SAMPLE COMPARTMENTALIZATION MODULE

BACKGROUND

[0001] Microscopy of sectioned tissue samples stained with fluorescent dyes and/or immunostaining (e.g., direct or indirect staining with primary or secondary antibodies conjugated to fluorophores) provides valuable histological, cellular and biomarker information. When multiple fluorescent reagents are used simultaneously for the same tissue sample spectral bleed-through i.e., detection of fluorescence from neighboring fluorescent channels in the channel of interest can hamper identification of actual targets. One workaround would be to limit the number of targets by staining samples with a few spectrally separate fluorophores. However, multiplexing the detection is often required since the availability of tissue samples can be limited, and the tissue and the detection reagents can be expensive. In addition, the process to prepare labeled tissue is laborious, particularly when multiple samples are processed in parallel. Even when multiple spectrally overlapping fluorophores are used, one must prepare additional control samples with appropriate single fluorescent reagents to separate targets computationally by linear unmixing.

[0002] What is needed is an apparatus that can divide tissue in smaller compartments providing individual sample environments for sectioned tissue samples on microscope slides or other applications.

SUMMARY

[0003] One embodiment described herein is a tissue sample compartmentalization module comprising: a first member defining a first plurality of apertures; and a second member defining a second plurality of apertures, the second member pivotably connected to the first member, wherein the second member is configured to engage with the first member to form a closed configuration or pivot relative to the first member between an open configuration and a closed configuration, wherein in the closed configuration the first plurality of apertures and the second plurality of apertures are aligned. In one aspect, the tissue sample compartmentalization module further comprises a locking arm coupled to the second member, the locking arm configured to rotate relative to the second member, wherein in the closed configuration the locking arm is configured to engage a portion of the first member to fasten the first and second members together. In another aspect, the locking arm defines a central channel, wherein in the closed configuration the central channel of the locking arm is configured to receive a portion of the first member. In another aspect, the tissue sample compartmentalization module further comprises an

insert defining a plurality of third apertures, wherein in the closed configuration the insert is positioned between the first and second members and the third plurality of apertures are aligned with the first plurality of apertures and the second plurality of apertures.

[0004] Another embodiment described herein is a method for labeling one or more analytes in a tissue sample, the method comprising: (a) placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module described herein; (b) aligning the tissue sample with the first member; (c) placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; (d) engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; (e) fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample; and (f) incubating a solution comprising an affinity molecule in an aligned second and third aperture, wherein the affinity molecule labels an analyte in the tissue sample. In one aspect, the sample is a tissue sample from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof. In another aspect, the analyte comprises one or more of metabolites, proteins, nucleic acids, carbohydrates, or lipids. In another aspect, the method further comprises labeling two or more distinct analytes, wherein each aligned second and third aperture comprises a solution comprising a distinct affinity molecule for each analyte. In another aspect, the affinity molecule is an antibody, a portion of an antibody, an antibody-like molecule, a ligand receptor, a ligand for a receptor, one member of a coupling pair, an aptamer, or an antigen. In another aspect, the affinity molecule is conjugated to a fluorescent molecule, conjugated to an enzyme, bound by another affinity molecule that is conjugated to a fluorescent molecule, or bound by another affinity molecule that is conjugated to an enzyme. In another aspect, the substrate is a microscope slide.

[0005] Another embodiment described herein is method for dividing a tissue sample into compartments for labeling two or more analytes in the tissue sample comprising: (a) placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module described herein; (b) aligning the tissue sample with the first member; (c) placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; (d) engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second

apertures with the plurality of third apertures; and (e) fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample. In one aspect, the sample is a tissue sample from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof. In another aspect, the substrate is a microscope slide. In another aspect, step (c) further comprises overlapping as many of the plurality of first apertures defined by the first member; as possible with the tissue sample.

[0006] Another embodiment described herein is a method of analyzing a tissue sample for a plurality of analytes comprising: (a) placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module described herein; (b) aligning the tissue sample with the first member; (c) placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; (d) engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; (e) fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample; and (f) incubating a solution comprising an affinity molecule in an aligned second and third aperture, wherein the affinity molecule labels an analyte in the tissue sample. (g) detecting a signal from each affinity molecule that is bound to the plurality of analytes, thereby detecting the presence or amount of each analyte in the plurality of analytes. In another aspect, the detecting is performed using a light or fluorescence microscope, a charge coupled device (CCD) camera or imager, a phosphorimager, or a combination thereof. In another aspect, the sample is a tissue from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof. In another aspect, the plurality of analytes comprises one or more of metabolites, proteins, nucleic acids, carbohydrates, or lipids. In another aspect, the affinity molecule is an antibody, a portion of an antibody, an antibody-like molecule, a ligand receptor, a ligand for a receptor, one member of a coupling pair, an aptamer, or an antigen. In another aspect, the affinity molecule is conjugated to a fluorescent molecule, conjugated to an enzyme, bound by another affinity molecule that is conjugated to a fluorescent molecule, or bound by another affinity molecule that is conjugated to an enzyme.

[0007] In another aspect, the affinity molecule is conjugated to an enzyme. In another aspect, the enzyme is horse radish peroxidase. In another aspect, the signal detected is from a tyramide

conjugate. In another aspect, the tyramide conjugate comprises on or more of Alexa Fluor 350, 488, 546, 588, 594, 647, or 750 tyramide reagents or Biotin-XX tyramide reagent.

[0008] Another embodiment described herein is a kit comprising: one or more tissue sample compartmentalization modules described herein; one or more affinity molecules; one or more signaling reagents; optionally, one or more reagents comprising deparaffinization reagents, enzyme activation reagents, antigen retrieval reagents, sample dilution reagents, reagent dilution buffers, blocking reagents, or endogenous enzyme activity blocking reagents; and optionally, one or more containers, packaging, instructions for use, MSDS sheets, reference or control tissue samples, or reference or control targets.

