the cDNA and add the other sequencing adapter closer to the TSO end of the fragment (Figure 5).

[0098] Another schematic workflow of a method of the disclosure is shown in Figure 6. Figure 6 shows a variation that uses ligation to add on the TSO sequence. After permeabilization of the tissue, reverse transcription is performed using a reverse transcriptase with or without terminal transferase activity. RNA is then removed from the surface (Figure 6A). Next, oligonucleotides complementary to the TSO sequence are hybridized to the surface to block the 3' ends of the spatially barcoded oligonucleotides. Then, the TSO' (where TSO' refers to a sequence that is complementary to the TSO sequence) sequence is ligated to the 3' ends of the cDNA molecules using annealed oligonucleotides with a 3' overhang comprising random bases (NNN). After ligation, the hybridized oligonucleotides are removed using alkaline or heat denaturation (Figure 6B). The workflow then proceeds with the steps outlined in Figure 1B. This step can be combined with template switching as described above.

[0099] Another schematic workflow of a method of the disclosure is shown in Figure 7. Figure 7 shows a variation that enables spatial barcoding of fragments within the transcript. Here, the surface contains two types of oligonucleotides, capture oligonucleotides and spatially barcoded oligonucleotides that contain a transposon ME sequence. ME' can be hybridized onto the surface oligos after the clusters have been generated. After template switching, second strand cDNA is generated. Then transposase is added to the surface. The transposase enzyme binds to the ME sequences and tagments the cDNA, covalently attaching the spatial barcode oligonucleotides. After tagmentation, Tn5 is removed with SDS and a 5'→3' exonuclease (*e.g.*, T7 exonuclease) is used to digest away the oligonucleotides that are not attached to the surface. Then sequencing adapters are hybridized and gap-fill ligation is performed to attach them to the 3' ends of the cDNA. The strands are then denatured, and polymerase extension is used to generate a copy that is dehybridized from the surface, followed by PCR amplification of the final library.

[0100] A further schematic workflow of a method of the disclosure is shown in Figure 11. Figure 11 shows a variation for establishing oligonucleotide clusters on a surface. Here, a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides are immobilized on the surface. Next, a plurality of template oligonucleotides comprising a cleavage site is hybridized to the plurality of first adapter oligonucleotides on the surface and the first adapter sequences are extended. These extended products are amplified (*e.g.*, by bridge amplification) and then the cleavage sites are cleaved. A plurality of capture oligonucleotides can then be immobilized on the surface after the second surface oligonucleotides have been amplified. This is shown, for example, in Figure 11B. Alternatively, a plurality of capture oligonucleotides may be immobilized on the surface at the same time as Adp1 and Adp2, as shown in Figure 11C.

[0101] At the point in the method that is shown on the right side of Figure 11B, surface oligonucleotides are prepared but do not yet comprise SMIs (*e.g.*, UMIs). Thus, as described herein, methods of the disclosure may further comprise the ligation of an oligonucleotide comprising a SMI and a TSO sequence (*e.g.*, via hybridization to a splint oligonucleotide) to a surface oligonucleotide, as shown in Figure 11D. In such methods, a plurality of modified template oligonucleotides is utilized as shown in Figure 11D. The modified template oligonucleotides comprise, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence.

[0102] Figure 11E shows a further variation for establishing oligonucleotide clusters on a surface. In particular, Figure 11E depicts the incorporation of unique dual indices (UDIs). Here, UDI pairs are incorporated into the template oligonucleotides as shown in Figure 11E. Following extension of the template oligonucleotides and eventual cleavage at the cleavage sites, the resulting capture oligonucleotides and spatial barcode oligonucleotides in a single cluster comprises paired UDI components.

EXAMPLE 2

[0103] The following example describes and demonstrates the generation of a surface comprising capture oligonucleotides and spatially barcoded oligonucleotides, preparing a library of target nucleic acids of a biological sample on the surface, and then performing spatial transcriptomic analysis on the prepared library.

