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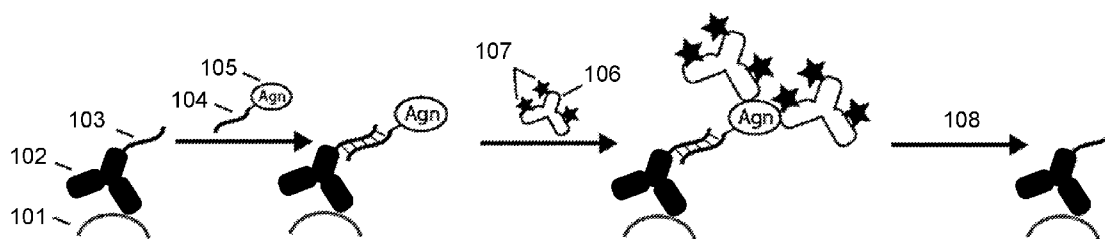


Fig. 1A

(57) Abstract: Methods for imaging are described, including, but not limited to a method comprising: (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners linked (directly or indirectly) to optical labels, wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels, (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners, (5) imaging the sample to detect bound labeled antigen-specific binding partners, (6) optionally removing/extinguishing signal from the optical labels, and (7) optionally repeating steps (1)-(7), or any subset thereof.



## DNA-ANTIGEN EXCHANGE AND AMPLIFICATION

### DESCRIPTION

#### FIELD

[001] DNA-Antigen Exchange and Amplification imaging methods capable of multiplexing and increasing detection sensitivity of targets by linking a target to an antigen, an antigen-specific binding partner, and optical labels.

#### BACKGROUND

[002] Secondary antibodies are raised to bind other antibodies, typically with species-specific affinity. The use of secondary antibodies in immunofluorescence applications is widespread. Secondary antibodies can be advantageous both for cost savings and signal amplification. Labeling secondary antibodies with fluorophores is more cost-effective than directly labeling primary antibodies because one type of fluorescently-labeled secondary antibody can be applied to image many different targets, provided the targets are detected on separate samples using primary antibodies from the same host species. Additionally, since multiple fluorescently-labeled secondary antibodies can bind to a single primary antibody, signal intensities generated from the sample are higher than if fluorescently-labeled primary antibodies were used. However, the use of fluorescently-labeled secondary antibodies severely limits the ability to carry out multiplexed detection. To multiplex several targets, each target-specific primary antibody must be raised in a different host species to ensure that the secondary antibodies bind to and are associated with the correct target complex. The most common host species for primary antibodies are mouse and rabbit, and the lack of high-quality alternative host species relegates the multiplexing power that is practically achievable with secondary antibodies to two targets per sample (i.e. using a labeled anti-mouse and labeled anti-rabbit secondary antibody to detect one target stained with a mouse antibody and one target stained with a rabbit antibody).

[003] Previous approaches have tried to overcome the limitation posed by species-specific detection with secondary antibodies. Immunoreagents comprising primary antibodies attached to an antigen or hapten have been described (WO2016127149) for detection with labeled anti-antigen or anti-hapten antibodies (i.e. a detection antibody). While this approach expands the number of targets that can be labeled simultaneously, the total number of targets that can be multiplexed is still limited by the number of spectrally distinct labels that can be used to modify the detection antibodies. Furthermore, once a sample is stained, the signal from the detection antibody is fixed and cannot be removed unless stringent conditions are applied that may damage the sample. In order to achieve higher levels of multiplexing, a sequential multiplexing approach is required that is gentle enough to maintain the integrity of the sample.

[004] Here, we present novel approaches for highly multiplexed target detection that achieves similar levels of convenience and improved amplification to that associated with labeled secondary antibodies. This approach is called DNA-Antigen Exchange and Amplification. DNA-Antigen Exchange and Amplification enables sequential multiplexing of targets and dynamic adjustments of amplification levels on a single sample.

## SUMMARY

[005] In accordance with the description, at least one method comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners linked (directly or indirectly) to optical labels, wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to

distinct optical labels, (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners, (5) imaging the sample to detect bound labeled antigen-specific binding partners, (6) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (7) optionally repeating steps (1)-(7), or any subset thereof (such as (1)-(6) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[006] In some embodiments a method comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to an antigen, and wherein target-specific binding partners of different specificity are linked to different antigens, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-specific binding partners linked to docking strands, wherein different antigen-specific binding partners are linked to different docking strands, (4) optionally removing unbound antigen-specific binding partners linked to docking strands; (5) adding imager strands; wherein the imager strands have complementarity to a docking strand, directly or indirectly, and wherein each imager strand is linked (directly or indirectly) to one or more optical labels, and wherein imager strands of different specificity are linked to distinct optical labels, (6) optionally removing unbound imager strands, (7) imaging the sample to detect bound labeled antigen-specific binding partners, (8) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (9) optionally repeating steps (1)-(9), or any subset thereof (such as (1)-(8) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[007] In some embodiments, a method comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a first docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-

bound imager strands and antigen-specific binding partners, wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and wherein each antigen-specific binding partner is linked to at least one second docking strand; (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners, (5) increasing the number of second docking strand strands by a DNA amplification reaction and labeling of the second docking strands using an optical label, (6) imaging the sample to detect bound labeled antigen-specific binding partners, (7) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (8) optionally repeating steps (1)-(8), or any subset thereof (such as (1)-(7) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[008] In some embodiments, a method comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to an antigen, and wherein target-specific binding partners of different specificity are linked to different antigens, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-specific binding partners linked docking strands, wherein different antigen-specific binding partners are linked to different docking strands, (4) optionally removing unbound antigen-specific binding partners linked to docking strands; (5) increasing the number of docking strands by a DNA amplification reaction and labeling of the docking strands using an optical label, (6) imaging the sample to detect bound labeled antigen-specific binding partners, (7) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (8) optionally repeating steps (1)-(8), or any subset thereof (such as (1)-(7) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[009] In some embodiments, a composition comprises (1) a first binding partner-oligonucleotide conjugate comprising a binding partner linked to an oligonucleotide that comprises a docking domain; (2) an antigen-bound oligonucleotide conjugate comprising an antigen linked to an oligonucleotide that

comprises an imager domain, wherein the imager domain of (2) is complementary to the docking domain of (1); and (3) a labeled antigen-specific binding partner comprising an optical label linked to an antigen-specific binding partner, wherein the antigen-specific binding partner of (3) specifically binds the antigen in (2).

{0010} Additional objects and advantages will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice. The objects and advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

{0011} It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

{0012} The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) embodiment(s) and together with the description, serve to explain the principles described herein.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

{0013} Figures 1A-F provide schemes of DNA-Antigen Amplification and Exchange. Figure 1A shows a target-specific binding partner (102) attached to a docking strand (103) binds to a target (101) in a sample. An imager strand (104) comprising an antigen (105) is added to bind to the docking strand. An antigen-specific binding partner (106) attached to optical labels (107) is added to the sample to bind the antigen that is complexed on the target. At this stage, the sample can be imaged to detect the signal and identify the target. Finally, an exchange reaction (108) can be carried out to remove the signal. Figure 1B shows DNA-Antigen amplification scheme via a layering approach wherein a target-specific binding partner (102) attached to a docking strand (103) binds to a target (101) in a sample. An imager strand (104) comprising an antigen (105) is added to bind to the docking strand. An antigen-specific binding partner (106) is added to the sample to bind the antigen that is complexed on the target. An anti-antigen-specific binding partner (109) attached to optical labels (107) is added to the

sample to bind to the target through the layered complex. At this stage, the sample can be imaged to detect the signal and identify the target. Finally, an exchange reaction (not shown) can be optionally carried out to remove the signal. Figure 1C shows a target-specific binding partner (102) attached to an antigen (105) binding to a target (101) in a sample. An antigen-specific binding partner (106) attached to docking strands (103) is added to the sample to bind the antigen that is complexed on the target. An imager strand (104) comprising an optical label (107) is added to bind to the docking strands. At this stage, the sample can be imaged to detect the signal and identify the target. Finally, an exchange reaction (not shown) can be optionally carried out to remove the signal. Figure 1D shows a target-specific binding partner (102) attached to an antigen (105) binding to a target (101) in a sample. An antigen-specific binding partner (106) attached to docking strands (103) is added to the sample to bind the antigen that is complexed on the target. Optionally, the number of docking strands complexed to the targets are increased by a DNA amplification reaction (110) such as rolling circle amplification. An imager strand (104) comprising an optical label (107) is added to bind to the docking strands. At this stage, the sample can be imaged to detect the signal and identify the target. Finally, an exchange reaction (108) can be optionally carried out to remove the signal. Figure 1E shows a target-specific binding partner (102) attached to a docking strand (103) binds to a target (101) in a sample. An imager strand (104) comprising an antigen (105) is added to bind to the docking strand. An antigen-specific binding partner (106) attached to docking strands (103) is added to the sample to bind the antigen that is complexed on the target. Optionally, the number of docking strands complexed to the targets are increased by a DNA amplification reaction (110) such as rolling circle amplification. An imager strand (104) comprising an optical label (107) is added to bind to the docking strands. At this stage, the sample can be imaged to detect the signal and identify the target. Finally, an exchange reaction (108) can optionally be carried out to remove the signal. Figure 1F shows a target-specific binding partner (102) attached to a docking strand (103) binds to a target (101) in a sample. An imager strand (104) comprising an antigen (105) is added to bind to the docking strand.

An antigen-specific binding partner (106) is added to the sample to bind the antigen that is complexed on the target. An anti-antigen-specific binding partner (109) attached to docking strands (103) is added to the sample to bind to the target through the layered complex. Optionally, the number of docking strands complexed to the targets are increased by a DNA amplification reaction (110) (product not shown). An imager strand (104) comprising an optical label (107) can be added to bind to the docking strands complexed to the target. At this stage, the sample can be imaged to detect the signal and identify the target. Finally, an exchange reaction (108) can optionally be carried out to remove the signal. While Figure 1F is shown as a variant of Figure 1E, it may also be carried out with primary antibodies directly conjugated to antigens, such as Figure 1D instead of primary antibodies conjugated to docking strands, such as in Figure 1E.

{0014} Figures 2A-D show data demonstrating the DNA Antigen Exchange results for a 2-plex assay on tonsil tissue using biotinylated imager strands and dye-labeled streptavidin. Figure 2A shows a blank image of prepared sample. Figure 2B shows an Image of Ki67 in sample following addition of imager strand I1-bio and SA-Cy5. Figure 2C shows a sample image after exchange step removing I1-bio and SA-Cy5. Figure 2D shows an image of cytokeratin in sample following addition of imager strand I2-bio and SA-Cy5.

{0015} Figures 3A-B illustrate amplification using DNA Antigen Exchange with HRP-labeled imager strands and dye-labeled anti-HRP antibodies (Figure 3A) compared to non-amplified image with fluorophore-labeled imager strand (Figure 3B). Brightness scale set from 0-3000 for both images.

{0016} Figure 4 shows data demonstrating DNA-antigen amplification on cytokeratin protein targets in FFPE tonsil tissue. Antigen-conjugated primary antibodies and DNA-conjugated anti-antigen antibodies were used to stain cytokeratin; rolling circle amplification was employed to increase the number of docking strands associated with the DNA-conjugated anti-antigen antibody; and fluorescently labeled imager strands were bound prior to imaging the sample.

{0017} The following reference numbers are employed in the application:

<b>Table 1: Reference Numbers</b>
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101	Target
102	Target-specific binding partner
103	docking strand
104	imager strand
105	antigen
106	antigen-specific binding partner
107	optical label
108	Exchange reaction
109	Secondary binding partner (such as but not limited to, an anti-antigen-specific binding partner)
110	DNA amplification reaction

## DESCRIPTION OF THE EMBODIMENTS

### I. Multiplexed Imaging using DNA-Antigen Exchange Amplification

{0018} This application relates to methods and compositions for testing for the presence of one or more target(s) with one or more specific binding partner(s). Components may be complexed together through a series of binding interactions to link a target to a detection element.