DESCRIPTION OF THE DRAWINGS

[0009] To describe the manner in which the above-recited and other advantages and features can be obtained, a more particular description of the subject matter briefly described above will be rendered by reference to specific embodiments which are illustrated in the appended drawings. Understanding that these drawings depict only typical embodiments and are not therefore to be limiting in scope, embodiments will be described and explained with additional specificity and detail through the use of the accompanying drawings.

[0010] FIG. 1A–B show an example of spatial transcriptomics. FIG. 1A shows the spectral emission profiles of 9 fluorophores illustrating the spectral overlap of different dyes (DAPI, eFluor 506, Alexa Fluor 488, Alexa Fluor 514, Alexa Fluor 555, eFluor 615, Alexa Fluor 647, Alexa Fluor 700, Alexa Fluor 750). FIG. 1B shows a spectrally mixed (left) and unmixed (right) composite images of human tonsil tissue sample stained with 9 fluorophores.

[0011] FIG. 2A shows a process diagram of linear unmixing of a 9-plex sample. The multiplex tissue sample was imaged for nine fluorescent dyes. Ten single color control slides were prepared to extract the spectral profiles of each fluorophore and tissue autofluorescence from which a unmixing matrix was produced. The unmixing matrix was employed to calculate the relative contribution from each fluorophore for every pixel of the mixed 9-plex image to produce the unmixed image.

[0012] FIG. 3 shows an exemplary illustration of a compartmentalized tissue sample on a microscope slide. A tissue section is affixed to the slide and divided into multiple sample area. In the illustration, the compartmentalization is a 4 × 4 array. The shape of the tissue sample controls the number of usable compartments. In the example, only 8 individual sample area are compartmentalized in the tissue.

[0013] FIG. 4 A–B show an exemplary prototype tissue compartmentalization module. FIG. 4A shows a typical size tissue samples drawn with a blue marker, with two tissues drawn per microscope slide. FIG. 4B shows two prototype compartmentalization apparata attached on the slide from FIG. 4A.

[0014] FIG. 5 A–E show fluorescent microscopy data obtained using a prototype tissue sample compartmentalization apparatus with three fluorescent dyes and an unstained control. FIG. 5A shows the compartmentalized tissue sample indicating the three dyes (eF506, AF532, AF488) and an unstained control. FIG. 5B–E shows the spectral data for eF506, AF532, AF488, and the unstained control, respectively, as compared to standard methodology in which a whole tissue section is stained without compartmentalization.

[0015] FIG. 6A–C illustrate an example of an embodiment of a tissue sample compartmentalization module. FIG. 6A is a perspective view of the module shown in an open configuration. FIG. 6B is a perspective view of the module shown in a closed configuration. FIG. 6C is a top-down view of the module in the closed configuration illustrating a plurality of aligned apertures.

[0016] FIG. 7A–D show a process workflow for using the tissue sample compartmentalization module. FIG. 7A illustrates a microscope slide is shown with tissue sections. FIG. 7B illustrates two tissue sample compartmentalization modules in an open configuration are centered on each tissue section to maximize the available tissue. FIG. 7C illustrates the modules in a closed and locked configuration, and immunohistochemical (or other) assays are performed. FIG. 7D illustrates the modules removed where the samples are imaged using fluorescence microscopy and spectral extraction is performed as needed.

[0017] FIG. 8A–B show fluorescence microscopy data for two fluorophores (AF488, AF555) obtained using a tissue sample compartmentalization module as compared to standard methodology.

[0018] FIG. 9A–D show a process workflow for using the tissue sample compartmentalization module with individual cell cultures. FIG. 9A shows a cell attachment enhancing surface coated microscope slide. FIG. 9B shows cell suspensions seeded in individual compartments created by the tissue sample compartmentalization module. FIG. 9C shows experiments being performed in individual sample environments of the tissue sample compartmentalization module (e.g., drug treatments, immunofluorescence staining). FIG. 9D shows a microscope slide ready for analysis after the issue sample compartmentalization modules have been removed.

DETAILED DESCRIPTION

[0019] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. For example, any nomenclatures used in connection with, and techniques of microscopy, biochemistry, molecular biology, immunology, microbiology, genetics, cell and tissue culture, and protein and nucleic acid chemistry described herein are well known and commonly used in the art. In case of conflict, the present disclosure, including definitions, will control. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the embodiments and aspects described herein.

[0020] As used herein, the terms "amino acid," "nucleotide," "polynucleotide," "vector," "polypeptide," and "protein" have their common meanings as would be understood by a biochemist of ordinary skill in the art. Standard single letter nucleotides (A, C, G, T, U) and standard single letter amino acids (A, C, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, or Y) are used herein.

[0021] As used herein, terms such as "include," "including," "contain," "containing," "having," and the like mean "comprising." The present disclosure also contemplates other embodiments "comprising," "consisting essentially of," and "consisting of" the embodiments or elements presented herein, whether explicitly set forth or not. As used herein, "comprising," is an "open-ended" term that does not exclude additional, unrecited elements or method steps. As used herein, "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim.

[0022] As used herein, the term "a," "an," "the" and similar terms used in the context of the disclosure (especially in the context of the claims) are to be construed to cover both the singular and plural unless otherwise indicated herein or clearly contradicted by the context. In addition, "a," "an," or "the" means "one or more" unless otherwise specified.

[0023] As used herein, the term "or" can be conjunctive or disjunctive.

[0024] As used herein, the term "and/or" refers to both the conjunctive and disjunctive.

[0025] As used herein, the term "substantially" means to a great or significant extent, but not completely.

[0026] As used herein, the term "about" or "approximately" as applied to one or more values of interest, refers to a value that is similar to a stated reference value, or within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, such as the limitations of the measurement

system. In one aspect, the term "about" refers to any values, including both integers and fractional components that are within a variation of up to ± 10% of the value modified by the term "about." Alternatively, "about" can mean within 3 or more standard deviations, per the practice in the art. Alternatively, such as with respect to biological systems or processes, the term "about" can mean within an order of magnitude, in some embodiments within 5-fold, and in some embodiments within 2-fold, of a value. As used herein, the symbol "~" means "about" or "approximately."