MATERIALS AND METHODS

Spatial Library Generation

[0104] Fresh frozen sections (10um) from mouse kidney were mounted onto a substrate containing both capture and spatially barcoded oligonucleotides. Tissues were methanol-fixed at -20°C for 30 minutes, after which they were stained with hematoxylin and eosin, and dried. The substrate was then placed in a proprietary device with sealable wells, which allowed heated incubations on a thermal cycler. Each well had an approximate surface area of 28mm² overlying each tissue section, enabling 80uL on-surface reaction volumes. Tissue sections were then permeabilized with a proprietary permeabilization reagent at 37°C for 7 minutes, followed by 3 room temperature washes in 0.1x SSC. Two different library preps (LP) were then performed as follows:

1. Template-switch (TSO), in which samples (permeabilized tissues) were incubated overnight at 42°C in first strand cDNA synthesis mix with a Rd1-containing template switch oligonucleotide (5' CCTACACGACGCTCTCCGATCTrGrGrG 3' (SEQ ID NO: 19); e.g., Figure 1A-B);
2. Single-stranded ligation (LIG), in which samples (permeabilized tissues) were incubated overnight at 42°C in first strand cDNA synthesis mix without a Rd1-containing template switch oligonucleotide (Figures 6A-B).

[0105] Following first strand cDNA synthesis, samples underwent a tissue removal step in a proprietary tissue removal mix at 37°C for 40 minutes, an RNA removal step comprising three 5-minute room temperature incubations in a proprietary RNA removal solution followed by one room temperature wash in spatial wash buffer.

[0106] For samples undergoing ligation, a proprietary ligation blocking mix was added and incubated for 10 minutes at 40°C, followed by one room temperature wash in spatial wash buffer. A proprietary ligation mix (with a Rd1-containing splinted adapter; Splint: 5' /5AmMC12/TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNN/3AmMO/ 3' (SEQ ID NO: 20) (5AmMC12 = 5'-Amino-modifier C12 (12-(4-Monomethoxytritylamino)dodecyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite; 3AmMO = a 3' amino modifier)); Donor: 5' /5Phos/CTGTCTCTTATACACATCTGACGCTGCCGACGA/3ddC 3' (SEQ ID NO: 21) (5Phos = 5' phosphorylation; 3ddC = 3' dideoxycytidine)) was then added and incubated for 2 hours at 37°C, followed by one room temperature wash in spatial wash buffer. This was followed by a splint removal step comprising three 5-minute room temperature incubations in a proprietary splint removal solution and one room temperature wash in spatial wash buffer.

[0107] For both LPs, a hybridization solution was added, which either contained (LIG LP) or did not contain (TSO-LP) a capture strand blocking reagent, for 10 minutes at 40°C, followed by one room temperature wash in spatial wash buffer. Thereafter a proprietary

extension mix (Figure 1B) was added and the mixture was incubated for 15 minutes at 65°C. After one room temperature wash in spatial wash buffer, the first complementary strands were cleaved off the surface, in a 50uL volume, using a cleavage method as described herein (Figure 4A-B). The second complementary strands were eluted into a PCR tube via two 5-minute room temperature incubations using 22uL of a proprietary elution solution (Figure 1C), followed by the addition of 6uL of a proprietary neutralizing solution. The first and second complementary strands were combined, after which a 1.5x SPRI purification was performed. A proprietary indexed primer was appended to 10% of the eluted, purified cDNA in a 50uL PCR reaction (98°C for 45 seconds, 11 cycles at 95°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute and a final incubation at 72°C for 2 minutes) using an Equinox-based PCR mix. A 0.7x SPRI purification step was used to clean up the PCR reaction. Libraries were sequenced on NovaSeq 6000 S4 flowcells. Each sample received approximately 300 million raw reads after which median UMI counts per 10um x 10um area were extracted using proprietary spatial software.

