therapeutic implications related to targeting DNA methylation, there remains a need to develop reliable and cost-effective methods to ascertain the methylation status of nucleic acids at spatial locations of a biological sample.

SUMMARY

This disclosure provides methods of spatial analysis with identification of DNA methylation status. The methods provided herein are applicable to normal physiological conditions, development and stem-cell studies, and in pathophysiological settings such as cancer. For example, in one example, the DNA methylation status (and changes of the same) of one or more particular analytes can be examined during therapeutic delivery. And, because the location of the analyte (or complement thereof) can be identified using spatial analysis methods (e.g., RNA-templated ligation) as disclosed herein, the location of the analyte and/or changes to the methylation status can be identified. In some instances, the methods disclosed herein can also be combined with imaging techniques that provide a correlation between a particular location of an image (e.g., location of a tumor in a biological sample) and both gene expression and methylation status at that location. Thus, the disclosure provides powerful methods of identifying the location of methylated DNA in a biological sample.

Accordingly, described herein is a method for identifying methylation status of an analyte in a biological sample, the method comprising: (a) contacting the biological sample with an array comprising a plurality of capture probes, wherein a capture probe in the plurality of capture probes comprises (i) a spatial barcode and (ii) a capture domain comprising at least one methylated cytosine; (b) deaminating the analyte in the biological sample; (c) contacting the analyte with a plurality of probes comprising a first probe and a second probe, wherein the first probe comprises (i) a sequence complementary to at least a first sequence of the analyte and (ii) a sequence complementary to the capture domain; and the second probe comprises a sequence complementary to at least a second sequence of the analyte; (d) ligating the first probe and the second probe, thereby generating a ligation product; (e) hybridizing the ligation product to the capture probe; (f) extending the capture probe using the ligation product as a template; thereby generating an extended capture probe; and (g) determining (i) all or a portion of the sequence of the spatial barcode or the complement thereof, and (ii) all or a portion of the sequence of the extended capture probe, or a complement thereof, and using the determined sequences of (i) and (ii) to identify the methylation status of the analyte in the biological sample.

In some examples, the first oligonucleotide and the second oligonucleotide hybridize to the analyte at sequences that are at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides apart.

In some examples, the method described herein further comprises extending the first oligonucleotide and/or the second oligonucleotide after step (c). In some examples, the first probe and/or second probe is extended using a polymerase.

In some examples, the method described herein further comprises releasing the ligation product from the analyte. In some examples, the releasing occurs before hybridizing the ligation product to the capture probe.

In some examples, the method further comprises, prior to step (b), contacting the biological sample with a permeabilization reagent.

In some examples, the first probe and/or the second probe comprises a sequence that binds to a sequence on the analyte that does not have a CG dinucleotide.

In some examples, the method further comprises washing the biological sample between step (b) and step (c).

In another example, described herein is a method for identifying a methylation status of DNA in a biological sample, the method comprising: (a) providing one or more transposomes to the biological sample, wherein the transposomes comprise methylated adaptors, under conditions wherein the one or more methylated adaptors is inserted into the DNA, thereby generating tagmented DNA fragments comprising methylated adaptors; (b) contacting the tagmented DNA fragments with an array comprising a plurality of capture probes, wherein a capture probe in the plurality of capture probes comprises a spatial barcode, wherein if the capture probe comprises one or more cytosines, wherein the one or more cytosines are methylated cytosines; (c) ligating the sequence comprising the tagmented DNA fragments and the one or more methylated adaptors to the capture probe, thereby creating a ligation product; (d) deaminating the ligation product; and (e) determining (i) all or a portion of the sequence of the spatial barcode or the complement thereof, and (ii) all or a portion of the sequence of the ligation product, or a complement thereof, and using the determined sequences of (i) and (ii) to identify the methylation status of the DNA, or a portion thereof, in the biological sample.

In some examples, the ligating comprises adding a splint oligonucleotide comprises (i) a sequence that binds specifically to a portion of the capture probe; and (ii) a sequence that binds specifically to a portion of the ligation product. In some examples,

the splint oligonucleotide comprises a sequence that binds specifically to a portion of the one or more methylated adaptors.

In some examples, the transposase enzyme is a Tn5 transposase enzyme, or a functional derivative thereof or a Tn7 transposase enzyme, or the functional derivative thereof.

In some examples, the one or more methylated adaptors comprises a first methylated adaptor and a second methylated adaptor.

In some examples, the method described herein further comprises incubating the ligation product with a terminal transferase and a plurality of deoxycytidine triphosphate (dCTP) molecules, thereby extending the ligation product at the 3' end.