[0027] All ranges disclosed herein include both end points as discrete values as well as all integers and fractions specified within the range. For example, a range of 0.1–2.0 includes 0.1, 0.2, 0.3, 0.4 . . . 2.0. If the end points are modified by the term "about," the range specified is expanded by a variation of up to ±10% of any value within the range or within 3 or more standard deviations, including the end points, or as described above in the definition of "about."

[0028] As used herein, the terms "active ingredient" or "active pharmaceutical ingredient" refer to a pharmaceutical agent, active ingredient, compound, or substance, compositions, or mixtures thereof, that provide a pharmacological, often beneficial, effect.

[0029] As used herein, the terms "control," or "reference" are used herein interchangeably. A "reference" or "control" level may be a predetermined value or range, which is employed as a baseline or benchmark against which to assess a measured result. "Control" also refers to control experiments or control cells.

[0030] "Antibody" as used herein means an immunoglobulin or a fragment thereof and encompasses any polypeptide comprising an antigen-binding site regardless of the source, method of production, and other characteristics.

[0031] An "analyte" or "antigen" as used herein refers to any substance recognized by an antibody, or another means of detection.

[0032] A "detectable label" as used herein refers to any molecule which may be detected directly or indirectly to reveal the presence of a target in the sample. A direct detectable label may be used. Direct detectable labels may be detected per se without the need for additional molecules. Examples include fluorescent dyes, radioactive substances, and metal particles. Indirect detectable labels may be used, which require the employment of one or more additional molecules. Examples include enzymes that affect a color change in a suitable substrate, as well as any molecule that may be specifically recognized by another substance carrying a label or react with a substance carrying a label. Other examples of indirect detectable labels thus include antibodies, antigens, nucleic acids and nucleic acid analogs, ligands, substrates, and haptens.

[0033] Examples of detectable labels which may be used herein include, but are not limited to, fluorophores, chromophores, electrochemiluminescent labels, bioluminescent labels, polymers,

polymer particles, bead or other solid surfaces, gold or other metal particles or heavy atoms, spin labels, radioisotopes, enzyme substrates, haptens, antigens, Quantum Dots, aminohexyl, pyrene, nucleic acids or nucleic acid analogs, or proteins, such as receptors, peptide ligands, or substrates, enzymes, and antibodies (including antibody fragments). Some detectable labels may comprise "color labels," in which the target is detected by the presence of a color, or a change in color in the sample. Examples of "color labels" include, but are not limited to, chromophores, fluorophores, chemiluminescent compounds, electrochemiluminescent labels, bioluminescent labels, and enzymes that catalyze a color change in a substrate. More than one type of color may be used, for instance, by attaching distinguishable color labels to a single detection unit or by using more than one detection unit, each carrying a different and distinguishable color label.

[0034] "Fluorophore" as used herein is a molecule that emits detectable electro-magnetic radiation upon excitation with electro-magnetic radiation at one or more wavelengths. A large variety of fluorophores are known in the art and are developed by chemists for use as detectable molecular labels and can be conjugated to affinity molecules described herein.

[0035] As used herein, the terms "inhibit," "inhibition," or "inhibiting" refer to the reduction or suppression of a given biological process, condition, symptom, disorder, or disease, or a significant decrease in the baseline activity of a biological activity or process.

[0036] The terms "recognize," "recognition," or "recognizing," etc., as used herein, mean an event in which one substance, such as an affinity molecule, directly or indirectly interacts with a target in any way such that the interaction with the target may be detected by an affinity molecule. In some nonlimiting examples, a probe may react with a target, or directly bind to a target, or indirectly react with or bind to a target by directly binding to another substance that in turn directly binds to or reacts with a target.

[0037] As used herein, the term "subject" refers to an animal. Typically, the subject is a mammal. A subject also refers to primates (e.g., humans, male or female; infant, adolescent, or adult), non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or archaea, among other life forms. In one embodiment, the subject is a primate. In one embodiment, the subject is a human.

[0038] "Target," also used interchangeably with "analyte," as used herein refers to any substance present in a sample that is capable of being detected.

[0039] Apparatus

[0040] With reference to FIG. 6A, an example of an embodiment of a tissue sample compartmentalization module **100** is illustrated. The module **100** includes a first member **104** and a second member **108**. The second member **108** is pivotably connected to the first member **104**

by a pivot assembly **112**. The pivot assembly **112** includes a first pivot member **116** coupled to the second member **108**. The first pivot member **116** is received by a first slot **120** defined by the first member **104**. The pivot assembly **112** is configured to facilitate pivoting movement of the second member **108** relative to the first member **104** between a first, open configuration (shown in FIG. 6A) and a second, closed configuration (shown in FIG. 6B). Stated another way, the second member **108** is configured to rotate relative to an axis defined by the first pivot member **116**.

[0041] The first member **104** includes a planar surface **124**. A plurality of first apertures **128** are arranged on the planar surface **124**. The first apertures **128** extend through the planar surface **124**, and entirely through the first member **104**. Thus, the first member **104** defines the plurality of first apertures **128**. In the illustrated embodiment, the plurality of first apertures **128** are arranged in a grid pattern. More specifically, the plurality of first apertures **128** are arranged in a 5 × 4 grid pattern. However, in other examples of embodiments, the plurality of first apertures **128** can be arranged in any suitable or desired pattern. In addition, the plurality of first apertures **128** are illustrated as having a square shape. In other examples of embodiment, the plurality of first apertures **128** can have any suitable polygonal shape (e.g., a circle, a triangle, a rectangle, a pentagon, etc.).

[0042] The first member **104** includes a pair of first projections **132** that extend away from the planar surface **124**. The first projections **132** each define the slot **120**. The first member **104** also includes a second projection **136**. The second projection **136** extend away from the planar surface **124** such that the planar surface **124** is recessed relative to the projections **132**, **136**. Stated another way, the first member **104** is offset from the second member **108**. The projections **132**, **136** are respectively positioned on opposing ends of the first member **104**. The projections **132**, **136** also serve as guides to facilitate alignment of a microscope slide on the planar surface **124** of the first member **104**.