Results

[0108] Spatial transcriptional profiling data for both TSO- and LIG- LPs are shown in Figures 13A, 13B and 13C, 13D, respectively. Examples are shown for *Kidney androgen regulated protein (Kap)* in Figures 13A and 13C and *Uromodulin (Umod)* in Figures 13B and 13D, respectively. *Kap* expression is predominantly confined to proximal convoluted tubules of the cortex, whereas *Umod* expression is restricted largely to the thick ascending Loop of Henle of the outer medulla. Also shown is the relative transcript abundance, with *Kap* (Figures 13A, 13C) more highly expressed than *Umod* (Figures 13B, 13D) in the mouse kidney for both TSO- and LIG- LPs. Thus, this example demonstrates that both TSO- and LIG- spatial LP methods recapitulate known mouse kidney biology.

WHAT IS CLAIMED IS:

1. A method of preparing an immobilized library of target nucleic acids of a biological sample, comprising:
 - (a) providing a surface comprising:
 - (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and
 - (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, and a template switching oligonucleotide (TSO) sequence;
 - (b) contacting the biological sample with the surface, the contacting resulting in hybridization of the target nucleic acids of the biological sample to the capture nucleotide sequences of the plurality of capture oligonucleotides to form hybridized capture oligonucleotides;
 - (c) extending the capture nucleotide sequence of the hybridized capture oligonucleotides to form first complementary strands of the target nucleic acids, wherein the extending comprises addition of a plurality of non-templated nucleotides to the end of the first complementary strands;
 - (d) hybridizing a plurality of template switching oligonucleotides to the plurality of non-templated nucleotides of the first complementary strands such that each of the plurality of template switching oligonucleotides that is hybridized to the plurality of non-templated nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal to the surface;
 - (e) extending the plurality of non-templated nucleotides on the first complementary strands using the template switching oligonucleotides as template, thereby generating a complementary template-switching oligonucleotide (TSO') sequence on the first complementary strands;
 - (f) hybridizing the complementary template-switching oligonucleotide (TSO') sequence on one or more of the first complementary strands to the template switching oligonucleotide (TSO) sequence of one or more of the plurality of spatially barcoded oligonucleotides, and extending the template-switching oligonucleotide binding site on the one or more of the first complementary strands using the one or more of the plurality of

spatially barcoded oligonucleotides as template, thereby generating a spatially barcoded first strand cDNA comprising a sequence complementary to the spatial barcode (SBC') and a sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands,

thereby preparing the immobilized library of target nucleic acids.

2. The method of claim 1, further comprising:

(g) extending an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby generating one or more second complementary strands.

3. The method of claim 1, further comprising:

(g) extending an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby generating one or more second complementary strands; and

(h) amplifying the one or more first complementary strands and/or the one or more second complementary strands, thereby generating a plurality of hybridized first complementary strands and second complementary strands, or portions thereof.

4. The method of claim 1, further comprising:

(g) extending an Adp2 primer that is hybridized to the sequence complementary to the second adapter (Adp2') sequence on the one or more of the first complementary strands, thereby generating one or more second complementary strands;

(h) amplifying the one or more first complementary strands and/or the one or more second complementary strands, thereby generating a plurality of hybridized first complementary strands and second complementary strands, or portions thereof; and

(i) performing tagmentation on the plurality of hybridized first complementary strands and second complementary strands, or portions thereof, to prepare a plurality of tagged fragments.

5. The method of claim 3 or claim 4, wherein the one or more second complementary strands are amplified in step (h) using a plurality of first primers that hybridize to the Adp2 sequence or the Adp2' sequence and a plurality of second primers that hybridize to the Adp1 sequence or the Adp1' sequence.

6. The method of claim 3 or claim 4, wherein the first complementary strands and/or the second complementary strands are amplified in step (h) using a plurality of first

primers that hybridize to the Adp2 sequence or the Adp2' sequence and a plurality of random primers comprising a random sequence.