{0019} Exchange imaging is a method to achieve high multiplexing capabilities, whereby a large number of targets can be imaged in the same sample, either simultaneously or sequentially. Exchange imaging relies on the ability to introduce detectable moieties that bind (e.g. through a molecular complex) specifically to one or more target(s) in a sample, wherein different target types are bound to distinct detectable moieties, and then subsequently the ability to remove the detectable moieties from the sample. A target in a sample that is bound to a target-specific binding agent attached to a docking strand that is attached to an antigen-bound imager strand can then be detected by introducing an antigen-specific binding partner that is attached to one or more detectable labels. The resulting signal can then be optionally removed. In one example, the signal is removed by disrupting the binding affinities between the docking and imager strands.

[0020] Signal amplification is often beneficial for low abundance targets, or when the sensitivity of the imaging equipment is low. Signal amplification covers the concept of increasing the number of detectable elements that are specifically bound to a target. In one non-limiting example, signal amplification results from increasing the number of fluorophores associated with a target. Signal amplification approaches can be combined with DNA-antigen Exchange methods to yield greater assay sensitivity, dynamic range, and greater content generation. Here, we discuss embodiments covering DNA-antigen exchange and signal amplification.

[0021] In some embodiments, a method of DNA-Antigen Exchange comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners linked (directly or indirectly) to optical labels, (a) wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and (b) wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels, (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners, (5) imaging the sample to detect bound labeled antigen-specific binding partners, (6) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (7) optionally repeating steps (1)-(7), or any subset thereof (such as (1)-(6) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[0022] By optionally removing/extinguishing the signal from the optical labels, we mean any step that causes the signal to terminate. This can include removing the antigen-bound imager strands from the docking strands, cleaving or degrading the imager strand and/or the docking strands, photobleaching the label

or otherwise extinguishing its signal, or removing the label from the moiety to which it has been attached. Such a signal termination step may completely eliminate the signal from the optical label or it may substantially reduce the signal from the optical label with a reduction of signal of at least 99%, 95%, 90%, 85%, 80%, 75%, 70%, 60%, or 50%.

{0023} In some methods, the antigen-specific binding partners and antigen-bound imager strands are added stepwise in the method herein. Namely, the method of DNA-Antigen Exchange comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with antigen-bound imager strands having complementarity to a docking strand, directly or indirectly, (4) optionally removing unbound antigen-bound imager strands, (5) contacting the sample with labeled antigen-specific binding partner, wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels, (6) optionally removing unbound antigen-specific binding partners, (7) imaging the sample to detect bound labeled antigen-specific binding partners, (8) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (9) optionally repeating steps (1)-(9), or any subset thereof (such as (1)-(8) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

{0024} In some other embodiments, the antigen-specific binding partners and antigen-bound imager strands are premixed before including them in the method herein. Namely, the method of DNA-Antigen Exchange comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally

removing unbound target-specific binding partners, (3) contacting the sample with labeled antigen-specific binding partners, wherein each antigen-specific binding partner is linked to one or more optical labels, wherein antigen-specific binding partners of different specificity are linked to distinct optical labels, and wherein the labeled antigen-specific binding partners are bound to antigen-bound imager strands having complementarity to a docking strand, directly or indirectly, (4) optionally removing unbound labeled antigen-specific binding partners and antigen-bound imager strands, (5) imaging the sample to detect bound labeled antigen-specific binding partners, (6) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (7) optionally repeating steps (1)-(7), or any subset thereof (such as (1)-(6) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[0025] In some embodiments set forth in at least paragraph [0021] and referencing the numbering of that paragraph, steps (3) and (4) together comprise: (a) first contacting the sample with antigen-bound imager strands, (b) second optionally removing unbound antigen-bound imager strands, (c) third contacting the sample with labeled antigen-specific binding partner, and (d) fourth optionally removing unbound antigen-specific binding partners.

[0026] In some embodiments set forth in at least paragraph [0021] and referencing the numbering of that paragraph, step (3) comprises contacting the sample with antigen-specific binding partners, wherein the antigen-specific binding partners are bound to antigen-bound imager strands. By bound, Applicant intends to include both covalent and noncovalent binding.

[0027] In some embodiments set forth in at least paragraph [0026], steps (3) and (4) together comprise (a) increasing the observable signal by contacting the sample with antigen-bound imager strands having complementarity to a docking strand, directly or indirectly, (b) optionally removing unbound antigen-bound imager strands, (c) contacting the sample with labeled antigen-specific binding partner, wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity

are linked to distinct optical labels, and (d) optionally removing unbound antigen-specific binding partners.

[0028] In some embodiments, the method includes removing antigen-bound imager strands not bound to docking strands. In some embodiments, the method includes removing antigen-specific binding partners not bound to antigen-bound imager strands.

## **II. DNA-Antigen Amplification**

[0029] The ability to dynamically adjust the level of signal amplification allows for greater flexibility in experimental workflow, sample preparation, and assay development. The level of target expression may vary from sample to sample. Thus, it is beneficial to be able to adjust the signal amplification level while an experiment is in progress. One may first prepare a sample and image without any amplification. Upon imaging, if the signal is found to be low, one may wish to exchange the unamplified detection reagents for amplified detection reagents. Alternatively, amplification may be used on a sample before any imaging has been conducted.

[0030] In one embodiment, DNA-antigen amplification can be used to functionally characterize assay reagents. For example, DNA-antigen amplification can be applied to validate or quality check the production of antibody-DNA conjugates.

[0031] In one embodiment, DNA-antigen amplification is employed to detect a plurality of targets in a sample. In another embodiment, target-specific binding partners from the same host-species are used to bind to a plurality of targets in a sample. In one non-limiting example, a plurality of primary antibodies derived from the same host species are bound to a plurality of targets and detected with DNA-antigen amplification.

### **A. DNA-Antigen Amplification for Dynamic Signal Adjustment**

[0032] The embodiments described herein can be used after an initial imaging step where the user determines whether the signal is strong enough or requires amplification. In an initial imaging step, the sample may be contacted

with one or more target-specific binding partner linked to a docking strand, wherein target-specific binding partners of different specificity are linked to different docking strands. As a “preview” or initial imaging step, the sample is then contacted with labeled imager strands having complementarity to a docking strand, directly or indirectly, the sample imaged, and the bound labeled imager strands removed. This initial “preview” imaging step would not employ antigen-bound imager strands and antigen-specific binding partners unless the user determined that the initial imaging step with the labeled imager strands did not produce a sufficient signal. If amplification is desired, the user can shift into an amplification mode using antigen-bound imager strands and labeled antigen-specific binding partners. The application terms this function “dynamic signal adjustment” because the antigen-bound imager strands and antigen-specific binding partners may be employed for amplification during the workflow of an exchange imaging method.

[0033] Thus, in some embodiments, a method of DNA-Antigen Amplification comprises (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands, (2) optionally removing unbound target-specific binding partners, (3) contacting the sample with labeled imager strands having complementarity to a docking strand, directly or indirectly, (4) optionally removing unbound labeled imager strands, (5) imaging the sample to detect bound labeled imager strands, (6) removing bound labeled imager strands, (7) increasing the observable signal by contacting the sample with antigen-bound imager strands having complementarity to a docking strand, directly or indirectly, (8) optionally removing unbound antigen-bound imager strands, (9) contacting the sample with labeled antigen-specific binding partner, wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels, (10) optionally removing unbound antigen-specific binding partners, (11) imaging the sample to detect

bound labeled antigen-specific binding partners, (12) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and (13) optionally repeating steps (1)-(13), or any subset thereof (such as (1)-(12) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[0034] In some embodiments set forth in at least paragraph [0021] and referencing the numbering of that paragraph, between steps (2) and (3), the method comprises: (a) contacting the sample with labeled imager strands having complementarity to a docking strand, directly or indirectly, (b) optionally removing unbound labeled imager strands, (c) imaging the sample to detect bound labeled imager strands, and (d) removing bound labeled imager strands.

#### **B. DNA-Antigen Amplification Composition**

[0035] In one embodiment, a composition is described comprising (a) a first binding partner-oligonucleotide conjugate comprising a binding partner linked to an oligonucleotide that comprises a docking domain; (b) an antigen-bound oligonucleotide conjugate comprising an antigen linked to an oligonucleotide that comprises an imager domain, wherein the imager domain of (b) is complementary to the docking domain of (a); and (c) a labeled antigen-specific binding partner comprising an optical label linked to an antigen-specific binding partner, wherein the antigen-specific binding partner of (c) has specificity for the antigen in (b).

#### **C. Employing Secondary Binding Partners for Amplification**

[0036] In some embodiments, secondary binding partners linked to optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, directly or indirectly. This allows for layering of additional binding partners linked to optical labels for a stronger signal. When the secondary binding partner binds directly, the secondary binding partner may be, thus, an anti-antigen-specific binding partner.

[0037] In some embodiments, the secondary binding partners and the antigen of the antigen-bound imager strands each comprise the same antigen. This

means that at least part of the secondary binding partner and the antigen are identical, even though the secondary binding partner is bound to an optical label and the antigen is bound to an imager strand. In other embodiments, the secondary binding partners and the antigen of the antigen-bound imager strands may comprise different antigens, in other words, the secondary binding partners may not, in this embodiment, comprise the antigen of the antigen-bound imager strands.

[0038] In some embodiments, the antigen and the secondary binding partner each comprise biotin. In some embodiments, the antigen-specific binding partner comprises streptavidin.

[0039] In some embodiments, the secondary binding partner binds the antigen-specific binding partner indirectly. For example, if the antigen is biotin and the antigen-specific binding partner is streptavidin, the secondary binding partner may also be streptavidin if a construct employing two biotin moieties conjugated by a linker was used. This embodiment may have advantages when an even stronger signal is desired because it may be possible to conjugate more copies of a label to streptavidin than biotin. The construct employing two biotin moieties can thus serve as a bridge enabling specific binding of the secondary binding partner to the antigen-specific binding partner.

[0040] In some embodiments, the antigen-specific binding partners and the secondary binding partners both comprise an antibody or an antigen-binding fragment thereof.

[0041] In some instances, the antigen-specific binding partner is a primary antibody or antigen-binding fragment thereof to the antigen and the secondary binding partner is a secondary antibody or antigen-binding fragment thereof.

[0042] In some embodiments, multiple antigen-specific binding partners bind to a single antigen. In some embodiments, the antigen-specific binding partners comprise a polyclonal antibody. In some embodiments, multiple types of antigen-specific binding partners are used. For instance, the multiple types of antigen-specific binding partners may comprise more than one monoclonal antibody directed to different epitopes on the antigen.

[0043] In some embodiments, multiple layers of amplification may be employed so that multiple secondary antibodies are layered on top of each other. For instance, the first secondary antibody could bind to the antigen and a second secondary antibody (i.e., a tertiary antibody) could bind to the first secondary antibody and so on. A third secondary antibody (i.e., a quaternary antibody) could bind to the second secondary antibody, and so on. The number of layers employed depends on the amplification desired and other factors, as follows.

[0044] When employing secondary binding partners for amplification, some layered complexes may be removable by washing steps. Other layered complexes may not be removable by washing steps. Those that are removable may be used for exchange imaging with amplification. Those that are not removable may be used for amplification and for the last imaging step in an exchange imaging reaction.