In some examples, the method described herein further comprises amplifying the ligation product. In some examples, the method described herein further comprises amplifying the ligation product using a primer sequence that is complementary to a poly-cysteine sequence at the 3' end of the ligation product.

In some examples, the ligating comprises enzymatic ligation or chemical ligation. In some examples, the enzymatic ligation utilizes a ligase. In some examples, the ligase is one or more of a splintR ligase, a single stranded DNA ligase, or a T4 DNA ligase.

In some examples, the method described herein further comprises a migration step wherein the analyte migrates to the substrate. In some examples, the migration step is an active migration step comprising applying an electric field to the genomic DNA. In some examples, the migration step is a passive migration step.

In another example, described herein is a method of identifying the methylation status of a nucleic acid in a biological sample on a first substrate, comprising: (a) deaminating the nucleic acid; (b) hybridizing a first probe and a second probe to the nucleic acid, wherein: the first probe comprises (i) a sequence complementary to at least a first sequence of the nucleic acid and (ii) a sequence complementary to a capture domain of a capture probe on an array; and the second probe comprises a sequence complementary to at least a second sequence of the nucleic acid; (c) ligating the first probe and the second probe to generate a ligation product; (d) aligning the first substrate with the second substrate comprising the array, such that at least a portion of the biological sample is aligned with at least a portion of the array, wherein the array comprises a plurality of capture probes, wherein a capture probe of the plurality of capture probes comprises: (i) a spatial barcode and (ii) the capture domain; (e) when the biological sample is aligned with at least a portion of the array, (i) releasing the

ligation product from the analyte and (ii) migrating the ligation product from the biological sample to the array; and (f) capturing the ligation product with the capture domain.

In some examples, the method described herein further comprises determining (i) all or a portion of the sequence of the spatial barcode or the complement thereof and (ii) all or a portion of the sequence of the ligation product, or a complement thereof.

In some examples, the method described herein further comprises using the determined sequences of (i) and (ii) to identify the methylation status of the nucleic acid in the biological sample.

In some examples, the first probe is at least 75%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary to the nucleic acid. In some examples, the first probe is about 15 to about 120 nucleotides in length. In some examples, the first sequence is about 10 to about 60 nucleotides in length.

In some examples, the second probe is at least 75%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary to the nucleic acid. In some examples, the second probe is about 15 to about 120 nucleotides in length. In some examples, the second sequence is about 10 to about 60 nucleotides in length.

In some examples, the first sequence and the second sequence are adjacent sequences.

In some examples, at least one deaminated nucleotide is located between the first sequence and the second sequence. In some examples, at least two, at least three, at least four, at least five, or more deaminated nucleotide is located between the first sequence and the second sequence.

In some examples, the method described herein further comprises generating an extended first probe and/or an extended second probe using a polymerase, wherein the extended first probe or the extended second probe comprises a sequence complementary to a sequence between the first sequence and the second sequence.

In some examples, at least one deaminated nucleotide is located in the first sequence. In some examples, the at least one deaminated nucleotide is located in the second sequence.

In some examples, the releasing comprises heating the biological sample. In some examples, step (e) further comprises contacting the biological sample with a reagent medium comprising a permeabilization reagent, optionally wherein the permeabilization reagent comprises a protease.

In some examples, the ligating the first probe and the second probe comprises ligating via a ligase the first probe and the second probe. In some examples, the ligating the first probe and the second probe comprises ligating via a ligase: (a) the first probe and the extended second probe; or (b) the extended first probe and the second probe. In some examples, the ligase is selected from a splintR ligase, a single stranded DNA ligase, or a T4 DNA ligase.

In some examples, the first probe and the second probe are on a contiguous nucleic acid. In some examples, the ligating utilizes a splint oligonucleotide. In some examples, the splint oligonucleotide comprises a first splint sequence that is substantially complementary to the first probe and a second splint sequence that is substantially complementary to the second probe.

In some examples, the capturing the ligation product comprises hybridizing the sequence complementary to a capture domain to the capture domain. In some examples, the capturing the ligation product comprises ligating the ligation product to the capture probe.

In some examples, the method described herein further comprises extending the capture probe using the ligation product as a template; thereby generating an extended capture probe.