7. The method of any one of claims 3-6, further comprising removing the one or more second complementary strands from the surface after step (g) and prior to step (h).

8. The method of any one of claims 3-6, further comprising removing the one or more first complementary strands and the one or more second complementary strands from the surface after step (g) and prior to step (h).

9. The method of any one of claims 1-8, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a single molecule identifier (SMI).

10. The method of claim 9, wherein the SMI is a unique molecular identifier (UMI).

11. The method of claim 9 or claim 10, wherein extending the template-switching oligonucleotide binding site on the one or more of the first complementary strands using the one or more of the plurality of spatially barcoded oligonucleotides as template in step (f) generates a sequence complementary to the SMI (SMI').

12. The method of any one of claims 1-11, further comprising removing the target nucleic acids from the surface after step (f).

13. The method of claim 12, wherein removing the target nucleic acids from the surface comprises denaturing the target nucleic acids.

14. The method of claim 12, wherein removing the target nucleic acids from the surface comprises digesting the target nucleic acids.

15. The method of any one of claims 1-14, further comprising removing the biological sample from the surface after step (c).

16. The method of any one of claims 1-14, further comprising removing the biological sample from the surface after step (e).

17. The method of any one of claims 1-16, wherein each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence.

18. The method of any one of claims 1-16, wherein the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences.

19. The method of claim 1-18, wherein the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof.

20. The method of any one of claims 1-18, wherein the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence.

21. The method of claim 19 or claim 20, wherein the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence.

22. The method of any one of claims 1-21, wherein the target nucleic acids are mRNA, gDNA, rRNA, tRNA, or a combination thereof.

23. The method of any one of claims 1-21, wherein the target nucleic acids are RNA, mRNA, or a combination thereof.

24. The method of any one of claims 1-23, wherein the extending of the capture nucleotide sequence in step (c) is carried out using a reverse transcriptase.

25. The method of any one of claims 1-24, wherein the target nucleic acids are polyadenylated prior to hybridization of the target nucleic acids to the capture nucleotide sequences.

26. The method of claim 25, wherein the target nucleic acids are polyadenylated using a poly(A) polymerase.

27. The method of claim 25, wherein the target nucleic acids are polyadenylated using chemical ligation or enzymatic ligation.

28. The method of any one of claims 3-27, wherein the one or more second complementary strands are amplified in step (h) by strand invasion of the one or more spatially barcoded oligonucleotides in the presence of recombinase.

29. The method of any one of claims 1-28, wherein prior to step (c) a capping step is performed to add a cap to the target nucleic acids.

30. The method of claim 29, wherein the cap is a m⁷G cap.

31. The method of claim 29 or claim 30, wherein the capping step is performed by a capping enzyme.

32. The method of claim 31, wherein the capping enzyme is a Faustovirus capping enzyme (FCE).

33. The method of any one of claims 1-32, wherein the first adapter (Adp1) sequence is immobilized on the surface through a cleavage site.

34. The method of any one of claims 2-32, wherein the first adapter (Adp1) sequence is immobilized on the surface through a cleavage site.

35. The method of claim 33 or claim 34, wherein the cleavage site is an enzymatic cleavage site.
36. The method of claim 35, wherein the enzymatic cleavage site comprises a restriction enzyme site, a uracil, an 8-oxoguanine, or a combination thereof.
37. The method of claim 33 or claim 34, wherein the cleavage site is a chemical cleavage site.
38. The method of any one of claims 33-36, wherein the cleavage site is cleaved after step (f).
39. The method of any one of claims 34-36, wherein the cleavage site is cleaved after step (g).
40. The method of any one of claims 7-39, wherein the one or more second complementary strands is removed by heat or alkaline denaturation.
41. The method of any one of claims 4-40, further comprising sequencing the plurality of tagged fragments to determine a sequence of the plurality of tagged fragments.
42. The method of claim 41, further comprising correlating the sequence of the plurality of tagged fragments to a position of the target nucleic acids in the biological sample.
43. A method of preparing an immobilized library of target nucleic acids of a biological sample, comprising:
- (a) providing a surface comprising:
 - (i) a plurality of capture oligonucleotides, wherein one or more of the plurality of capture oligonucleotides comprises a first adapter (Adp1) sequence that is immobilized on the surface and a capture nucleotide sequence that is configured to bind to the target nucleic acids of the biological sample; and
 - (ii) a plurality of spatially barcoded oligonucleotides, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a second adapter (Adp2) sequence that is immobilized on the surface, a spatial barcode, and a mosaic end (ME) transposase recognition sequence;
 - (b) contacting the biological sample with the surface, the contacting resulting in hybridization of the target nucleic acids of the biological sample to the capture nucleotide sequences of the plurality of capture oligonucleotides to form hybridized capture oligonucleotides;