### III. Components

#### A. Targets

[0045] In certain embodiments, the target-specific binding partner is specific for a cellular marker. Cellular markers may include: 4-1BB, AFP, ALK1, Amyloid A, Amyloid P, Androgen Receptor, Annexin A1, ASMA, BCA225, BCL-1, BCL-2, BCL-6, BerEP4, Beta-Catenin, Beta-HCG, BG-8, BOB-1, CA19-9, CA125, Calcitonin, Caldesmon, Calponin-1, Calretinin, CAM 5.2, CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD15, CD19, CD20, CD21, CD22, CD23, CD25, CD30, CD31, CD33, CD34, CD38, CD42b, CD43, CD45 LCA, CD45RO, CD56, CD57, CD61, CD68, CD79a, CD99, CD117, CD138, CD163, CDX2, CEA, Chromogranin A, CMV, c-kit, c-MET, c-MYC, Collagen Type IV, Complement 3c (C3c), COX-2, CXCR5, CK1, CK5, CK6, CK7, CK8, CK14, CK18, CK17, CK19, CK20, CK903, CK AE1, CK AE1/AE3, D2-40, Desmin, DOG-1, E-Cadherin, EGFR, EMA, ER, ERCC1, Factor VIII-RA, Factor XIIIa, Fascin, FoxP1, FoxP3, Galectin-3, GATA-3, GCDFP-15, GCET1, GFAP, Glycophorin A, Glypican 3, Granzyme B, HBME-1, Helicobacter Pylori, Hemoglobin A, Hep Par 1, HER2, HHV-8, HMB-45, HSV 1/11, ICOS,

IFN $\gamma$ , IgA, IgD, IgG, IgM, IL17, IL4, Inhibin, iNOS, Kappa Ig Light Chain, Ki67, LAG-3, Lambda Ig Light Chain, Lysozyme, Mammaglobin A, MART-1/Melan A, Mast Cell Tryptase, MLH1, MOC-31, MPO, MSA, MSH2, MSH6, MUC1, MUC2, MUM1, MyoD1, Myogenin, Myoglobin, Napsin A, Nestin, NSE, Oct-2, OX40, OX40L, p16, p21, p27, p40, p53, p63, p504s, PAX-5, PAX-8, PD-1, PD-L1, PHH3, PIN-4, PLAP, PMS2, Pneumocystis jirovecii (carinii), PR, PSA, PSAP, RCC, 5-100, SMA, SMM, Smoothelin, SOX10, SOX11, Surfactant Apoprotein A, Synaptophysin, TAG 72, TdT, Thrombomodulin, Thyroglobulin, TIA-1, TIM3, TRAcP, TTF-1, Tyrosinase, Uroplakin, VEGFR-2, Villin, Vimentin, and WT-1. In other embodiments, the target-specific binding partner is specific for an immunoglobulin from a different species.

#### **B. Target-specific binding partners**

{0046} The target specific binding partner refers to antibodies and antibody-like molecules that can be used to detect the target molecule. Antibody refers to any immunoglobulin from any species that can specifically recognize a target molecule. Antibody-like molecule refers to (Class A) any engineered variation or fragment of an antibody such as Fab, Fab', F(ab')<sub>2</sub>, single heavy chain, diabody, and the like (antigen binding fragments of antibodies) (Class B) any known binding partner of a target molecule and engineered variants of such binding partner, (Class C) any binding partner of the target molecule engineered via directed evolution (e.g., peptides and aptamers), and (Class D) any molecule that selectively forms covalent bond(s) with a target (e.g., a suicide substrate of an enzyme of interest). References to specific types of antibodies throughout the specification encompass both full length antibodies and any antibody-like molecules that include any engineered variation or fragments of an antibody such as Fab, Fab', F(ab')<sub>2</sub>, single heavy chain, diabody, and the like (antigen binding fragments of antibodies). Thus, for example, in Table 2, when the target recognition moiety references antibody, it also includes antigen binding fragments of those antibodies.

[0047] The target-specific binding partner may be provided in a liquid medium or buffer solution. Target-specific binding partners for different targets may be contacted with a sample in a single step or in multiple steps, such as after a prior imaging step.

[0048] Table 2 provides a representative listing of targets and corresponding target recognition moieties.

<b>Target</b>	<b>Target Recognition Moiety</b>	<b>Source or Sequence</b>
Any protein	Antibody (Class A)	Variable
Fluorescein (chemical compound)	Antibody (Class A)	Abcam, product # ab7253
Digoxigenin (chemical compound)	Antibody (Class A)	Abcam, product # ab76907
Biotin	Avidin/Streptavidin (Class B)	
Epidermal growth factor receptor (EGFR, protein)	Epidermal growth factor (EGF, Class B)	
Platelet-derived growth factor receptor (PDGFR, protein)	Platelet-derived growth factor (PDGF, Class B)	
Epidermal growth factor receptor (EGFR, protein)	E07 aptamer (Class C)	Li et al., PloS ONE, 2011;6(6):e20299
Integrins (protein)	RGD-containing peptides (Class B)	
TNF- $\alpha$ (protein)	T09.12 peptide (Class C)	Xu et al., Chem Biol. 2002 Aug;9(8):933-42.
HaloTag (enzyme)	Halogenated compounds (Class D)	Bioconjug Chem. 2015 Jun 17;26(6):975-86.
Oxidosqualene cyclase (OSC, enzyme)	[3H]29-methylidene-2,3-oxidosqualene ([3H]29-MOS, Class D)	Biochem Biophys Res Commun. 1992 Aug 31;187(1):32-8.

[0049] Table 3 provides a listing of additional targets. Antibodies and other known binding partners of these targets may be used as target recognizing moieties.

Actin	EGFR	MMP
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AIF	EpCAM	mTOR
AKT	ER	MYC
alpha-synuclein	ERK	NeuN
amyloid precursor protein	ERK	p21
annexin	FOXA	p53
Arrestin	FOXP3	PAX
BAD	GABA	PD-1
BAX	GAPDH	PD-L1
Bcl-2	GFP	PI3K
Bcl-2	GranzymeB	PR
beta-catenin	GRB2	PSD95
BRCA1	HER2	RAS
cAMP	HER3	SOX
Caveolin	HIF-1	STAT
CD20	HistoneH3	synapsin
CD3	HSP27	Tau
CD4	HSP70	TOM20
CD45	HSP90	Tubulin
CD68	keratin	Ubiquitin
CD8	Ki67	VEGF
collagen	Lamin	Vimentin
CREB	MAPK	WNT
DNA	MEK	
E-Cadherin	MET	

### C. Docking strands

[0050] In some embodiments, the docking moiety or docking strand is a nucleic acid, a protein, a peptide, or a chemical compound. Many proteins and domains of proteins are known to interact with other proteins, domains or peptides. Some of the best-known domains include SH2, SH3, and WD40 domains. In many cases the binding partner of these proteins and domains are known and can be engineered to have the desired affinity. For example, biotin and avidin/streptavidin interact with sufficient specificity. Many other chemical compounds, such as digoxigenin, fluorescein, tacrolimus and rapamycin also have well known binding partners.

[0051] In some embodiments, the docking strand comprises nucleic acids. In some embodiments, the nucleic acids are single stranded nucleic acids such as

single stranded DNA, RNA, or a nucleic acid analog. A nucleic acid analog (also known as non-natural nucleic acid) may include an altered phosphate backbone, an altered pentose sugar, and/or altered nucleobases. Nucleic acid analogs may include, but are not limited to, 2'-O-Methyl ribonucleic acid, 2'-fluoro ribonucleic acid, peptide nucleic acid, morpholino and locked nucleic acid, glycol nucleic acid, and threose nucleic acid.

[0052] In some embodiments, the docking strand is attached to the imager strand covalently and in other embodiments noncovalently.

[0053] In some embodiments, the docking strand comprises single-stranded nucleic acids and may be from about 5 to 20 nucleic acids long, from about 8 to 15, or from about 10 to 12 nucleic acids long. In some embodiments, the docking strand is about 5, 8, 9, 10, 11, 12, 13, 14, 15, 18, or 20 nucleic acids long.

[0054] The docking strand may be an independent element or it may be part of the target recognizing moiety. For example, if the target recognizing moiety is an antibody, part of the Fc domain of the antibody may be the docking strand and a peptide or protein that binds the Fc domain may be used, such as protein A or protein G.

[0055] The docking strand may be provided in a liquid medium or buffer solution.

#### **D. Imager strands**

[0056] The imager strand may be any molecule that is complementary to the docking strand (i.e., capable of specific binding to the docking strand) and attached (either directly or indirectly) to an antigen. In some embodiments, the docking strand may be a nucleic acid strand, a protein, a peptide, or a chemical compound. In such cases, the observable moiety or label may be conjugated to an imager moiety, which may be a nucleic acid strand that is complementary to the docking strand. In other words, the imager strand specifically binds the docking strand. In such a case, the label may be conjugated to an imager moiety that may be from about 5 to 20 nucleic acids long, from about 8 to 15, or from about 10 to

12 nucleic acids long. In some embodiments, the imager moiety is about 5, 8, 9, 10, 11, 12, 13, 14, 15, 18, or 20 nucleic acids long.

[0057] In some embodiments, the complementary portions between the imager moiety and the docking strand may be from about 5 to 20 nucleic acids long, from about 8 to 15, or from about 10 to 12 nucleic acids long. In some embodiments, the complementary portions between the imager moiety and the docking strand may be about 5, 8, 9, 10, 11, 12, 13, 14, 15, 18, or 20 nucleic acids long.

[0058] In some embodiments, the nucleic acid imager strand comprises single stranded nucleic acids such as single stranded DNA, RNA, or a nucleic acid analog. A nucleic acid analog (also known as non-natural nucleic acid) may include an altered phosphate backbone, an altered pentose sugar, and/or altered nucleobases. Nucleic acid analogs may include, but are not limited to, 2'-O-Methyl ribonucleic acid, 2'-fluoro ribonucleic acid, peptide nucleic acid, morpholino and locked nucleic acid, glycol nucleic acid, and threose nucleic acid.

[0059] In some embodiments, the imager moiety is a protein, peptide, or a chemical compound, as a partner to the docking strand options discussed above in Section III.C above.

[0060] In some embodiments, the docking strand may bind to the imager moiety indirectly, such as through an intermediate moiety. For instance, when the docking strand and the imager moiety are nucleic acids, an intermediate moiety comprising nucleic acids may be used as long as the intermediate moiety has a first region complementary to the docking strand and a second region complementary to the imager moiety. In this embodiment, it is not necessary for the docking strand to be complementary to the imager moiety. The intermediate moiety may serve only a bridging function or it may also serve an amplification function.

[0061] The imager strand may be provided in a liquid medium or buffer solution.

### E. Intermediate Strands

[0062] In some instances, the docking strand binds to the imager strand through an intermediate moiety (or intermediate strand). For instance, when the docking moiety and the imager moiety comprise nucleic acids, the intermediate strand comprising nucleic acids may be used as long as the intermediate strand has a first region complementary to the docking strand and a second region complementary to the imager strand. In such embodiments, it is not necessary for the docking strand to be complementary to the imager moiety.

[0063] In some embodiments, the intermediate strand is added as a first step to a sample comprising the target-specific binding partner linked to a docking strand, either directly or indirectly, and the imager strands added as a second step. In another embodiment, the intermediate strand and imager strand are not added in discrete steps. In some instances, the intermediate strand and imager strand are hybridized together before being added in a single step.

[0064] In some embodiments, the intermediate strand comprises nucleic acids. In some embodiments, the nucleic acids are single stranded nucleic acids such as single stranded DNA, RNA, or a nucleic acid analog. A nucleic acid analog (also known as non-natural nucleic acid) may include an altered phosphate backbone, an altered pentose sugar, and/or altered nucleobases. Nucleic acid analogs may include, but are not limited to, 2'-O-Methyl ribonucleic acid, 2'-fluoro ribonucleic acid, peptide nucleic acid, morpholino and locked nucleic acid, glycol nucleic acid, and threose nucleic acid.