In one aspect, the present disclosure refers to a method for identifying methylation status of a nucleic acid in a biological sample, the method comprising:

(a) contacting the biological sample with a substrate;

(b) deaminating the nucleic acid in the biological sample;

(c) contacting the nucleic acid with a plurality of probes comprising a first probe and a second probe, wherein

the first probe comprises (i) a sequence complementary to at least a first sequence of the nucleic acid and (ii) a sequence complementary to a capture domain of a capture probe; and

the second probe comprises a sequence complementary to a second sequence of the nucleic acid;

(d) ligating the first probe and the second probe, thereby generating a ligation product;

(e) hybridizing the ligation product to the capture probe; and

(f) determining (i) the sequence of a spatial barcode of the capture probe or a complement thereof, and (ii) all or a portion of the sequence of the ligation product, or a complement thereof, and using the determined sequences of (i) and (ii) to identify the methylation status of the nucleic acid in the biological sample.

In another example, described herein is a method of identifying a methylation status of a nucleic acid in a biological sample on a first substrate, the method comprising: (a) ligating a methylated adaptor to a nucleic acid, generating an adapted nucleic acid fragment; (b) deaminating the adapted nucleic acid fragment; (c) aligning the first substrate with a second substrate comprising an array, such that at least a portion of the biological sample is aligned with at least a portion of the array, wherein the array comprises a plurality of capture probes, wherein a capture probe of the plurality of capture probes comprises: (i) a spatial barcode and (ii) a capture domain; (d) when the biological sample is aligned with at least a portion of the array, migrating the adapted nucleic acid fragment from the biological sample to the array; and (e) capturing the adapted nucleic acid fragment with the capture domain.

In some examples, the method described herein further comprises determining (i) all or a portion of the sequence of the spatial barcode or the complement thereof, and (ii) all or a portion of the sequence of the ligation product, or a complement thereof.

In some examples, the method described herein further comprises using the determined sequences of (i) and (ii) to identify the methylation status of the nucleic acid, or a portion thereof, in the biological sample.

In some examples, the methylated adaptor is ligated to the nucleic acid by tagmentation. In some examples, the methylated adaptor is ligated to the nucleic acid at a 5' end of the nucleic acid. In some examples, the methylated adaptor is ligated to the nucleic acid at a 3' end of the nucleic acid.

In some examples, the methylated adaptor is at least about 10 nucleotides to about 50 nucleotides long.

In some examples, the methylated adaptor comprises at least one, at least two, at least three, at least four, at least five, or more methylated cytosines.

In some examples, the spatial barcode comprises at least one, at least two, at least three, at least four, at least five, or more methylated cytosines.

In some examples, the ligating the methylated adaptor to the nucleic acid comprises attaching the one or more methylated adaptors to the 5' and/or 3' end of the nucleic acid using a transposase enzyme complexed with a transposon, wherein the transposase enzyme is a Tn5 transposase enzyme, or the functional derivative thereof.

In some examples, the capturing the adapted nucleic acid fragment comprises hybridizing the adapted nucleic acid fragment with the capture domain. In some examples, the capturing the adapted nucleic acid fragment comprises ligating the adapted nucleic acid fragment with the capture domain, wherein the ligating comprises enzymatic ligation or chemical ligation.

In some examples, the ligating utilizes a splint oligonucleotide, optionally wherein the splint oligonucleotide comprises a first splint sequence that is substantially complementary to the adapted nucleic acid fragment and a second splint sequence that is substantially complementary to the capture probe.

In some examples, the method described herein further comprises extending the capture probe using the adapted nucleic acid fragment as a template; thereby generating an extended capture probe.

In some examples, the method described herein further comprises incubating the adapted nucleic acid fragment with a terminal transferase and a plurality of deoxycytidine triphosphate (dCTP) molecules, thereby generating an extended adapted nucleic acid fragment.

In some examples, the method described herein further comprises amplifying the extended adapted nucleic acid fragment. In some examples, the method described herein further comprises amplifying the extended adapted nucleic acid fragment using a primer

sequence that is complementary to a poly-cysteine sequence at the 3' end of the adapted nucleic acid fragment.

In some examples, the biological sample is a cancer tissue sample. In some examples, the biological sample is from a subject treated with a cancer therapy. In some examples, the nucleic acid is DNA. In some examples, the DNA is genomic DNA.

In some examples, the biological sample is a tissue sample, optionally wherein the tissue sample is a solid tissue sample, optionally wherein the tissue sample is a tissue section. In some examples, the biological sample is a fixed sample, a frozen sample, a fresh sample, or a fresh frozen sample. In some examples, the biological sample is a formalin fixed paraffin embedded (FFPE) sample.

In some examples, the first substrate is a slide. In some examples, the first substrate is a glass slide. In some examples, the first substrate does not comprise an array of capture probes.