[0043] With reference now to FIG. 6B, the second member **108** includes a plurality of second apertures **140**. The second apertures **140** extend entirely through the second member **108**. Thus, the second member **108** defines the plurality of second apertures **140**. Each of the plurality of second apertures **140** are vertically aligned with one of the plurality of first apertures **128**. Accordingly, the plurality of second apertures **140** are arranged in a similar configuration as the first apertures **128**. In the illustrated embodiment, the plurality of second apertures **140** are arranged in a grid pattern, and more specifically in a 5 × 4 grid pattern. However, in other examples of embodiments, the plurality of second apertures **140** can be arranged in any suitable or desired pattern such that each second aperture **140** is vertically aligned with an associated first

aperture **128**. In addition, the plurality of second apertures **140** are illustrated as having a square shape. In other examples of embodiments, the plurality of second apertures **140** can have any suitable polygonal shape (e.g., a circle, a triangle, a rectangle, a pentagon, etc.). The shape of the second apertures **140** is preferably the same as the shape of the first apertures **128**.

[0044] The second member **108** includes a locking arm **144**. The locking arm **144** is positioned on an end of the second member **108** opposite the pivot member **116**. The locking arm **144** includes a second pivot member **148**. The second pivot member **148** is received by a second slot **152** defined by the second member **108**. The locking arm **144** is accordingly configured to engage with the first member to form a closed configuration or pivot (or rotate) relative to the second member **108**. Stated another way, the locking arm **144** is configured to rotate relative to an axis defined by the second pivot member **148**.

[0045] With reference back to FIG. 6A, the locking arm **144** defines a C-shaped (or U-shaped) member. The C-shaped member includes a central channel that is configured to receive a portion of the first member **104** in the second, closed configuration (shown in FIG. 6B). Accordingly, the second, closed configuration can also be referred to as a locked configuration.

[0046] An insert **156** is configured to be positioned between the first member **104** and the second member **108**. More specifically, the insert **156** is configured to be trapped between (or sandwiched between) the first and second members **104**, **108**. The insert **156** is configured to be received by the offset space between the first and second members **104**, **108**. The insert **156** defines a plurality of third apertures **160**. The third apertures **160** extend entirely through the insert **156**. Thus, the insert **156** defines the plurality of third apertures **160**. Each of the plurality of third apertures **156** is configured to be vertically aligned with one of the plurality of first apertures **128** and one of the plurality of second apertures **140**. Accordingly, the plurality of third apertures **156** are arranged in a similar configuration as both the first and apertures **128**, **140**. In the illustrated embodiment, the plurality of third apertures **160** are arranged in a grid pattern, and more specifically in a 5 × 4 grid pattern. However, in other examples of embodiments, the plurality of third apertures **160** can be arranged in any suitable or desired pattern such that each third aperture **160** is vertically aligned with an associated first aperture **128** and second aperture **140**. Exemplary embodiments include 4 × 4, 5 × 4, 5 × 5, 5 × 8, 5 × 8, 6 × 6, 6 × 8, 8 × 8, and other iterations. In addition, the plurality of third apertures **160** are illustrated as having a square shape. In other examples of embodiments, the plurality of third apertures **160** can have any suitable polygonal shape (e.g., a circle, a triangle, a rectangle, a pentagon, etc.). The shape of the third apertures **160** is preferably the same (or complimentary) as the shape of the first and second

apertures **128**, **140**. In the illustrated embodiment, the insert **156** is a silicon insert that is configured to be removable from the module **100**.

[0047] With reference to FIG. 6C, it should also be appreciated that in the closed configuration, each first, second, and third aperture **128**, **140**, **160** is aligned (or vertically aligned). The aligned first, second, and third apertures **128**, **140**, **160** define a passage that extends entirely thought the module **100**. The first, second, and third apertures **128**, **140**, **160** can also be referred to as first, second, and third wells **128**, **140**, **160**.

[0048] Methods of Manufacture

[0049] The tissue sample compartmentalization modules described herein may be manufactured using standard manufacturing methods including injection molding, 3D-printing, casting, laser ablation, punching, other means, or combinations thereof.

[0050] The tissue sample compartmentalization modules may comprise materials such as plastics (polyethylene, polypropylene, polystyrene, polyoxymethylene, polytetrafluoroethylene, copolymers), metals (aluminum, titanium, stainless steel), glass, composite materials (graphite, carbon fibers, fiberglass), or combinations thereof. The insert or gasket may comprise water-tight materials such as silicone, closed cell foams, open cell foams, polytetrafluoroethylene, rubber, fiberglass, aramid fibers, felt, cork, paper, or combinations thereof.

[0051] Methods of Use

[0052] Provided herein are methods of dividing a tissue sample into compartments for labeling two or more analytes in the tissue sample. The methods may comprise: placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module, wherein the tissue sample compartmentalization module comprises a first member defining a first plurality of apertures; and a second member defining a second plurality of apertures, the second member pivotably connected to the first member, the second member is configured to engage with the first member to form a closed configuration or pivot relative to the first member between an open configuration and a closed configuration, wherein in the closed configuration the first plurality of apertures and the second plurality of apertures are aligned; aligning the tissue sample with the first member; placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; and fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample. In one embodiment, aligning the tissue sample with the first member may further include overlapping as

many of the plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member as possible with the tissue sample to create a plurality of sample compartments.

[0053] With reference to FIGS. 7A–7D, a process for using the tissue sample compartmentalization module **100** is illustrated. With specific reference to FIG. 7A, a microscope slide **200** is shown with a plurality of tissue sections **204a**, **204b** positioned thereon.