[0065] In some embodiments, the intermediate strand comprises single-stranded nucleic acids and may be from about 5 to 30 nucleic acids long, from about 8 to 15, or from about 10 to 12 nucleic acids long. In some embodiments, the intermediate strand is about 5, 8, 9, 10, 11, 12, 13, 14, 15, 18, 20, 25, or 30 nucleic acids long.

[0066] The intermediate strand may be provided in a liquid medium or buffer solution.

## F. Antigens

[0067] Antigens could include haptens, small molecules, proteins, or immunogenic molecules. For example, antigens could include but not be limited to digoxigenin, biotin, avidin, streptavidin, horseradish peroxidase (HRP), albumin, bovine serum albumin, keyhole limpet hemocyanin, *Concholepas concholepas* hemocyanin, Ovalbumin, alkaline phosphatase, protein-C, or derivatives thereof. Antigens could include molecules such as nitrophenyl, dinitrophenyl, trinitrophenyl, digitoxin, 5-bromodeoxyuridine, 3-nitrotyrosine, benzylguanine, benzylcytosine, melamine, small-molecule drugs, and any other similar chemical tag. Antigens could include a Myc tag, a FLAG tag, a SNAP tag, a CLIP tag, an HA tag, an S tag, a Streptag, a His tag, or a V5 tag. Antigens could be antibodies (e.g. IgG, IgM, IgA, IgD, IgE, etc), antibody fragments (Fab, Fab', F(ab')<sub>2</sub>, Fc, pFc', single-chain variable fragment (scFv), di-scFv, single-domain antibody (sdAb), nanobody). Antigens could include any cellular marker listed as a target in this application, for example those proteins listed in Table 3.

[0068] Antigens could be nucleic acids or aptamers, including but not limited to, non-natural nucleic acids and oligomers. Antigens could be polymers (e.g. amino acid oligomers, peptides, cellulose, pectin, polyethylene, polyethylene glycol (PEG), dextran, cyclodextrin, nylon, Teflon, polystyrene, PVC, polypropylene) or molecular imprints (MIPs). Antigens could be organic fluorophores (e.g. Alexa Fluor dyes, Atto dyes, fluorescein (FITC)), fluorescent or chemiluminescent proteins (e.g. GFP, RFP, YFP, mCherry, phycoerythrin). Antigens could be mis-folded or denatured proteins. Antigens could be nanoparticles or coated nanoparticles. Antigens may be produced through chemical synthesis, be naturally occurring, be recombinantly expressed or genetically engineered.

[0069] Antigens may be linear, circular, branched, polymeric, or dendritic. Antigens may have one or more epitopes and may bind to one or more antigen-specific binding partners. One or more distinct species of antigen-binding partners may bind to the same antigen at different epitopes.

**G. Antigen-specific binding partners**

[0070] The antigen-specific binding partner may refer to antibodies and antibody-like molecules that can be used to detect an antigen. Antibody refers to any immunoglobulin from any species that can specifically recognize a target molecule or antigen. Antibody-like molecule refers to (Class A) any engineered variation or fragment of an antibody such as Fab, Fab', F(ab')<sub>2</sub>, single heavy chain, diabody, and the like (antigen binding fragments of antibodies) (Class B) any known binding partner of an antigen molecule and engineered variants of such binding partner, (Class C) any binding partner of the antigen molecule engineered via directed evolution (e.g., peptides and aptamers), and (Class D) any molecule that selectively forms covalent bond(s) with an antigen (e.g., a suicide substrate of an enzyme of interest).

[0071] An antigen-specific binding partner may also be a protein with high affinity for an antigen. For example, streptavidin may be used as a binding partner in the case where biotin is used as the antigen. If the antigen is a metal ion, an antigen-specific binding partner may be a chelating agent, such as EDTA, ethylenediamine, polyhistidine, heme, porphine, crown ether, cryptand, or another polydentate ligand.

[0072] An antigen-specific binding partner may form a covalent or non-covalent association with an antigen.

[0073] Antigen-specific binding partners corresponding to different targets may be contacted with a sample in a single step or in multiple steps, such as after a prior imaging step.

[0074] Antigens of antigen-bound imager strands and antigen-specific binding partners are described in pairs in Table 4.

<b>Table 4: Pairings of Antigens of Antigen-Bound Imager Strands and Antigen-Specific Binding Partners</b>	
<b>Antigens of Antigen-Bound Imager Strands</b>	<b>Antigen-Specific Binding Partners</b>
Any protein or peptide	Immunoglobulin (namely an antibody or antigen binding fragment thereof that specifically binds the protein or peptide)

Any protein or peptide	Aptamer
Biotin	Streptavidin
Streptavidin	Biotin
Horseradish Peroxidase (HRP)	Anti-HRP antibody or antigen binding fragment thereof
Anti-HRP antibody or antigen binding fragment thereof	Horseradish Peroxidase (HRP)
Alkaline Phosphatase (AP)	Anti-AP antibody or antigen binding fragment thereof
Any epitope tag	Anti-epitope antibody or antigen binding fragment thereof
Myc tag	Anti-myc antibody or antigen binding fragment thereof
FLAG tag	Anti-FLAG antibody or antigen binding fragment thereof
SNAP tag	benzylguanine
CLIP tag	benzylcytosine
benzylguanine	SNAP tag
benzylcytosine	CLIP tag
Histidine tag	Metal ions
Metal ion	Chelating agent (such as EDTA, ethylenediamine, polyhistidine, heme, porphine, crown ether, cryptand, or other polydentate ligand)
IgG (namely a primary antibody)	Anti-IgG antibody or antigen binding fragment thereof (namely a secondary antibody that specifically binds to the primary antibody and using the terminology herein this antibody binds to the antigen antibody of the antigen-bound imager strand)
Fc fragment	Anti-Fc antibody or antigen binding fragment thereof
Any fluorophore	Antibody or antigen binding fragment thereof
GFP	Anti-GFP antibody or antigen binding fragment thereof
Anti-GFP antibody or antigen binding fragment thereof	GFP
Fluorescein	Anti-fluorescein antibody or antigen binding fragment thereof
PEG	Anti-PEG antibody or antigen binding fragment thereof
Cyclodextrin	Anti-cyclodextrin or antigen binding fragment thereof

Any hapten	Immunoglobulin or antigen binding fragment thereof
Digitoxin	Anti-digitoxin antibody or antigen binding fragment thereof
Digoxigenin	Anti-digoxigenin antibody or antigen binding fragment thereof
Molecular imprint template	Molecular imprint
Dinitrophenyl	Immunoglobulin or antigen binding fragment thereof
Nitrophenyl	Immunoglobulin or antigen binding fragment thereof

### H. Nucleic Acid Amplification Methods

[0075] Additional nucleic acid amplification methods may be employed by amplifying the docking strands described herein. Thus, methods and constructs herein may benefit from both antigen:antigen-specific binding partner amplification, but they may also benefit from nucleic acid amplification.

[0076] In nucleic acid amplification methods, an oligonucleotide (such as a docking strand) bound (directly or indirectly) to the target-recognizing moiety is amplified using an amplifier strand (in some instances a circular DNA template), followed by extension of the docking strand by a DNA polymerase to create a concatemeric repeat of the reverse complement of the amplifier strand (i.e. an amplified strand or rolling circle amplification (RCA) product).

[0077] The use of nucleic acid amplification, such as RCA, is shown in Figures 1D-1F. The docking strand may, in fact, serve as a primer molecule for a polymerization or dendrimerization reaction. One example of such polymerization reactions is RCA where the primer of the RCA is linked (directly or indirectly, for example see Figure 1D and 1E) to the target-recognizing molecule and is converted to a long repetitive single-stranded DNA. Fluorescent molecules can be either directly incorporated into the RCA product via fluorescent-labeled nucleotides or be bound to the RCA product as a part of a fluorescent-labeled oligonucleotide that is designed to hybridize to the RCA product. Other examples of such polymerization or dendrimerization reactions include branched DNA toehold-based strand displacement (Schweller et al. PMID: PMC3517005),

hybridization chain reaction (HCR) (Dirks et al., 2014, PMID: 15492210, 24712299) and a similar DNA hairpin-based dendrimerization reaction (Yin et al., 2008, PMID 18202654), which here we call HDR. Other hairpin-based concatemerization methods may be used.

[0078] One may use RCA, HCR or HDR to generate a polymeric or dendrimeric product from the primer molecule linked to the antibody. In some embodiments, the product may contain many (e.g., greater than 2, 5, 10, 15, 20, 25, 50, 100, etc.) copies of single-stranded DNA domains that can serve as the docking strand and thus be recognized by oligonucleotides serving as the imager strand. Such DNA domains may be long enough (e.g. 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, or more nucleotides long, or be able to bind its complementary strand with  $K_d < 1$  nM at imaging condition). For RCA, this is achieved regularly. For HCR and HDR, if necessary one can include, at the loop or tail of the substrate hairpin, DNA domains that do not participate in the strand-displacement cascades but constitute part or the entirety of the imager strand-binding site. In some embodiments, signal amplification involves linking (covalently or non-covalently) the target-recognizing molecule to a docking strand that serves as a primer molecule of a polymerization or dendrimerization reaction.

### 1. Rolling Circle Amplification

[0079] In rolling circle amplification, the docking strand that serves as a primer of the RCA is linked (directly or indirectly) to the target-recognizing molecule and is converted to a long repetitive single-stranded DNA. Fluorescent molecules can be either directly incorporated into the RCA product via fluorescent-labeled nucleotides or be bound to the RCA product as a part of a fluorescent-labeled oligonucleotide that is designed to hybridize to the RCA product.

[0080] In some embodiments, imager strands may be hybridized to the RCA product (e.g. the concatemeric repeat of the reverse complement of the amplifier strand) linked (directly or indirectly) to the target-recognizing moiety during the RCA reaction. In some embodiments, therefore, amplification occurs using rolling circle amplification, while in the presence of labeled imager strands

having complementarity to the amplified strand. For example, a sample may be contacted with an oligonucleotide docking strand conjugated to a target-recognizing moiety that is either prehybridized to an amplifier strand or the amplifier strand may be hybridized in a later step. Then, all additional components for the RCA reaction may be added in one step including proteins (e.g. DNA polymerases, optionally BSA), nucleotides, buffer solution, salts, and imager strands. In some embodiments, a user may wish to prevent the imager strand from being amplified. This can be accomplished by several means, including but not limited to employing a 3'-modified imager strand having a modification on the 3' end. For example, the 3' modification on the imager strand may include a label (such as a fluorophore), a modified base, a stop code or terminator, a 3'-O-modification, a dideoxy-C, a dideoxy-G, a dideoxy-A, a dideoxy-T, an inverted nucleotide, any modification that eliminates the presence of a 3' hydroxyl group, or a single-stranded extension of the 3' end that is not complimentary to the amplifier strand.

[0081] In addition to HCR and RCA, other examples of such polymerization or dendrimerization reactions include, but are not limited to, DNA hairpin-based dendrimerization reaction (HDR) (Yin et al., 2008, PMID 18202654), and toe-hold mediated strand displacement.

[0082] DNA strand displacement is a method for the isothermal and dynamic exchange of DNA complexes. Strand displacement can be designed and intentionally controlled based on an understanding of DNA hybridization interactions and thermodynamics and can be facilitated by introducing engineered handles which are known as "toehold domains." The ability to modulate binding interactions and exchange hybridization partners gives rise to a series of potential signal amplification applications.

[0083] The amplification of multiple targets can be carried out sequentially. Alternatively, the amplification of multiple targets can be carried out simultaneously. Imaging steps can be carried out between rounds of amplification or following all rounds of amplification.