(c) extending the capture nucleotide sequence of the hybridized capture oligonucleotides to form first complementary strands of the target nucleic acids, wherein the extending comprises addition of a plurality of non-templated nucleotides to the end of the first complementary strands;

(d) hybridizing a plurality of template switching oligonucleotides (TSOs) to the plurality of non-templated nucleotides of the first complementary strands such that each of the plurality of template switching oligonucleotides (TSOs) that is hybridized to the plurality of non-templated nucleotides of the first complementary strands is positioned at the terminus of the target nucleic acids that is distal to the surface;

(e) extending the plurality of non-templated nucleotides on the first complementary strands using the template switching oligonucleotides (TSOs) as template, thereby generating a complementary template-switching oligonucleotide sequence (TSO') on the first complementary strands;

(f) removing the target nucleic acids from the surface;

(g) hybridizing an additional template-switching oligonucleotide (TSO) primer to the complementary template switching oligonucleotide sequence of one or more of the first complementary strands to generate a hybridized TSO primer, and extending the hybridized TSO primer to generate one or more second complementary strands, thereby generating a plurality of clusters comprising hybridized first complementary strands and second complementary strands;

(h) hybridizing a plurality of oligonucleotides comprising a sequence complementary to the ME sequence (ME') to the ME transposase recognition sequence of one or more of the plurality of spatially barcoded oligonucleotides;

(i) performing tagmentation on the plurality of clusters comprising hybridized first complementary strands and second complementary strands to prepare a plurality of tagged fragments,

(j) adding an enzyme having 5' to 3' exonuclease activity;

(k) hybridizing a plurality of oligonucleotides comprising a sequence complementary to the ME sequence (ME') and a sequence complementary to a third adapter (Adp3') sequence to the ME transposase recognition sequence of one or more of the plurality of spatially barcoded oligonucleotides and performing gap-fill ligation, thereby attaching the ME' and Adp3' sequences to the 3' end of one or more of the first complementary strands and the 3' end of one or more of the second complementary strands;

(l) subjecting the surface to denaturing conditions to form one or more single-stranded first complementary strands and one or more single-stranded second complementary strands; hybridizing a plurality of primers comprising the third sequencing adapter (Adp3) sequence to the Adp3' sequence attached to the 3' ends of one or more of the single-stranded first complementary strands and one or more of the single-stranded second complementary strands; and extending the primers to form a plurality of clusters comprising Adp3'-tagged immobilized strands hybridized to Adp3-tagged non-immobilized strands,

thereby preparing the immobilized library of target nucleic acids.

44. The method of claim 43, further comprising the step of dehybridizing the Adp3-tagged non-immobilized strands and amplifying the dehybridized Adp3-tagged non-immobilized strands using a plurality of first primers comprising the Adp2 sequence or the Adp2' sequence and a plurality of second primers comprising the Adp3 sequence or the Adp3' sequence.

45. The method of claim 43, wherein one or more of the plurality of spatially barcoded oligonucleotides comprises a single molecule identifier (SMI).