[0054] Next, in FIG. 7B, a plurality of tissue sample compartmentalization modules **100** are placed into engagement with the microscope slide **200**. In the illustrated example, a pair of modules **100** are positioned into engagement with the slide **200**, such that one module **100** is associated with each tissue sections **204a**, **204b**. Each module **100** is positioned in an open configuration, as shown in FIG. 6A. The microscope slide **200** is positioned on the planar surface **124** of the first member **104** of each module **100**. The projections **132**, **136** of each member facilitate alignment of the slide **200** relative to the first member **104**. Once the slide **200** is positioned on the planar surface **124** of the first member **104**, the slide **200** can be laterally moved relative to the first member **104** to align the respective tissue section **204a**, **204b** over the plurality of first apertures **128**. The insert **156** is then positioned onto the slide **200**. The insert **156** is positioned over each respective tissue section **204a**, **204b** with the plurality of third apertures **160** being vertically aligned with the plurality of first apertures **128**. The insert **156** can be manually positioned on the slide **200** or can be configured to be positioned on the slide **200** in response to pivoting movement of the second member **108** relative to the first member **104** from the open configuration (*see* FIG. 6A) to the closed configuration (*see* FIG. 6B).

[0055] With reference now to FIG. 7C, each module **100** is actuated into the closed configuration and locked. Once positioned on the slide, the second member **108** of each module **100** is pivoted relative to the first member **104** to align the plurality of second apertures **140** with the plurality of third apertures **160** of the insert **156**. The locking arm **144** can then pivot relative to the second member **108** into engagement with the first member **104**, locking the members **104**, **108** together. It should be appreciated that the slide **200** and the insert **156** are sandwiched (or positioned between) the members **104**, **108**. In addition, the second and third apertures **140**, **160** cooperate to form a plurality of wells that are separated from the first apertures **128** by the slide **200**. The wells provide access to the tissue samples **204a**, **204b** on the slide **200**. Thus, one or more materials can be introduced into each well to interact with a portion of the tissue sample **204a**, **204b** positioned in the well.

[0056] With reference to FIG. 7D, the modules **100** have been unlocked and removed from engagement with the slide **200**. The samples **204a**, **204b** on the slide are imaged using fluorescence microscopy and linear unmixing is performed as needed.

[0057] The methods described herein may be used in an immunohistochemistry assay, an immunocytochemistry assay, an in-situ hybridization (ISH) assay, enzyme immuno-assays (EIA), enzyme linked immuno-assays (ELISA), blotting methods (e.g., Western, Southern, and Northern), labeling inside electrophoresis systems or on surfaces or arrays, or other general detection assays known in the art.

[0058] For example, IHC provides a method of detecting targets in a sample or tissue specimen *in situ*. *See e.g.,* Mokry, *Acta Medica* 39(4): 129-140 (1996). The overall cellular integrity of the sample is maintained in IHC, thus allowing detection of both the presence and location of the targets of interest. Typically, a sample is fixed with formalin, embedded in paraffin, and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labeling or secondary antibody-based or hapten-based labeling. Examples of known IHC systems include, but are not limited to, EnVision™ (DakoCytomation), Powervision® (Immunovision, Springdale, Ariz.), the NBA™ kit (Zymed Laboratories Inc., South San Francisco, Calif.), HistoFine® (Nichirei Corp, Tokyo, Japan). The apparatus and methods disclosed herein may allow for enhancement of signal, increased flexibility in IHC detection platforms, or a combination thereof.

[0059] Many types of samples are compatible with the apparatus and methods disclosed herein. Samples may comprise a solid, for example, containing targets in a tissue slice from an organ. Samples may be derived from living matter taken from any living organism, such as an animal, such as mammals (e.g., humans), plants, fungi, archaea, or bacteria. Thus, samples may comprise eukaryotic cells, archaeal cells, or prokaryotic cells. The tissue sample may be from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof.

[0060] Samples may comprise a cell sample, such as a cell smear or colony, or a tissue specimen derived from a living organism, such as a tissue sample from an organ. Samples may also comprise other naturally obtained samples such as plant tissue samples, and synthetically derived samples such as chemical or industrial products and food products.

[0061] Tissue or cell samples may be prepared by a variety of methods known to those of ordinary skill in the art, depending on the type of sample and the assay format. For instance, tissue or cell samples may be fresh or preserved, and may be, for example, flash-frozen, smeared, dried,

embedded, or fixed on slides or other supports. Samples may be prepared and stained using a free-floating technique. For example, a tissue section may be brought into contact with different reagents and wash buffers in suspension or freely floating in appropriate containers, for example microcentrifuge tubes, before being mounted on slides for further treatment and examination by the methods described herein.

[0062] A tissue section may be mounted on a slide or other substrate after an incubation with immuno-specific reagents. The remains of the staining process may then be conducted after mounting. For example, for microscopic inspection in IHC and ISH, samples may be comprised in a tissue section mounted on a suitable solid substrate. For photomicrographs, sections comprising samples may be mounted on a glass slide or other planar substrate, to highlight by selective staining certain morphological indicators of disease states or detection of detectable targets. The substrate may be a glass or plastic microscope slide (e.g., 26 × 75 × 1 mm or 1 × 3 × 0.04 in).

[0063] For IHC, a sample may be taken from an individual, fixed, and exposed to, for example, antibodies which specifically bind to the detectable target of interest. Sample processing steps may include, for example, antigen retrieval, exposure to a primary antibody, washing, exposure to a secondary antibody (optionally coupled to a suitable detectable label), washing, and exposure to a tertiary antibody linked to a detectable label. Washing steps may be performed with any suitable buffer or solvent, e.g., phosphate-buffered saline (PBS), Tris-buffered saline (TBS), or distilled water. The wash buffer may optionally contain a detergent, e.g., polyoxyethylene sorbitan monolaurate (TWEEN®20) or octylphenoxypolyethoxyethanol (Nonidet P-40).

[0064] IHC samples may include, for instance: preparations comprising un-fixed fresh tissues and/or cells or solution samples; fixed and embedded tissue specimens, such as archived material; and frozen tissues or cells. An IHC staining procedure may comprise steps such as: cutting and trimming tissue, fixation, dehydration, paraffin infiltration, cutting in thin sections, mounting onto glass slides, baking, deparaffination, rehydration, antigen retrieval, blocking steps, applying primary antibody, washing, applying secondary antibody-enzyme conjugate, washing, applying a tertiary antibody conjugated to a polymer and linked with an enzyme, applying a chromogen substrate, washing, counter staining, applying a cover slip, and microscopic examination using a fluorescence microscope and processed using imaging and unmixing software.