[0084] Multiple types of signal amplification can even be used in combination. For example, Gusev et al reported combining rolling circle amplification and HRP-based signal amplification (PMID: 11438455).

## 2. Nonlinear Amplification

[0085] An alternative method of RCA involves the use of a nonlinear amplifier or template strand, wherein an oligonucleotide (such as a docking strand) bound (directly or indirectly) to the target-recognizing moiety is hybridized to a circular DNA template (amplifier strand), followed by extension of the docking strand by a DNA polymerase to create a concatemeric repeat of the reverse complement of the amplifier strand (i.e. an amplified strand or RCA product). The hybridization of the amplifier strand to the oligonucleotide conjugated target-recognizing moiety may occur before (preassembly or prehybridization) or after the oligonucleotide conjugated target-recognizing moiety contacts the sample.

[0086] A nonlinear DNA template could be employed for signal amplification as a circular amplification strand. A circular oligo, with complementarity to a docking strand, can be generated separately from the amplification method. For example, ex situ ligation could be performed on a template DNA strand to form a circular strand of DNA. A circular strand could be hybridized to a docking strand that is attached to a target-specific binding partner before contacting the sample. Alternatively, the target-specific binding partner could first be used to stain the sample, and then subsequently the circular strand could be introduced to the sample to hybridize with the docking strand on the target-specific binding partner. Following the formation of a complex wherein a circular strand is attached to a docking strand that is linked to a target-specific binding partner, rolling circle amplification (RCA) could be carried out. This method offers certain advantages as it can be used to circumvent issues with inefficient in situ ligation steps.

[0087] In some embodiments, a polymerase may be used for RCA. In some instances, the labeled imager strands are linear strands. In some instances, the nonlinear amplifier strands are circular strands. In some instances, the

nonlinear amplifier strands are branched strands. In some instances, the nonlinear amplifier strand becomes circular after ligation.

[0088] In some embodiments, amplification products may comprise a geometric shape, such as a triangle, quadrilateral, pentagon, hexagon, and the like.

### **I. Optical Labels**

[0089] Various optical labels, also known as observable moieties, may be employed for signaling purposes. The optical labels may be bound to the antigen-specific binding partner (see Figure 1A). The optical labels may be bound to the secondary binding partner that specifically binds to the antigen-specific binding partner (see Figure 1B). The optical label may be bound to an imager strand (see Figures 1C, 1D, and 1E). The optical label may be bound to a nucleotide and incorporated into a nucleic acid amplification reaction (similar to Figures 1D and 1E but without an imager strand). Thus, the optical labels may be bound directly or indirectly to the antigen-specific binding partner.

[0090] In some embodiments, any observable moiety may be employed and, in some embodiments, the moiety is optically observable. The moiety may be signal absorbing or signal emitting. Of signal emitting molecules, molecules that fluoresce may be used, such as organic small molecules, including, but not limited to fluorophores, such as, but not limited to, fluorescein, Rhodamine, cyanine dyes, Alexa dyes, DyLight dyes, Atto dyes, etc.

[0091] In some embodiments, organic polymers, such as p-dots may be employed. In some embodiments, the observable moiety may be a biological molecule, including but not limited to a fluorescent protein or fluorescent nucleic acid (including fluorescent RNAs including Spinach and its derivatives). In some embodiments, the observable moiety may be an inorganic moiety including Q-dots. In some embodiments, the observable moiety may be a moiety that operates through scattering, either elastic or inelastic scattering, such as nanoparticles and Surface Enhanced Raman Spectroscopy (SERS) reporters (e.g., 4-Mercaptobenzoic acid, 2,7-mercapto-4-methylcoumarin). In some embodiments, the observable moiety may be chemiluminescence/ electrochemiluminescence

emitters such as ruthenium complexes and luciferases. The observable moiety may generate an optical signal, an electromagnetic signal (across the entire electromagnetic spectrum), atomic/molecular mass (e.g. detectable by mass spectrometry), tangible mass (e.g. detectable by atomic force microscope), current or voltage.

[0092] In some embodiments, the antigen-specific binding partner with the optical label may have multiple labels or observable moieties. For example, if the antigen-specific binding partner is streptavidin, it may be able to carry at least 5 or 6 fluorophores. Depending on the degree of signal desired, larger antigen-specific binding partners may be used to generate additional signaling capacity.

#### **J. Sample**

[0093] The reagents and techniques described herein may be useful for interrogating a plurality of different samples. In some instances, the sample is a cell, cell lysate, tissue, tissue lysate, a bodily fluid and/or a whole organism.

### **EXAMPLES**

#### **Example 1. DNA-Antigen Exchange with biotin antigen and dye-labeled streptavidin in tissue**

[0094] Formalin-fixed paraffin-embedded (FFPE) tonsil tissue sections were dewaxed and antigen-retrieved using PT buffer (pH 6) in a Lab Vision PT-module. Tissue sections were blocked in 3% BSA and 0.2% Triton-X 100 for 1.5 hours, then rinsed in 1x PBS. Tissue sections were stained with mouse anti-cytokeratin and rabbit anti-Ki67 primary antibodies overnight at 4C in a humidity chamber. Tissue sections were then washed with 1x PBS and stained for 1.5 hours at room temperature with a goat anti-mouse secondary antibody conjugated to a DNA docking strand (D1) and a goat anti-rabbit antibody conjugated to a different DNA docking strand (D2). Tissue sections were washed again in 1x PBS and stained for DAPI. A fluorescence microscope was used to image the tissue section in the DAPI and Cy5 channels to serve as a blank. (See figure 2A)

[0095] An imager strand (I1-bio), comprising a biotinylated DNA strand hybridized to a DNA strand that includes a domain complementary to a docking strand D1, was added to the prepared tissue section and allowed to hybridize for 25 minutes at room temperature. Sections were washed to remove unbound I1-bio. Then, a solution of Cy5 labeled streptavidin (SA-Cy5) was added and allowed to incubate for 30 minutes at room temperature. The tissue was washed in 1x PBS with 0.1% Tween-20. After washing, fluorescence images were captured in the DAPI and Cy5 channels using a 10x objective. (See figure 2B)

[0096] The imager strand I1-bio was then removed by applying 10 units of USER enzyme to the tissue sections for 15 minutes at room temperature, washing with 1x PBS. A fluorescence microscope was used to confirm the complete removal of fluorescent signal in the Cy5 channel. (See figure 2C).

[0097] An imager strand (I2-bio), comprising a biotinylated DNA strand hybridized to a DNA strand that includes a domain complementary to a docking strand D2, was added to the prepared tissue section and allowed to hybridize for 25 minutes at room temperature. Sections were washed to remove unbound I2-bio. Then, a solution of Cy5 labeled streptavidin (SA-Cy5) was added and allowed to incubate for 30 minutes at room temperature. The tissue was washed in 1x PBS with 0.1% Tween-20. After washing, fluorescence images were captured in the DAPI and Cy5 channels using a 10x objective. (See figure 2D).

### **Example 2. DNA-Antigen Exchange with HRP antigen and dye-labeled anti-HRP antibody**

[0098] Formalin-fixed paraffin-embedded (FFPE) tonsil tissue sections were dewaxed and antigen-retrieved using PT buffer (pH 6) in a Lab Vision PT-module. Tissue sections were blocked in 3% BSA and 0.2% Triton-X 100 for 1.5 hours, then rinsed in 1x PBS. Tissue sections were stained with mouse anti-cytokeratin and rabbit anti-Ki67 primary antibodies overnight at 4C in a humidity chamber. Tissue sections were then washed with 1x PBS and stained for 1.5 hours at room temperature with a goat anti-mouse secondary antibody conjugated to a DNA docking strand (D1) and a goat anti-rabbit antibody conjugated to a

different DNA docking strand (D2). Tissue sections were washed again in 1x PBS and stained for DAPI. A fluorescence microscope was used to image the tissue section in the DAPI and Cy5 channels to serve as a blank.

[0099] An imager strand (I2-HRP), comprising an HRP-conjugated DNA strand hybridized to a DNA strand that includes a domain complementary to a docking strand D2, was added to the prepared tissue section and allowed to hybridize for 25 minutes at room temperature. Sections were washed to remove unbound I2-HRP. Then, the tissue section was stained with a goat anti-HRP antibody conjugated to Alexa-647 for 1.5 hours at room temperature, protected from light. Unbound material was removed by washing in 1x PBS with 0.1% Tween-20. After washing, fluorescence images were captured in the DAPI and Cy5 channels using a 10x objective. (See figure 3).

### **Example 3. DNA-Antigen Exchange with directly labeled antigen and DNA-labeled anti-antigen antibody**

[00100] Formalin-fixed paraffin-embedded (FFPE) tonsil tissue sections were dewaxed and antigen-retrieved using PT buffer (pH 9) in a Lab Vision PT-module. Tissue sections were blocked in 3% BSA and 0.2% Triton-X 100 for 1.5 hours, then rinsed in 1x PBS. Tissue sections were stained with mouse anti-cytokeratin primary antibody conjugated to dinitrophenyl (DNP) for 1 hour at room temperature, then washed to remove unbound primary antibody DNP conjugates. Tissue sections were then stained for 1.5 hours at room temperature with an anti-DNP KLH polyclonal antibody conjugated to a docking strand. Amplification of the docking strands was carried out using rolling circle amplification (RCA). Following the RCA reaction, imager strands with complementarity to the docking strands and labeled with Cy5.5 fluorophores were added and allowed to incubate for 25 minutes at room temperature to bind to the sample. Unbound imager strands were washed away and the sample was imaged with a fluorescent microscope (Figure 4).

**Example 4: Certain Embodiments**

[00101] Item A. In some embodiments, the antigen-specific binding partners and antigen-bound imager strands are added stepwise in the method herein. Namely, a method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with antigen-bound imager strands having complementarity to a docking strand, directly or indirectly,
- (4) optionally removing unbound antigen-bound imager strands,
- (5) contacting the sample with labeled antigen-specific binding partner, wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels,
- (6) optionally removing unbound antigen-specific binding partners,
- (7) imaging the sample to detect bound labeled antigen-specific binding partners,
- (8) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and
- (9) optionally repeating steps (1)-(9), or any subset thereof (such as (1)-(8) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[00102] Item B. In some embodiments, the antigen-specific binding partners and antigen-bound imager strands are premixed before including them in the method herein. Namely, a method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with labeled antigen-specific binding partners, wherein each antigen-specific binding partner is linked to one or more optical labels, wherein antigen-specific binding partners of different specificity are linked to distinct optical labels, and wherein the labeled antigen-specific binding partners are bound to antigen-bound imager strands having complementarity to a docking strand, directly or indirectly,
- (4) optionally removing unbound labeled antigen-specific binding partners and antigen-bound imager strands,
- (5) imaging the sample to detect bound labeled antigen-specific binding partners,
- (6) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and
- (7) optionally repeating steps (1)-(7), or any subset thereof (such as (1)-(6) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

{00103} Item C. A method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,

- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with labeled imager strands having complementarity to a docking strand, directly or indirectly,
- (4) optionally removing unbound labeled imager strands,
- (5) imaging the sample to detect bound labeled imager strands,
- (6) removing bound labeled imager strands,
- (7) increasing the observable signal by contacting the sample with antigen-bound imager strands having complementarity to a docking strand, directly or indirectly,
- (8) optionally removing unbound antigen-bound imager strands,
- (9) contacting the sample with labeled antigen-specific binding partner, wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels,
- (10) optionally removing unbound antigen-specific binding partners,
- (11) imaging the sample to detect bound labeled antigen-specific binding partners,
- (12) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and
- (13) optionally repeating steps (1)-(13), or any subset thereof (such as (1)-(12) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

**Example 5: Additional Embodiments**

[00104] The following numbered items provide additional support for and descriptions of the embodiments herein.

