capture domain. In some instances, the techniques disclosed herein facilitate downstream processing, such as sequencing of the probes and/or barcodes bound to a capture domain.

In some examples, the methods, compositions, devices, and systems disclosed herein utilize RNA-templated ligation (RTL) for analyzing an analyte (e.g., RNA) in a biological sample. In some examples, RTL is used in combination with a "sandwich process," wherein the analyte is transferred from a first substrate to a second substrate for further downstream processing. In some examples, analyte capture agents are used for analyzing an analyte (e.g., protein) in a biological sample. In some examples, the methods disclosed herein allow spatial analysis of two different types of analytes.

In some instances, disclosed herein is a method for analyzing an analyte in a biological sample mounted on a first substrate comprising: hybridizing a first probe oligonucleotide and a second probe oligonucleotide to the analyte, wherein the first probe oligonucleotide and the second probe oligonucleotide each comprise a sequence that is substantially complementary to adjacent sequences of the analyte, and wherein the second probe oligonucleotide comprises a capture probe binding domain; coupling the first probe oligonucleotide and the second probe oligonucleotide, thereby generating a connected probe; 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; when the biological sample is aligned with at least a portion of the array, (i) releasing the connected probe from the analyte and (ii) migrating the connected probe from the biological sample to the array; and hybridizing the connected probe to the capture domain. In one aspect, the present disclosure refers to a method for analyzing an analyte in a biological sample mounted on a first substrate, the method comprising: (a) hybridizing a first probe oligonucleotide and a second probe oligonucleotide to the analyte, wherein the first probe oligonucleotide and the second probe oligonucleotide each comprise a sequence that is substantially complementary to adjacent sequences of the analyte, and wherein the second probe oligonucleotide comprises a capture probe binding domain; (b) coupling the first probe oligonucleotide and the second probe oligonucleotide, thereby generating a connected probe; (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, (i) releasing the connected probe from the analyte and (ii) migrating the connected probe from the biological sample to the array; and (e) hybridizing the connected probe to the capture domain.

In some instances, the first probe oligonucleotide and the second probe oligonucleotide are on a contiguous nucleic acid sequence. In some instances, the first probe oligonucleotide is on the 3' end of the contiguous nucleic acid sequence. In some instances, the second probe oligonucleotide is on the 5' end of the contiguous nucleic acid sequence. In some instances, the adjacent sequences abut one another. In some instances, the adjacent sequences are at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides away from one another.

In some instances, the methods further include generating an extended first probe oligonucleotide, wherein the extended first probe oligonucleotide comprises a sequence complementary to a sequence between the sequence hybridized to the first probe oligonucleotide and the sequence hybridized to the second probe oligonucleotide.

analyte capture agents comprises an analyte binding moiety and a capture agent barcode domain, wherein the analyte binding moiety specifically binds to the analyte, and wherein the capture agent barcode domain comprises an analyte binding moiety barcode and an capture handle sequence; 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; when the biological sample is aligned with at least a portion of the array, (i) releasing the capture agent barcode domain from the analyte and (ii) passively or actively migrating the capture agent barcode domain from the biological sample to the array; and coupling the capture handle sequence to the capture domain. In one aspect, the present disclosure refers to a method for analyzing an analyte in a biological sample mounted on a first substrate, the method comprising: (a) contacting the biological sample with a plurality of analyte capture agents, wherein an analyte capture agent of the plurality of analyte capture agents comprises an analyte binding moiety and a capture agent barcode domain, wherein the analyte binding moiety specifically binds to the analyte, and wherein the capture agent barcode domain comprises an analyte binding moiety barcode and an capture handle sequence; (b) 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; (c) when the biological sample is aligned with at least a portion of the array, (i) releasing the capture agent barcode domain from the analyte and (ii) passively or actively migrating the capture agent barcode domain from the biological sample to the array; and (d) coupling the capture handle sequence to the capture domain. In some instances, the methods further include determining (i) all or a part of the capture agent barcode domain, or a complement thereof; and (ii) all or a part of the sequence of the spatial barcode, or a complement thereof. In some instances, the methods further include using the determined sequence of (i) and (ii) to determine the location and abundance of the analyte in the biological sample.