[0065] ISH samples, for instance, may be taken from an individual and fixed before being exposed to a nucleic acid or nucleic acid analog probe on a recognition unit. The nucleic acid in the sample may first be denatured to expose the target binding sites. Various counterstains or

paints may further be used to locate nucleic acid molecules or chromosomes within an ISH sample.

[0066] Tissue or cell samples may be fixed or embedded. Fixatives may be needed, for example, to preserve cells and tissues in a reproducible and life-like manner. Fixatives may also stabilize cells and tissues, thereby protecting them from the rigors of processing and staining techniques. For example, samples comprising tissue blocks, sections, or smears may be immersed in a fixative fluid, or in the case of smears, dried.

[0067] Many methods of fixing and embedding tissue specimens are known, for example, alcohol fixation and formalin-fixation and subsequent paraffin embedding (FFPE). Any suitable fixing agent may be used. Examples include ethanol, acetic acid, picric acid, 2-propanol, 3,3′-diaminobenzidine tetrahydrochloride dihydrate, acetoin (mixture of monomer) and dimer, acrolein, crotonaldehyde (*cis* + *trans*), formaldehyde, glutaraldehyde, glyoxal, potassium dichromate, potassium permanganate, osmium tetroxide, paraformaldehyde, mercuric chloride, tolylene-2,4-diisocyanate, trichloroacetic acid, and tungstic acid. Other examples include formalin (aqueous formaldehyde), neutral buffered formalin, glutaraldehyde, carbodiimide, imidates, benzoequinone, osmic acid, and osmium tetraoxide. Fresh biopsy specimens, cytological preparations (including touch preparations and blood smears), frozen sections, and tissues for IHC analysis may be fixed in organic solvents, including ethanol, acetic acid, methanol and/or acetone.

[0068] It may be useful to pre-treat the samples to increase the reactivity or accessibility of a detectable target and to reduce nonspecific interactions. If the target is an antigen, for example, a process called “antigen retrieval” may be used (and which is also known in the art as target retrieval, epitope retrieval, target unmasking, or antigen unmasking). *See, e.g.*, Shi et al., *J. Histochem. Cytochem.* 45(3): 327-343 (1997). Antigen retrieval encompasses a variety of methods including enzymatic digestion with proteolytic enzymes, such as proteinase, pronase, pepsin, papain, trypsin, or neuraminidase. Heat may be used, such as heat-induced epitope retrieval or HIER. Heating may involve microwave irradiation, a water bath, a steamer, a regular oven, an autoclave, or a pressure cooker in an appropriately pH stabilized buffer, usually containing EDTA, EGTA, Tris-HCl, citrate, urea, glycin-HCl, or boric acid. Detergents may be added to the HIER buffer to increase the epitope retrieval, or to the dilution media and/or rinsing buffers to lower non-specific binding. Combinations of different antigen retrieval methods may be used. The antigen retrieval buffer may be aqueous, but may also contain other solvents, including solvents with a boiling point above that of water such as glycerol. This allows for treatment of the tissue at greater than 100 °C at standard pressure.

[0069] Signal-to-noise ratio may be increased by different physical methods, including application of vacuum, ultrasound, or freezing and thawing tissue samples before or during incubation of the reagents.

[0070] Treatments may be performed to reduce nonspecific binding. For example, carrier proteins, carrier nucleic acid molecules, salts, or detergents may reduce or prevent non-specific binding. Non-specific binding sites may be blocked in some embodiments with inert proteins like, HSA, BSA, ovalbumin, with fetal calf serum or other sera, or with detergents like polyoxyethylene sorbitan monolaurate (TWEEN®20), octylphenoxypolyethoxyethanol (Nonidet P-40), *t*-octylphenoxypolyethoxyethanol (TRITON™ X-100), triterpene glycosides (Saponin), nonionic polyoxyethylene surfactants (BRIJ®-35), or nonionic triblock copolymers (PLURONICS®). Alternatively, non-specific binding sites may be blocked with unlabeled competitors for the recognition event between the target and the affinity molecule. For example, in the case of a nucleic acid interaction, non-specific binding may be reduced by adding unlabeled competitor nucleic acids or nucleic acid analogs such as digested, total human DNA, or unlabeled versions of the affinity molecule. In addition, repetitive sequences may be blocked, for example, using nucleic acids or nucleic acid analogs that specifically recognize those sequences, or sequences derived from a total DNA preparation. Salt, buffer, and temperature conditions may also be modified to reduce non-specific binding.

[0071] Cross reactivity of different components of the detection methods may be avoided, for example, by using antibodies derived from different species. Furthermore, combinations of, for example, secondary antibodies against primary antibodies and haptens may also be used to avoid unwanted cross reactivity. Endogenous biotin binding sites or endogenous enzyme activity (for example phosphatase, catalase, or peroxidase) may be removed as a step in the staining procedure. Endogenous biotin and peroxidase activity may be removed by treatment with peroxides, while endogenous phosphatase activity may be removed by treatment with levamisole. Heating may destroy endogenous phosphatase and esterase activity.

[0072] Methods for Growing Cells on Substrates

[0073] Described herein are methods for growing cells on a substrate such as a glass microscope slide. The method may comprise treating the substrate with a cell adherence facilitating substance; placing the treated substrate onto the first member of the tissue sample compartmentalization module, wherein the tissue sample compartmentalization module comprises a first member defining a first plurality of apertures; and a second member defining a second plurality of apertures, the second member pivotably connected to the first member, the second member is configured to engage with the first member to form a closed configuration or

pivot relative to the first member between an open configuration and a closed configuration, wherein in the closed configuration the first plurality of apertures and the second plurality of apertures are aligned; aligning the treated substrate on the first member; placing an insert on the treated substrate to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; engaging or pivoting the second member relative to the first member to sandwich the insert and the treated substrate between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; fastening the first member and the second member together by a locking arm, sealing insert to the treated substrate; dispensing a solution comprising cells and a medium into one or more of the aligned second and third apertures; sealing the apertures; and incubating the tissue sample compartmentalization module/treated substrate comprising cell cultures under conditions to facilitate grown of the cell culture. After incubation for a period of time, the cells can be imaged or fixed and stained with immunohistochemical reagents as described herein. The sample can be preserved by disassembling the tissue sample compartmentalization module and covering the cells with mounting medium and cover slip.