[00105] Item 1. A method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners linked (directly or indirectly) to optical labels,
  - wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and
  - wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels,
- (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners,
- (5) imaging the sample to detect bound labeled antigen-specific binding partners,
- (6) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and
- (7) optionally repeating steps (1)-(8), or any subset thereof (such as (1)-(6) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

{00106} Item 2. A method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to an antigen, and wherein target-specific binding partners of different specificity are linked to different antigens,

- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with antigen-specific binding partners linked to docking strands, wherein different antigen-specific binding partners are linked to different docking strands,
- (4) optionally removing unbound antigen-specific binding partners linked to docking strands;
- (5) adding imager strands;
  - wherein the imager strands have complementarity to a docking strand, directly or indirectly, and
  - wherein each imager strand is linked (directly or indirectly) to one or more optical labels, and wherein imager strands of different specificity are linked to distinct optical labels,
- (6) optionally removing unbound imager strands,
- (7) imaging the sample to detect bound labeled antigen-specific binding partners,
- (8) optionally removing/extinguishing signal from the optical labels (i.e., signal termination), and
- (9) optionally repeating steps (1)-(8), or any subset thereof (such as (1)-(7) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[00107] Item 3. A method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a first docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
- (2) optionally removing unbound target-specific binding partners,

(3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners,

wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and

wherein each antigen-specific binding partner is linked to at least one second docking strand;

(4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners,

(5) increasing the number of second docking strand strands by a DNA amplification reaction and labeling of the second docking strands using an optical label,

(6) imaging the sample to detect bound labeled antigen-specific binding partners,

(7) optionally extinguishing the signal from the optical label (i.e., signal termination), and

(8) optionally repeating steps (1)-(8), or any subset thereof (such as (1)-(7) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

[00108] Item 4. The method of item 3, wherein the first docking strand and the second docking strand have a different sequence.

[00109] Item 5. The method of item 3, wherein the first docking strand and the second docking strand have the same sequence.

[00110] Item 6. A method comprising:

(1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to an antigen, and wherein target-specific binding partners of different specificity are linked to different antigens,

- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with antigen-specific binding partners linked docking strands, wherein different antigen-specific binding partners are linked to different docking strands,
- (4) optionally removing unbound antigen-specific binding partners linked to docking strands;
- (5) increasing the number of docking strands by a DNA amplification reaction and labeling of the docking strands using an optical label,
- (6) imaging the sample to detect bound labeled antigen-specific binding partners,
- (7) optionally extinguishing the signal from the optical label (i.e., signal termination), and
- (8) optionally repeating steps (1)-(8), or any subset thereof (such as (1)-(7) or other subset, keeping in mind some steps may not be repeated, especially the signal termination step).

{00111} Item 7. The method of any one of items 3-6, wherein the docking strands are directly labeled using labeled nucleotides in the DNA amplification reaction.

{00112} Item 8. The method of item 7, wherein the labeled nucleotides are fluorescently-labeled nucleotides.

{00113} Item 9. The method of any one of items 3-6, wherein the docking strands are labeled using a labeled nucleic acid that binds to the docking strand.

{00114} Item 10. The method of any one of items 3-9, wherein the DNA amplification reaction comprises rolling circle amplification.

{00115} Item 11. The method of items 3-9, wherein the DNA amplification reaction comprises hybridization chain reaction.

[00116] Item 12. The method of items 3-9, wherein the DNA amplification reaction comprises hairpin-based concatemerization (including dendrimerization) reaction.

[00117] Item 13. The method of any one of items 1, 3-5, or 7-12, wherein steps (3) and (4) together comprise:

- a. first contacting the sample with antigen-bound imager strands,
- b. second optionally removing unbound antigen-bound imager strands,
- c. third contacting the sample with labeled antigen-specific binding partner, and
- d. fourth optionally removing unbound antigen-specific binding partners.

[00118] Item 14. The method of any one of items 1, 3-5, or 7-13, wherein between steps (2) and (3), the method comprises:

- a. contacting the sample with labeled imager strands having complementarity to a docking strand, directly or indirectly,
- b. optionally removing unbound labeled imager strands,
- c. imaging the sample to detect bound labeled imager strands, and
- d. removing the bound labeled imager strands from the docking strands.

[00119] Item 15. The method of any one of items 1, 3-5, or 7-14, wherein step (3) comprises contacting the sample with antigen-specific binding partners, wherein the antigen-specific binding partners are bound to antigen-bound imager strands.

[00120] Item 16. The method of any one of items 1-15, wherein the method includes removing antigen-bound imager strands not bound to docking strands.

[00121] Item 17. The method of any one of items 1, 3-5, 7-12, or 14-16, wherein the method includes removing antigen-specific binding partners not bound to antigen-bound imager strands.

[00122] Item 18. The method of any one of items 1-19, wherein the target-specific binding partner comprises an antibody or antigen-binding fragment thereof.

[00123] Item 19. The method of any one of items 1-18, wherein the target-specific binding partner comprises:

- a. a known binding partner of a target molecule or a variant of such binding partner,
- b. any binding partner of the target molecule engineered via directed evolution (e.g., peptides and aptamers), or
- c. any molecule that selectively forms at least one covalent bond with a target (e.g., a suicide substrate of an enzyme of interest).

[00124] Item 20. The method of any one of items 1-19, wherein the docking strand is a nucleic acid strand.

[00125] Item 21. The method of any one of items 1-20, wherein the imager strand is a nucleic acid strand.

[00126] Item 22. The method of any one of items 1, 3-5, 7-12, 14-16, or 18-21, wherein the antigen of the antigen-bound imager strand comprises HRP.

[00127] Item 23. The method of any one of items 1-22, wherein the antigen-specific binding partner comprises an anti-HRP antibody or antigen binding fragment thereof.

[00128] Item 24. The method of any one of items 1, 3-5, 7-12, 14-16, 18-21, or 23, wherein the antigen of the antigen-bound imager strands comprises biotin.

[00129] Item 25. The method of any one of items 1-24, wherein the antigen-specific binding partner comprises streptavidin.

[00130] Item 26. The method of any one of items 1, 3-5, 7-12, 14-16, 18-21, 23, or 25, wherein the antigen-specific binding partner comprises an

antibody or antigen binding fragment thereof that specifically binds the antigen of the antigen-bound imager strand.

[00131] Item 27. The method of any one of items 1, 3-5, 7-12, 14-16, 18-21, 23, or 25, wherein the antigen of the antigen-bound imager strand and the antigen-specific binding partner are both antibodies or antigen binding fragments thereof and the antigen-specific binding partner antibody or antigen binding fragment thereof binds to the antigen antibody or antigen binding fragment thereof of the antigen-bound imager strand.

[00132] Item 28. The method of any one of items 1-27, wherein the optical label comprises a signal-emitting molecule.

[00133] Item 29. The method of any one of items 1-38, wherein the optical label comprises a signal-absorbing molecule.

[00134] Item 30. The method of any one of items 1-29, wherein the sample is a cell, cell lysate, tissue, tissue lysate, a bodily fluid and/or a whole organism.

[00135] Item 31. The method of any one of items 1-30, wherein secondary binding partners linked to optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, either directly or indirectly.

[00136] Item 32. The method of any one of items 1, 3-5, 7-12, 14-16, 18-21, 23, 25, or 28-31, wherein secondary binding partners linked to optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, either directly or indirectly, and wherein the secondary binding partners and the antigen of the antigen-bound imager strands each comprise the same antigen.

[00137] Item 33. The method of any one of items 31-32, wherein the antigen and the secondary binding partner each comprise biotin.

[00138] Item 34. The method of any one of items 31-33, wherein the antigen-specific binding partner comprises streptavidin.

[00139] Item 35. The method of any one of items 1, 3-5, 7-12, 14-16, 18-21, 23, 25, 28-31, or 33-34, wherein secondary binding partners linked to

optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, either directly or indirectly, and wherein the secondary binding partners do not comprise the antigen of the antigen-bound imager strands.

[00140] Item 36. The method of any one of items 31-35, wherein the antigen-specific binding partners and the secondary binding partners both comprise an antibody or an antigen-binding fragment thereof.

[00141] Item 37. The method of item 36, wherein the antigen-specific binding partner is a primary antibody or antigen-binding fragment thereof to the antigen and the secondary binding partner is a secondary antibody or antigen-binding fragment thereof.

[00142] Item 38. The method of any one of items 1-37, wherein multiple antigen-specific binding partners bind to a single antigen.

[00143] Item 39. The method of any one of items 1-38, wherein the antigen-specific binding partners comprise a polyclonal antibody.

[00144] Item 40. The method of any one items 44-41, wherein multiple types of antigen-specific binding partners are used.

[00145] Item 41. The method of item 40, wherein the multiple types of antigen-specific binding partners comprise more than one monoclonal antibody directed to different epitopes on the antigen.

[00146] Item 42. A composition comprising:

- (1) a first binding partner-oligonucleotide conjugate comprising a binding partner linked to an oligonucleotide that comprises a docking domain;
- (2) an antigen-bound oligonucleotide conjugate comprising an antigen linked to an oligonucleotide that comprises an imager domain, wherein the imager domain of (2) is complementary to the docking domain of (1); and
- (3) a labeled antigen-specific binding partner comprising an optical label linked to an antigen-specific binding partner, wherein the antigen-specific binding partner of (3) specifically binds the antigen in (2).

## EQUIVALENTS

[00147] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the embodiments. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiment may be practiced in many ways and should be construed in accordance with the appended claims and any equivalents thereof.

[00148] As used herein, the term about refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. Each number in the specification or claims may be considered modified by the term about. The term about generally refers to a range of numerical values (e.g., +/-5-10% of the recited range) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). When terms such as at least and about precede a list of numerical values or ranges, the terms modify all of the values or ranges provided in the list. In some instances, the term about may include numerical values that are rounded to the nearest significant figure.

**What is Claimed is:**

1. A method comprising:
  - (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
  - (2) optionally removing unbound target-specific binding partners,
  - (3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners linked (directly or indirectly) to optical labels,  
wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and  
wherein each antigen-specific binding partner is linked to one or more optical labels, and wherein antigen-specific binding partners of different specificity are linked to distinct optical labels,
  - (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners,
  - (5) imaging the sample to detect bound labeled antigen-specific binding partners,
  - (6) optionally removing/extinguishing signal from the optical labels, and
  - (7) optionally repeating steps (1)-(7), or any subset thereof.
2. A method comprising:
  - (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to an antigen, and wherein target-specific binding partners of different specificity are linked to different antigens,

- (2) optionally removing unbound target-specific binding partners,
  - (3) contacting the sample with antigen-specific binding partners linked to docking strands, wherein different antigen-specific binding partners are linked to different docking strands,
  - (4) optionally removing unbound antigen-specific binding partners linked to docking strands;
  - (5) adding imager strands;
    - wherein the imager strands have complementarity to a docking strand, directly or indirectly, and
    - wherein each imager strand is linked (directly or indirectly) to one or more optical labels, and wherein imager strands of different specificity are linked to distinct optical labels,
  - (6) optionally removing unbound imager strands,
  - (7) imaging the sample to detect bound labeled antigen-specific binding partners,
  - (8) optionally removing/extinguishing signal from the optical labels, and
  - (9) optionally repeating steps (1)-(9), or any subset thereof.
3. A method comprising:
- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to a first docking strand, and wherein target-specific binding partners of different specificity are linked to different docking strands,
  - (2) optionally removing unbound target-specific binding partners,
  - (3) contacting the sample with antigen-bound imager strands and antigen-specific binding partners,

wherein the antigen-bound imager strands have complementarity to a docking strand, directly or indirectly, and

wherein each antigen-specific binding partner is linked to at least one second docking strand;

- (4) optionally removing unbound antigen-bound imager strands and/or antigen-specific binding partners,
- (5) increasing the number of second docking strand strands by a DNA amplification reaction and labeling of the second docking strands using an optical label,
- (6) imaging the sample to detect bound labeled antigen-specific binding partners,
- (7) optionally extinguishing the signal from the optical label, and
- (8) optionally repeating steps (1)-(8), or any subset thereof.