In some instances, the aligning step comprises: (i) mounting the first substrate on a first member of a support device, the first member configured to retain the first substrate; (ii) mounting the second substrate on a second member of the support device; (iii) applying a reagent medium to the first substrate and/or the second substrate, wherein the reagent medium comprises a permeabilization agent and optionally an agent for releasing the connected probe; and (iv) operating an alignment mechanism of the support device to move the first member and/or the second member such that at least a portion of the biological sample is aligned with at least a portion of the array, and such that the portion of the biological sample and the portion of the array contact the reagent medium.

In some instances, the alignment mechanism is coupled to the first member, the second member, or both the first member and the second member. In some instances, the alignment mechanism comprises a linear actuator, optionally wherein: the linear actuator is configured to move the second member along an axis orthogonal to the plane or the first member and/or the second member, and/or the linear actuator is configured to move the first member along an axis orthogonal to the plane of the first member and/or the second member, and/or the linear actuator is configured to move the first member, the second member, or both the first member and the second member at a velocity of at least 0.1 mm/sec, and/or the linear actuator is configured to move the first member, the second member, or both the first member and the second member with an amount of force of at least 0.1 lbs.

first substrate, and wherein the second substrate 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; (b) (b1) a first probe oligonucleotide and a second probe oligonucleotide, wherein the first probe oligonucleotide and the second probe oligonucleotide each comprise a sequence that is substantially complementary to adjacent sequences of the analyte, wherein the second probe oligonucleotide comprises a capture probe binding domain, and wherein the first probe oligonucleotide and the second probe oligonucleotide are capable of being ligated together to form a connected probe; or (b2) a plurality of analyte capture agents, wherein an analyte capture agent of the plurality of analyte capture agents comprises an analyte binding moiety and a capture agent barcode domain, wherein the analyte binding moiety specifically binds to the analyte, and wherein the capture agent barcode domain comprises an analyte binding moiety barcode and an capture handle sequence; (c) a reagent medium comprising a permeabilization agent and optionally an agent for releasing the connected probe; and (d) instructions for performing any one of the methods disclosed herein. In one aspect, the present disclosure refers to a system or kit for analyzing an analyte in a biological sample, the system or the kit comprising: (a) a support device configured to retain a first substrate and a second substrate, wherein the biological sample is placed on the first substrate, and wherein the second substrate 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; (b) (b1) a first probe oligonucleotide and a second probe oligonucleotide, wherein the first probe oligonucleotide and the second probe oligonucleotide each comprise a sequence that is substantially complementary to adjacent sequences of the analyte, wherein the second probe oligonucleotide comprises a capture probe binding domain, and wherein the first probe oligonucleotide and the second probe oligonucleotide are capable of being ligated together to form a connected probe; or (b2) a plurality of analyte capture agents, wherein an analyte capture agent of the plurality of analyte capture agents comprises an analyte binding moiety and a capture agent barcode domain, wherein the analyte binding moiety specifically binds to the analyte, and wherein the capture agent barcode domain comprises an analyte binding moiety barcode and an capture handle sequence; (c) a reagent medium comprising a permeabilization agent and optionally an agent for releasing the connected probe; and (d) instructions for performing the method as disclosed herein. In some instances, the permeabilization agent is pepsin or proteinase K. In some instances, the agent for releasing the connected probe is an RNAse, optionally wherein the RNAse is RNase A, RNase C, RNase H, or RNase I. In some instances, the system or the kit further comprises an alignment mechanism on the support device to align the first substrate and the second substrate. In some instances, the alignment mechanism comprises a linear actuator, wherein the first substrate comprises a first member and the second substrate comprises a second member, and optionally wherein: the linear actuator is configured to move the second member along an axis orthogonal to the plane or the first member and/or the second member, and/or the linear actuator is configured to move the first member along an axis orthogonal to the plane of the first member and/or the second member, and/or the linear actuator is configured to move the first member, the second member, or both the first member and the second member at a velocity of at least 0.1 mm/sec, and/or the linear actuator is configured to move the first member, the second member, or both the first member and the second member with an amount of force of at least 0.1 lbs.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, patent application, or item of information was specifically and individually indicated to be incorporated by reference. To the extent publications, patents, patent applications, and items of information incorporated by reference contradict the disclosure contained in the

specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

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

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

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 diagram of an exemplary analyte capture agent.

FIG. 5 is a schematic diagram depicting an exemplary interaction between a feature-immobilized capture probe **524** and an analyte capture agent **526**.

FIGs. 6A, **6B**, and **6C** are schematics illustrating how streptavidin cell tags can be utilized in an array-based system to produce a spatially-barcoded cells or cellular contents.