[0074] Methods for Performing Immunohistochemistry

[0075] Described herein are methods for labeling one or more analytes in a tissue sample described herein. The method may comprise: placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module, wherein the tissue sample compartmentalization module comprises a first member defining a first plurality of apertures; and a second member defining a second plurality of apertures, the second member pivotably connected to the first member, the second member is configured to engage with the first member to form a closed configuration or pivot relative to the first member between an open configuration and a closed configuration, wherein in the closed configuration the first plurality of apertures and the second plurality of apertures are aligned; aligning the tissue sample with the first member; placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample; and incubating a solution comprising an affinity molecule in an aligned second and third aperture, wherein the affinity molecule labels an analyte in the tissue sample.

[0076] The method may also include labeling two or more distinct analytes. Each aligned second and third aperture of tissue sample compartmentalization module described herein may comprise a solution comprising a distinct affinity molecule for each analyte. The methods described herein may further comprise multiplexing performed for two or more targets simultaneously, wherein the two or more target molecules are each separately bound by affinity molecules. Multiplexing may be performed for 10 or more targets simultaneously, wherein the 10 or more target molecules are each separately bound by affinity molecules. Multiplexing may be performed for 100 or more targets simultaneously, wherein the 100 or more target molecules are each separately bound by affinity molecules.

[0077] Also described herein are methods of analyzing a tissue sample for a plurality of analytes. The method may include: placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module, wherein the tissue sample compartmentalization module comprises a first member defining a first plurality of apertures; and a second member defining a second plurality of apertures, the second member pivotably connected to the first member, the second member is configured to engage with the first member to form a closed configuration or pivot relative to the first member between an open configuration and a closed configuration, wherein in the closed configuration the first plurality of apertures and the second plurality of apertures are aligned; aligning the tissue sample with the first member; placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member; engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample; incubating a solution comprising an affinity molecule in an aligned second and third aperture, wherein the affinity molecule labels an analyte in the tissue sample; and detecting a signal from each affinity molecule that is bound to the plurality of analytes, thereby detecting the presence or amount of each analyte in the plurality of analytes.

[0078] A tissue sample described herein may be from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof.

[0079] An analyte or plurality of analytes described herein may include one or more of metabolites, proteins, nucleic acids, carbohydrates, or lipids.

[0080] An affinity molecule described herein may be an antibody, a portion of an antibody, an antibody-like molecule, a ligand receptor, a ligand for a receptor, one member of a coupling pair, an aptamer, or an antigen. The affinity molecule may comprise a primary antibody. The affinity molecule may comprise a secondary antibody. The affinity molecule may be conjugated to a fluorescent molecule, conjugated to an enzyme, bound by another affinity molecule that is conjugated to a fluorescent molecule, or bound by another affinity molecule that is conjugated to an enzyme. In the methods described herein, the method may comprise use of more than one affinity molecule, for example, two affinity molecules, three affinity molecules, four affinity molecules, five affinity molecules, six affinity molecules, seven affinity molecules, eight affinity molecules, nine affinity molecules, or 10 affinity molecules. More than 10 affinity molecules may be used in any of the methods described herein.

[0081] An affinity molecule described herein may be detected by a signal from a detectable label associated with the affinity molecule which may be directly or indirect associated with the affinity molecule. A detectable label used herein may be a fluorophore, polymer particle, metal particle, hapten, enzyme, luminescent label, radioactive label, and the like.

[0082] Examples of fluorophores include fluorescein or its derivatives, such as fluorescein-5-isothiocyanate (FITC), 5-(and 6)-carboxyfluorescein, 5- or 6-earboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid, fluorescein isothiocyanate, rhodamine or its derivatives such as tetramethylrhodamine and tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC). Other example fluorophores that could be conjugated to affinity molecules include, but are not limited to: coumarin dyes such as (diethyl-amino)coumarin or 7-amino-4-methylcoumarin-3-acetic acid, succinimidyl ester (AMCA); sulforhodamine 101 sulfonyl chloride (TexasRed™ or TexasRed™ sulfonyl chloride; 5-(and-6)-carboxyrhodamine 101, succinimidyl ester, also known as 5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (CXR); lissamine or lissamine derivatives such as lissamine rhodamine B sulfonyl Chloride (LisR); 5-(and-6)-carboxyfluorescein, succinimidyl ester (CFI); fluorescein-5-isothiocyanate (FITC); 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester (DECCA); 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (CTMR); 7-hydroxycoumarin-3-carboxylic acid, succinimidyl ester (HCCA); 6-fluorescein-5-(and-6)-carboxamidolhexanoic acid (FCHA); *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacenepropionic acid, succinimidyl ester; also known as 5,7-dimethylBODIPY™ propionic acid, succinimidyl ester (DMBP); "activated fluorescein derivative" (FAP), available from Molecular Probes, Inc.; eosin-5-isothiocyanate (EITC); erythrosin-5-Isothiocyanate (ErlTC); and Cascade™ Blue acetylazide (CBAA) (the *O*-acetylazide derivative of 1-hydroxy-3,6,8-pyrenetrisulfonic acid). Yet other potential fluorophores useful herein include, but are not limited

to, fluorescent proteins such as green fluorescent protein (GFP) and its analogs or derivatives, fluorescent amino acids such as tyrosine and tryptophan and their analogs, fluorescent nucleosides, and other fluorescent molecules such as Cy2, Cy3, Cy 3.5, Cy5, Cy5.5, Cy 7, IR dyes, Dyomics dyes, phycoerythrins, Oregon green 488, pacific blue, rhodamine green, and Alexa dyes. Yet other examples of fluorescent labels which may be used herein include conjugates of phycoerythrin, inorganic fluorescent labels such as particles based on semiconductor material like coated CdSe nanocrystallites. Several the fluorophores above, as well as others, are available commercially from companies such as Molecular Probes, Inc. (Eugene, Oreg.), Pierce Chemical Co. (Rockford, Ill.), and Sigma-Aldrich Co. (St. Louis, Mo.).