4. The method of claim 3, wherein the first docking strand and the second docking strand have a different sequence.

5. The method of claim 3, wherein the first docking strand and the second docking strand have the same sequence.

6. A method comprising:

- (1) contacting a sample being tested for the presence of one or more targets with one or more target-specific binding partners, wherein each target-specific binding partner is linked to an antigen, and wherein target-specific binding partners of different specificity are linked to different antigens,
- (2) optionally removing unbound target-specific binding partners,
- (3) contacting the sample with antigen-specific binding partners linked docking strands, wherein different antigen-specific binding partners are linked to different docking strands,

- (4) optionally removing unbound antigen-specific binding partners linked to docking strands;
  - (5) increasing the number of docking strands by a DNA amplification reaction and labeling of the docking strands using an optical label,
  - (6) imaging the sample to detect bound labeled antigen-specific binding partners,
  - (7) optionally extinguishing the signal from the optical label, and
  - (8) optionally repeating steps (1)-(8), or any subset thereof.
7. The method of claim 3, wherein the docking strands are directly labeled using labeled nucleotides in the DNA amplification reaction.
  8. The method of claim 7, wherein the labeled nucleotides are fluorescently-labeled nucleotides.
  9. The method of claim 3, wherein the docking strands are labeled using a labeled nucleic acid that binds to the docking strand.
  10. The method of claim 3, wherein the DNA amplification reaction comprises rolling circle amplification.
  11. The method of claim 3, wherein the DNA amplification reaction comprises hybridization chain reaction.
  12. The method of claim 3, wherein the DNA amplification reaction comprises hairpin-based concatemerization (including dendrimerization) reaction.
  13. The method of claim 1, wherein steps (3) and (4) together comprise:
    - a. first contacting the sample with antigen-bound imager strands,
    - b. second optionally removing unbound antigen-bound imager strands,
    - c. third contacting the sample with labeled antigen-specific binding partner, and

- d. fourth optionally removing unbound antigen-specific binding partners.
14. The method of claim 1, wherein between steps (2) and (3), the method comprises:
- a. contacting the sample with labeled imager strands having complementarity to a docking strand, directly or indirectly,
  - b. optionally removing unbound labeled imager strands,
  - c. imaging the sample to detect bound labeled imager strands, and
  - d. removing the bound labeled imager strands from the docking strands.
15. The method of claim 1, wherein step (3) comprises contacting the sample with antigen-specific binding partners, wherein the antigen-specific binding partners are bound to antigen-bound imager strands.
16. The method of claim 1, wherein the method includes removing antigen-bound imager strands not bound to docking strands.
17. The method of claim 1, wherein the method includes removing antigen-specific binding partners not bound to antigen-bound imager strands.
18. The method of claims 1, wherein the target-specific binding partner comprises an antibody or antigen-binding fragment thereof.
19. The method of claim 1, wherein the target-specific binding partner comprises:
- a. a known binding partner of a target molecule or a variant of such binding partner,
  - b. any binding partner of the target molecule engineered via directed evolution (e.g., peptides and aptamers), or
  - c. any molecule that selectively forms at least one covalent bond with a target (e.g., a suicide substrate of an enzyme of interest).

20. The method of claim 1, wherein the docking strand is a nucleic acid strand.
21. The method of claim 1, wherein the imager strand is a nucleic acid strand.
22. The method of claim 1, wherein the antigen-specific binding partner comprises an antibody or antigen binding fragment thereof that specifically binds the antigen of the antigen-bound imager strand.
23. The method of claim 1, wherein the antigen of the antigen-bound imager strand and the antigen-specific binding partner are both antibodies or antigen binding fragments thereof and the antigen-specific binding partner antibody or antigen binding fragment thereof binds to the antigen antibody or antigen binding fragment thereof of the antigen-bound imager strand.
24. The method of claim 1, wherein secondary binding partners linked to optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, either directly or indirectly.
25. The method of claim 1, wherein secondary binding partners linked to optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, either directly or indirectly, and wherein the secondary binding partners and the antigen of the antigen-bound imager strands each comprise the same antigen.
26. The method of claim 1, wherein secondary binding partners linked to optical labels are introduced, wherein the secondary binding partners specifically bind the antigen-specific binding partners, either directly or indirectly, and wherein the secondary binding partners do not comprise the antigen of the antigen-bound imager strands.
27. The method of claim 24, wherein the antigen-specific binding partners and the secondary binding partners both comprise an antibody or an antigen-binding fragment thereof.
28. The method of claim 27, wherein the antigen-specific binding partner is a primary antibody or antigen-binding fragment thereof to the antigen and the

secondary binding partner is a secondary antibody or antigen-binding fragment thereof.