46. The method of claim 45, wherein the SMI is a unique molecular identifier (UMI).

47. The method of any one of claims 43-46, wherein removing the target nucleic acids from the surface comprises denaturing the target nucleic acids.

48. The method of claim 47, wherein removing the target nucleic acids from the surface comprises digesting the target nucleic acids.

49. The method of any one of claims 43-48, further comprising removing the biological sample from the surface after step (c).

50. The method of any one of claims 43-48, further comprising removing the biological sample from the surface after step (e).

51. The method of any one of claims 43-50, wherein each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence.

52. The method of any one of claims 43-50, wherein the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences.

53. The method of claim 52, wherein the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof.

54. The method of any one of claims 43-52, wherein the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence.

55. The method of claim 53 or claim 54, wherein the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence.

56. The method of any one of claims 43-55, wherein the target nucleic acids are mRNA, gDNA, rRNA, tRNA, or a combination thereof.

57. The method of any one of claims 43-55, wherein the target nucleic acids are RNA, mRNA, or a combination thereof.

58. The method of any one of claims 43-57, wherein the extending of the capture nucleotide sequence in step (c) is carried out using a reverse transcriptase.

59. The method of any one of claims 43-58, wherein the target nucleic acids are polyadenylated prior to hybridization of the target nucleic acids to the capture nucleotide sequences.

60. The method of claim 59, wherein the target nucleic acids are polyadenylated using a poly(A) polymerase.

61. The method of claim 59, wherein the target nucleic acids are polyadenylated using chemical ligation or enzymatic ligation.

62. The method of any one of claims 43-61, further comprising sequencing the plurality of tagged fragments to determine a sequence of the plurality of tagged fragments.

63. The method of claim 62, further comprising correlating the sequence of the plurality of tagged fragments to a position of the target nucleic acids in the biological sample.

64. A method of preparing a spatial array, comprising:

(a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence;

(b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence;

(c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface;

(d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence;

(e) optionally releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides;

(f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface;

(g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence;

(h) releasing the plurality of first surface oligonucleotides from the surface;

(i) cleaving the cleavage site of the cluster of second surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of second surface oligonucleotides,

thereby preparing the spatial array.

65. The method of claim 64, further comprising:

(j) providing a second plurality of template oligonucleotides, wherein one or more of the second plurality of template oligonucleotides comprises, from 5' to 3': the second adapter (Adp2) sequence, a second spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to and capable of hybridizing to an Adp1 sequence;

(k) hybridizing the Adp1' sequence of one or more of the second plurality of template oligonucleotides to a second Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface;

(l) extending one or more of the second Adp1 sequence using the one or more of the second plurality of template oligonucleotides as a template to generate a plurality of third surface oligonucleotides each third surface oligonucleotide comprising: the second Adp1 sequence that is immobilized on the surface, the sequence that is complementary to the cleavage site, the sequence that is complementary to the TSO sequence, a sequence that is complementary to the second spatial barcode, and the Adp2' sequence that is complementary to the Adp2 sequence;

(m) releasing the one or more of the second plurality of template oligonucleotides from the plurality of third surface oligonucleotides;

(n) hybridizing the Adp2' sequence of one or more of the plurality of third surface oligonucleotides to a second Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface;

(o) extending the second Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of fourth surface oligonucleotides comprising: the second Adp2 sequence immobilized on the surface, the second spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to the first adapter (Adp1) sequence;

(p) releasing the plurality of third surface oligonucleotides from the surface;

(q) cleaving the cleavage site of the cluster of fourth surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of fourth surface oligonucleotides.