In some examples, the deaminating comprises contacting the biological sample with a composition comprising sodium bisulfite. In some examples, the deaminating comprises treating the biological sample with an enzyme. In some examples, the enzyme is a cytidine deaminase or a demethylase.

In some examples, the second substrate is a glass slide. In some examples, a 5' end of the capture probe is attached to the second substrate.

In some examples, the array is a bead array. In some examples, a 5' end of the capture probe is attached to a bead of the bead array.

In some examples, the capture probe further comprises a unique molecular identifier (UMI).

In some examples, the capture probe further comprises one or more functional domains, a unique molecular identifier, a cleavage domain, and combinations thereof.

In some examples, the migrating is active. In some examples, the migrating is passive.

In some examples, the capture probe is extended using a polymerase.

In some examples, the determining comprises sequencing (i) all or a part of the sequence of the nucleic acid, or a complement thereof, and (ii) all or a part of the sequence of the spatial barcode, or a complement thereof.

In some examples, the methylation status comprises identifying that about 1% to about 100% of cytosines comprises a methyl group.

In some examples, the method described herein further comprises imaging the biological sample. In some examples, the imaging occurs prior to deaminating the biological sample.

In one aspect, the present disclosure refers to a method for identifying a methylation status of a nucleic acid in a biological sample, the method comprising:

(a) contacting the biological sample with a substrate;

(b) providing one or more transposomes to the biological sample, wherein the one or more transposomes comprise one or more methylated adaptors, under conditions wherein the nucleic acid is fragmented and one or more methylated adaptors from the one or more transposomes is inserted into the nucleic acid , thereby generating a plurality of tagmented nucleic acid fragments, wherein a tagmented nucleic acid fragment of the plurality of tagmented nucleic acid fragments comprises the one or more methylated adaptors;

(c) ligating the tagmented nucleic acid fragment to a capture probe, thereby creating a ligation product;

(d) deaminating the ligation product; and

(e) determining (i) the sequence of a spatial barcode of the capture probe or a complement thereof, and (ii) all or part of the sequence of the ligation product, or a complement thereof, and using the determined sequences of (i) and (ii) to identify the methylation status of the nucleic acid in the biological sample.

In another example, described herein is a kit comprising comprising parts and instruction for performing any one of the methods described herein.

In some examples, the kit comprises a substrate comprising a plurality of capture probes, wherein a capture probe of the plurality of first capture probes comprises (i) a spatial barcode and (ii) a capture domain.

In some examples, the kit comprises one or more enzyme. In some examples, the one or more enzyme is selected from the group consisting of a transposase, a ligase, a polymerase; a reverse transcriptase, a cytidine deaminase and a demethylase.

In some examples, the kit further comprises a transposome complex comprising a transposase, a transposon sequence, and/or an adaptor sequence.

In another example, described herein is a composition comprising reagents for performing any one of the methods described herein.

In some examples, the composition comprises a substrate comprising a plurality of capture probes, wherein a capture probe of the plurality of first capture probes comprises (i) a spatial barcode and (ii) a capture domain.

In some examples, the composition comprises one or more enzyme. In some examples, the one or more enzyme is selected from the group consisting of a transposase, a ligase, a polymerase; a reverse transcriptase, a cytidine deaminase and a demethylase.

In some examples, the composition further comprises a transposome complex comprising a transposase, a transposon sequence, and/or an adaptor sequence.

All publications, patents, patent applications, and information available on the internet and mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, patent application, or item of information was specifically and individually indicated to be incorporated by reference. To the extent publications, patents, patent applications, and items of information incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

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

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

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

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

DESCRIPTION OF DRAWINGS

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

FIG. 1 is a schematic diagram showing an example of a barcoded capture probe, as described herein.

FIG. 2 is a schematic illustrating a cleavable capture probe, wherein the cleaved capture probe can enter into a non-permeabilized cell and bind to target analytes within the sample.

FIG. 3 is a schematic diagram of an exemplary multiplexed spatially-barcoded feature.

FIG. 4 is a schematic showing the arrangement of barcoded features within an array.

FIG. 5 is a schematic illustrating a side view of a diffusion-resistant medium, e.g., a lid.

FIG. 6A and **6B** are schematics illustrating expanded **FIG. 6A** and side views **FIG. 6B** of an electrophoretic transfer system configured to direct transcript analytes toward a spatially-barcoded capture probe array.

FIG. 7 is a schematic illustrating an exemplary workflow protocol utilizing an electrophoretic transfer system.