29. The method of claim 1, wherein multiple antigen-specific binding partners bind to a single antigen.

30. The method of claim 1, wherein multiple types of antigen-specific binding partners are used.

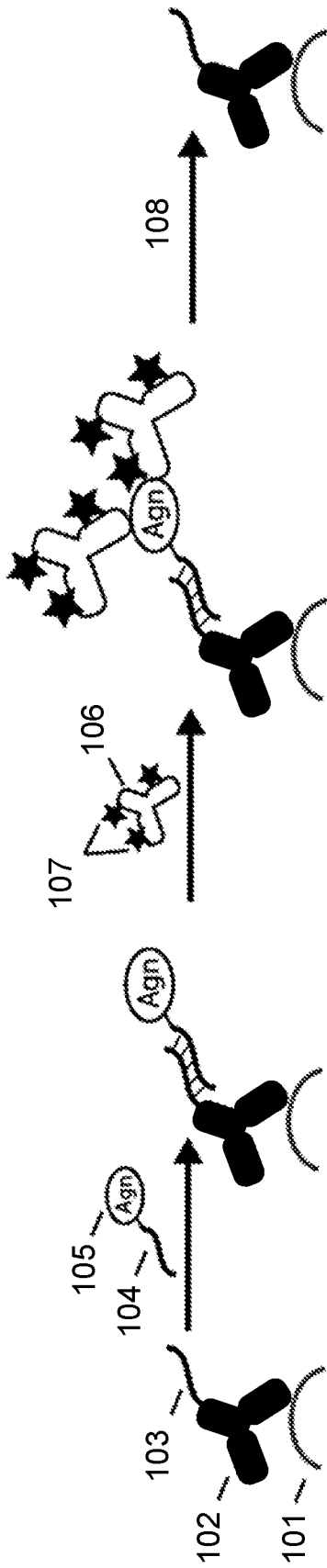


Fig. 1A

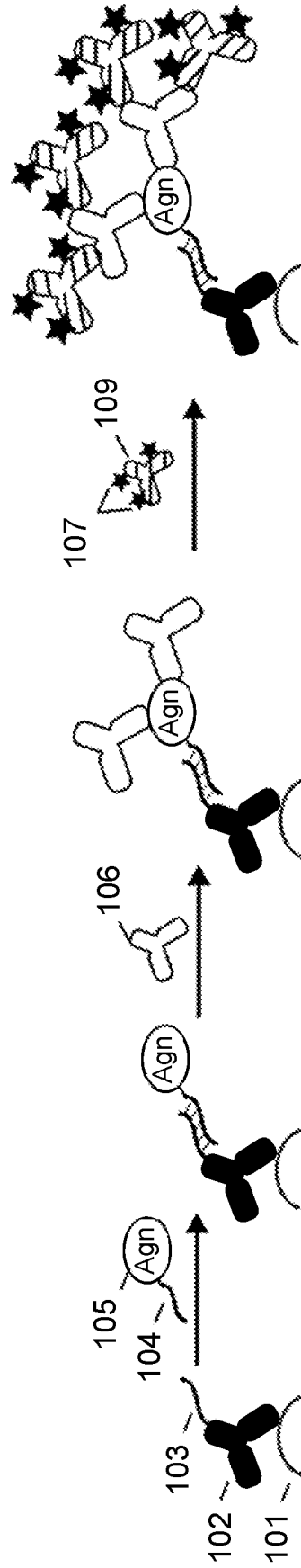


Fig. 1B

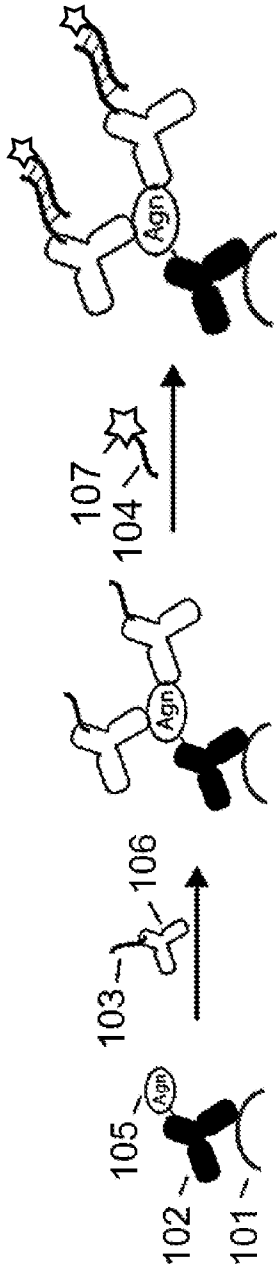


Fig. 1C

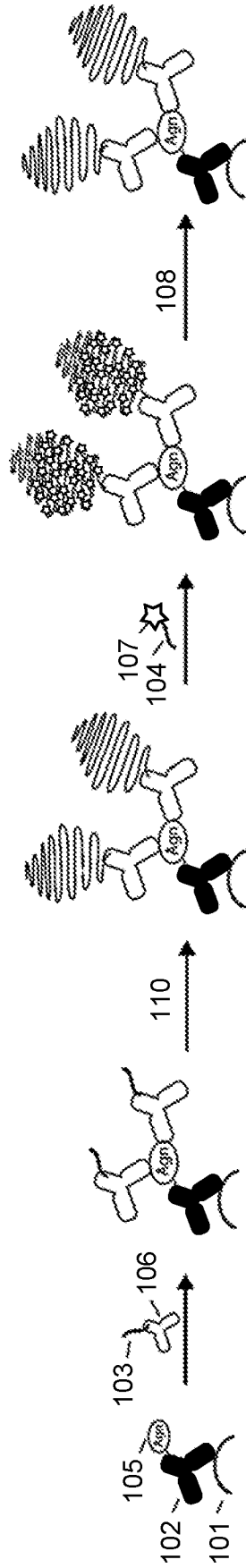


Fig. 1D

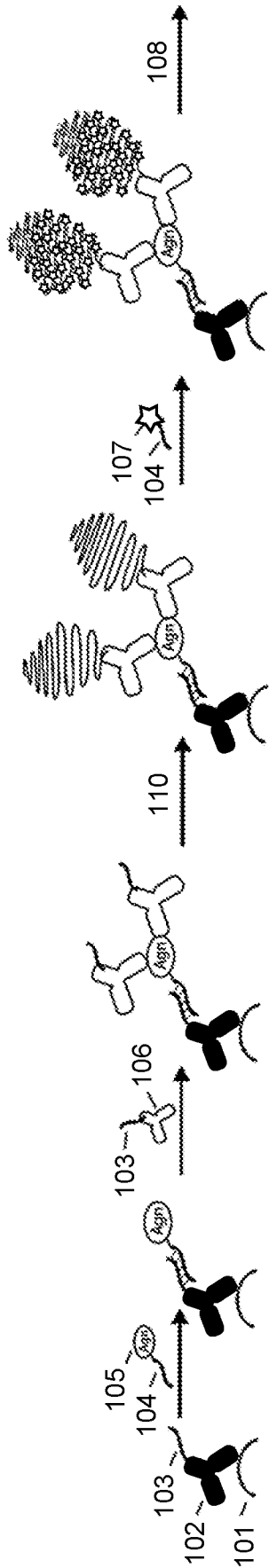


Fig. 1E

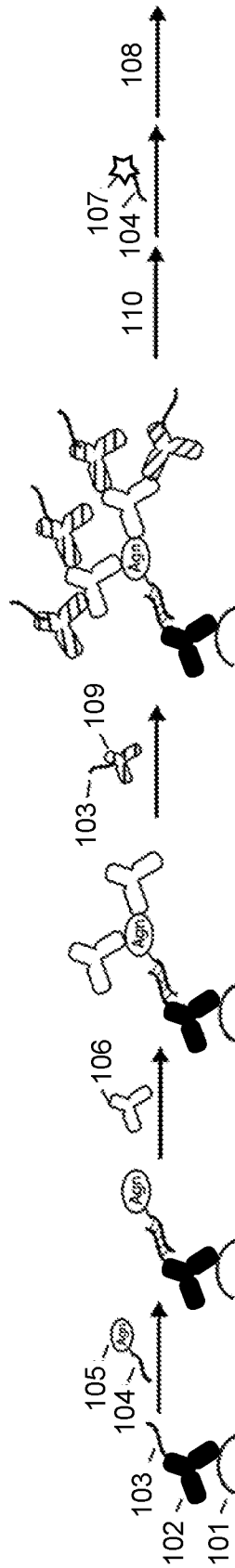
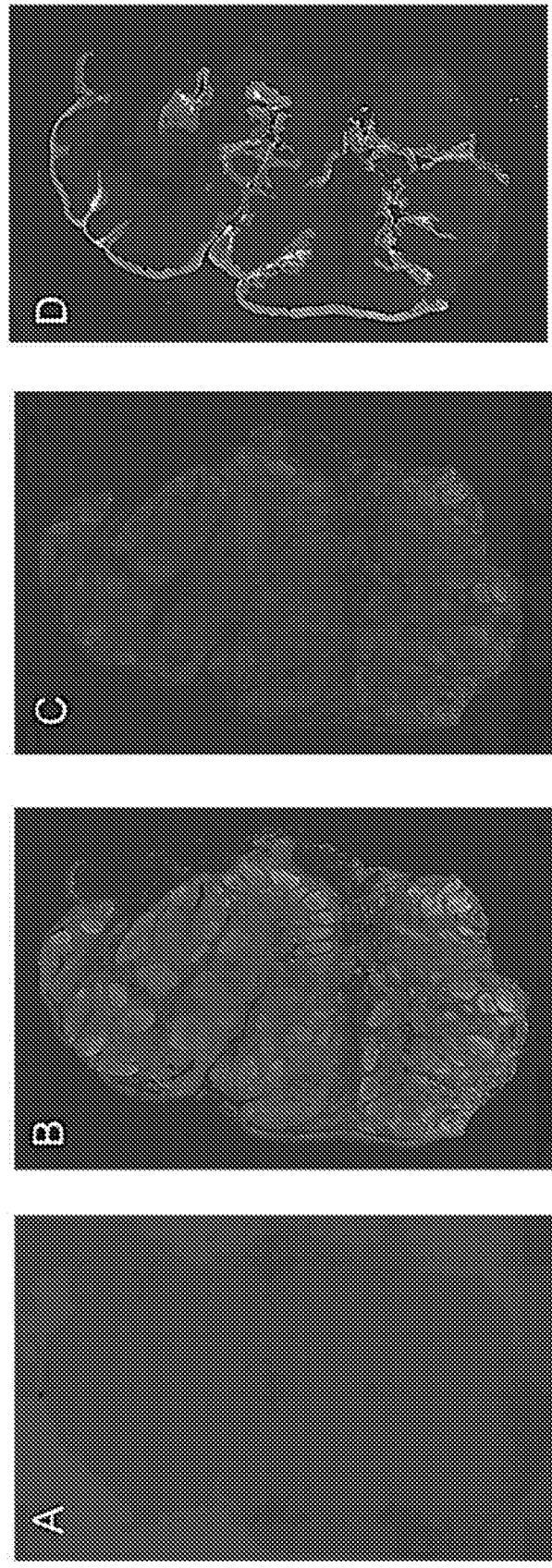


Fig. 1F



Blank

Ki67

Blank

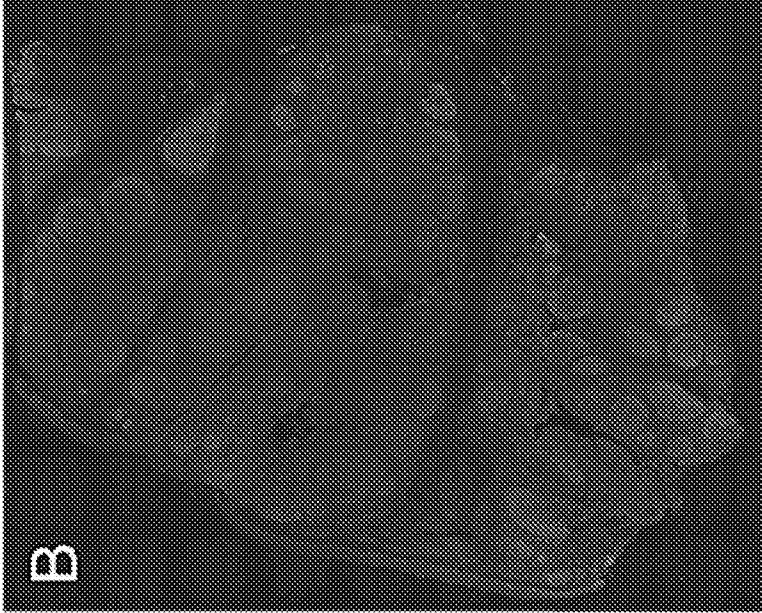
Keratin

**Fig. 2A**

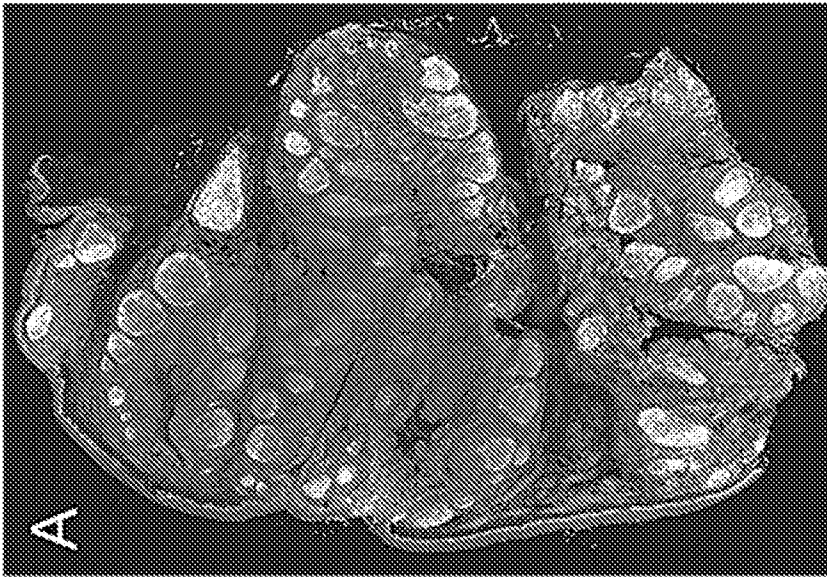
**Fig. 2B**

**Fig. 2C**

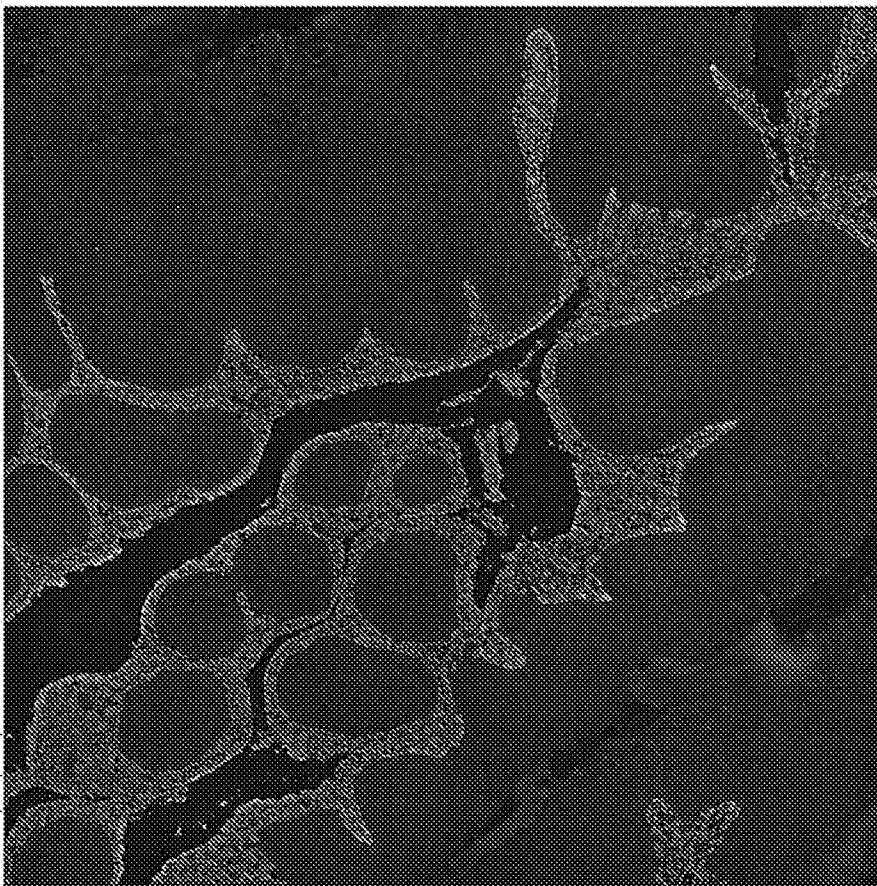
**Fig. 2D**



*Fig. 3B*



*Fig. 3A*



*Fig. 4*

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/025444

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 16/18; C07K 19/00; C12Q 1/68; G01N 33/53; G01N 33/533; G01N 33/58 (2018.01)

CPC - C07K 16/18; C12Q 1/6804; C12Q 1/6841; G01N 33/582; G01N 2458/10 (2018.05)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 435/6.11; 436/501; 506/9; 506/16; 506/18 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0258956 A1 (CELL IDX, INC.) 08 September 2016 (08.09.2016) entire document	2, 6
Y	US 2016/0319328 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 03 November 2016 (03.11.2016) entire document	2
Y	US 2016/0002704 A1 (WILLIAM MARSH RICE UNIVERSITY) 07 January 2016 (07.01.2016) entire document	6
A	US 2016/0161472 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 09 June 2016 (09.06.2016) entire document	1-30
A	US 2014/0309134 A1 (UNIVERSITY OF HOUSTON SYSTEM) 16 October 2014 (16.10.2014) entire document	1-30
A	AGASTI et al. "DNA-barcoded labeling probes for highly multiplexed exchange-PAINT imaging," Chemical Science, 30 January 2017 (30.01.2017), Vol. 8, No. 4, Pgs. 3080-3091. entire document	1-30
A	WANG et al. "Rapid Sequential in Situ Multiplexing with DNA Exchange Imaging in Neuronal Cells and Tissues," Nano Letters, 20 March 2017 (20.03.2017), Vol. 17, Pgs. 6131-6139. entire document	1-30

 Further documents are listed in the continuation of Box C. See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

14 May 2018

Date of mailing of the international search report

15 JUN 2018

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