66. The method of claim 65, further comprising:

(r) providing a third plurality of template oligonucleotides, wherein one or more of the third plurality of template oligonucleotides comprises, from 5' to 3': the second adapter (Adp2) sequence, a third spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to and capable of hybridizing to an Adp1 sequence;

(s) hybridizing the Adp1' sequence of one or more of the third plurality of template oligonucleotides to a third Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface;

(t) extending one or more of the third Adp1 sequence using the one or more of the third plurality template oligonucleotides as a template to generate a plurality of fifth surface oligonucleotides each fifth surface oligonucleotide comprising: the third Adp1 sequence that is immobilized on the surface, the sequence that is complementary to the cleavage site, the sequence that is complementary to the TSO sequence, a sequence that is complementary to the third spatial barcode, and the Adp2' sequence that is complementary to the Adp2 sequence;

(u) releasing the one or more of the third plurality of template oligonucleotides from the plurality of fifth surface oligonucleotides;

(v) hybridizing the Adp2' sequence of one or more of the plurality of fifth surface oligonucleotides to a third Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface;

(w) extending the third Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of sixth surface oligonucleotides comprising: the third Adp2 sequence immobilized on the surface, the third spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, and the Adp1' sequence that is complementary to the first adapter (Adp1) sequence;

(x) releasing the plurality of fifth surface oligonucleotides from the surface;

(y) cleaving the cleavage site of the cluster of sixth surface oligonucleotides to release the cleavage site and the Adp1' sequence from the cluster of sixth surface oligonucleotides.

67. The method of claim 66, further comprising immobilizing a plurality of capture oligonucleotides on the surface, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample.

68. The method of any one of claims 64-66, wherein the surface of (a) further comprises a plurality of capture oligonucleotides on the surface, wherein one or more of the plurality of capture oligonucleotides comprises from 5' to 3': a first adapter (Adp1) sequence and a capture nucleotide sequence that is configured to bind to a target nucleic acid of a biological sample.

69. The method of claim 67 or claim 68, wherein the plurality of capture oligonucleotides is immobilized on the surface through a spacer.

70. The method of any one of claims 67-69, wherein each of the plurality of capture oligonucleotides comprises the same capture nucleotide sequence.

71. The method of any one of claims 67-69, wherein the plurality of capture oligonucleotides comprises multiple, different capture nucleotide sequences.

72. The method of claim 71, wherein the multiple, different capture nucleotide sequences comprise one or more gene-specific capture sequences, one or more universal capture sequences, or a combination thereof.

73. The method of any one of claims 67-71, wherein the capture nucleotide sequence is a poly-T sequence, a poly-A sequence, a gene-specific capture sequence, or a universal capture sequence.

74. The method of claim 72 or claim 73, wherein the universal capture sequence is a random nucleotide sequence or a non-self complementary semi-random sequence.

75. The method of any one of claims 64-74, wherein one or more of the first plurality of template oligonucleotides, one or more of the second plurality of template oligonucleotides, and/or one or more of the third plurality of template oligonucleotides comprises a sequencing primer domain.

76. The method of claim 75, wherein the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

77. A method of preparing a spatial array, comprising:

(a) providing a surface comprising a plurality of first adapter oligonucleotides and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the plurality of first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the plurality of second adapter oligonucleotides comprises a second adapter (Adp2) sequence;

(b) providing a first plurality of template oligonucleotides, wherein one or more of the first plurality of template oligonucleotides comprises, from 5' to 3': a second adapter (Adp2) sequence, a first unique dual index (UDI1) sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, a sequence that is complementary to and capable of hybridizing to a capture nucleotide sequence, a second UDI (UDI2) sequence, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence;

(c) hybridizing the Adp1' sequence of one or more of the first plurality of template oligonucleotides to a first Adp1 sequence of the plurality of first adapter oligonucleotides immobilized on the surface;

(d) extending one or more of the first Adp1 sequence using the one or more of the first plurality of template oligonucleotides as a template to generate a plurality of first surface oligonucleotides, each first surface oligonucleotide comprising: the first Adp1 sequence that is immobilized on the surface, the UDI1 sequence, the capture nucleotide sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, the UDI2 sequence, and an Adp2' sequence that is complementary to the Adp2 sequence;

(e) optionally releasing the one or more of the first plurality of template oligonucleotides from the plurality of first surface oligonucleotides;

(f) hybridizing the Adp2' sequence of one or more of the plurality of first surface oligonucleotides to a first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface;

(g) extending the first Adp2 sequence of the plurality of second adapter oligonucleotides immobilized on the surface to generate a cluster of second surface oligonucleotides comprising: the first Adp2 sequence immobilized on the surface, the UDI1 sequence, the first spatial barcode, the template switching oligonucleotide (TSO) sequence, the cleavage site, the sequence that is complementary to the capture nucleotide sequence, the UDI2 sequence, and the Adp1' sequence that is complementary to a first adapter (Adp1) sequence;

(h) cleaving the cleavage site of the cluster of second surface oligonucleotides,

thereby preparing the spatial array.

78. The method of claim 77, wherein one or more of the first plurality of template oligonucleotides comprises a sequencing primer domain.

79. The method of claim 78, wherein the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

80. A method of preparing a spatial array, comprising:

(a) providing a surface comprising a plurality of first adapter oligonucleotides immobilized on the surface and a plurality of second adapter oligonucleotides immobilized on the surface, wherein each of the first adapter oligonucleotides comprises a first adapter (Adp1) sequence, and wherein each of the second adapter oligonucleotides comprises a second adapter (Adp2) sequence;

(b) applying a library of template oligonucleotides to one or more regions of the surface, wherein the library comprises at least one first template oligonucleotide comprising, from 5' to 3': the Adp2 sequence, a first spatial barcode, a template switching oligonucleotide (TSO) sequence, a cleavage site, and a Adp1' sequence that is complementary to and capable of hybridizing to the Adp1 sequence;

(c) hybridizing the Adp1' sequence of the at least one first template oligonucleotide to the Adp1 sequence of at least one first adapter oligonucleotide and extending the hybridized Adp1 sequence to prepare at least one first double-stranded nucleic acid, wherein each first double-stranded nucleic acid comprises a first extended Adp1 strand immobilized on the surface and a first template strand, wherein the first extended Adp1 strand comprises, from 5' to 3': the Adp1 sequence, a sequence that is complementary to the cleavage site, a sequence that is complementary to the TSO sequence, a sequence that is complementary to the first spatial barcode, and an Adp2' sequence that is complementary to the Adp2 sequence, and wherein the first template strand comprises, from 5' to 3': the Adp2 sequence, the first spatial barcode, the TSO sequence, the cleavage site, and the Adp1' sequence;

(d) hybridizing the Adp1 sequence of at least one first adapter oligonucleotide to the Adp1' sequence of at least one first template strand and extending the hybridized Adp1 sequence to prepare at least one additional first extended Adp1 strand immobilized on the surface;

(e) hybridizing the Adp2 sequence of at least one second adapter oligonucleotide to the Adp2' sequence of at least one first extended Adp1 strand immobilized on the surface and extending the hybridized Adp2 sequence to prepare at least one first extended Adp2 strand immobilized on the surface, wherein each first extended Adp2 strand comprises, from 5' to 3': the Adp2 sequence, the first spatial barcode, the TSO sequence, the cleavage site, and the Adp1' sequence;

(f) repeating steps (d) and (e) to produce a first cluster comprising a plurality of first extended Adp1 strands immobilized on the surface and a plurality of first extended Adp2 strands immobilized on the surface;

(g) releasing the first extended Adp1 strands in the first cluster from the surface;

(h) cleaving the cleavage sites of the first extended Adp2 strands in the first cluster to release the cleavage sites and the Adp1' sequences from the surface,

thereby preparing the spatial array.

81. The method of claim 80, wherein one or more of the first plurality of template oligonucleotides comprises a sequencing primer domain.

82. The method of claim 81, wherein the sequencing primer domain is situated between the first spatial barcode and the template switching oligonucleotide (TSO) sequence.

83. The method of any one of claims 64-79, wherein steps (f) and (g) are repeated 0, 1, 2, 3, 4, 5, or more times.