[0083] Examples of polymer particles labels include, but are not limited to, microparticles, beads, or latex particles of polystyrene, PMMA or silica, which can be embedded with fluorescent dyes, or polymer micelles or capsules which contain dyes, enzymes, or substrates.

[0084] Examples of metal particles include, but are not limited to, gold particles and coated gold particles, which can be converted by silver stains.

[0085] Examples of haptens include, but are not limited to, fluorophores, myc, nitrotyrosine, biotin, avidin, strepavidin, 2,4-dinitrophenyl, digoxigenin, bromodeoxyuridine, sulfonate, acetylaminofluorene, mercury trinitrophenol, and estradiol.

[0086] Examples of enzymes include, but are not limited to, horse radish peroxidase (HRP), alkaline phosphatase (AP), beta-galactosidase (GAL), glucose-6-phosphate dehydrogenase, beta-N-acetylglucosaminidase, β-glucuronidase, invertase, Xanthine Oxidase, firefly luciferase, and glucose oxidase (GO).

[0087] Examples of commonly used substrates for horse radish peroxidase (HRP) include, but are not limited to, Alexa Fluor™ 350, Alexa Fluor™ 488, Alexa Fluor™ 546, Alexa Fluor™ 555, Alexa Fluor™ 568, Alexa Fluor™ 594, and Alexa Fluor™ 647 Tyramide Reagents or Biotin-XX Tyramide Reagent (Thermo Fisher), 3,3′-diaminobenzidine (DAB), diaminobenzidine with nickel enhancement, 3-amino-9-ethylcarbazole (AEC), Benzidine dihydrochloride (BDHC), Hanker-Yates reagent (HYR), Indophane blue (IB), tetramethylbenzidine (TMB), 4-chloro-1-naphtol (CN), α-naphtol pyronin (α-NP), *o*-dianisidine (OD), 5-bromo-4-chloro-3-indolylphosphate (BCIP), Nitro blue tetrazolium (NBT), 2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT), tetranitro blue tetrazolium (TNBT), and 5-bromo-4-chloro-3-indoxyl-β-D-galactoside/ferro-ferricyanide (BCIG/FF).

[0088] Examples of commonly used substrates for Alkaline Phosphatase include, but are not limited to, Naphthol-AS-B1-phosphate/fast red TR(NABP/FR), Naphthol-AS-MX-phosphate/fast red TR(NAMP/FR), Naphthol-AS-B1-phosphate/fast red TR(NABP/FR), Naphthol-AS-MX-

phosphate/fast red TR(NAMP/FR), Naphthol-AS-B1-phosphate/new fuschin (NABP/NF), bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT), and 5-Bromo-4-chloro-3-indolyl-b(beta)-d (delta)-galactopyranoside (BCIG).

[0089] Examples of luminescent labels include, but are not limited to, luminol, isoluminol, acridinium esters, 1,2-dioxetanes, and pyridopyridazines. Examples of electrochemiluminescent labels include, but are not limited to, ruthenium derivatives.

[0090] Examples of radioactive labels include, but are not limited to, radioactive isotopes of iodide, cobalt, selenium, hydrogen, carbon, sulfur, and phosphorous.

[0091] A labeled analyte can be detected by numerous methods, including, for example, optional labels may be detected by reflectance, transmittance, light scatter, optical rotation, fluorescence, or combinations thereof; radioactive labels may be detected by film, scintillation counting, or phosphorimaging. *See*, *e.g.*, Larsson, *Immunocytochemistry: Theory and Practice*, CRC Press, Boca Raton, FL 1988); *Methods in Molecular Biology* 80, J. Pound (ed.), Humana Press, Totowa, NJ (1998). More than one detectable label may be employed. When more than one color label is used, the different colors may have different, distinguishable colors. Both colors can be detected simultaneously, such as by fusion or juxtaposition of the signals, signal enhancement or quenching, or detection of multiple colors in the sample. The exact choice of detectable label or combinations of detectable labels may be based on personal preferences in combinations with restrictions of the sample type, sample preparation method, detection method and equipment, and optional contrasting labels used in the sample.

[0092] The apparatus and methods disclosed herein can be applied to a variety of targets. Any target which can be recognized by a suitable affinity molecule is compatible with the apparatus and methods described herein. The recognition may be direct or indirect, via another affinity molecule, such as at least one primary, secondary, or higher order affinity molecule.

[0093] A target or analyte may comprise a protein, such as a glycoprotein or lipoprotein, phosphoprotein, methylated protein, or a protein fragment, a peptide, or a polypeptide. A target or analyte may comprise a nucleic acid segment or a nucleic acid analog segment.

[0094] A target or analyte may comprise one or more of lipids; glyco-lipids; carbohydrates; polysaccharides; salts; ions; or a variety of other organic and inorganic substances. A target or analyte may be expressed on the surface of the sample, such as on a membrane or interface. Alternatively, a target or analyte may be contained in the interior of the sample. In the case of a cell sample, for instance, an interior target or analyte may comprise a target or analyte located within the cell membrane, periplasmic space, cytoplasm, or nucleus, or within an intracellular compartment or organelle.

[0095] A target or analyte may also include viral particles, or portions thereof, such as nucleic acids or proteins. The viral particle may be a free viral particle, i.e., not associated with any other molecule, or it may be associated with any sample described above. A target or analyte may be an antigen or an antibody.

[0096] An approximate amount of a target in a sample may be determined. For instance, a control target within the sample may be assayed as well as an experimental target. In the case of a nucleic acid target, for example, a chromosomal paint or counterstain may be used. For example, if the target is a locus on a larger piece of nucleic acid such as a plasmid or chromosome, the intensity of a contrasting label for the plasmid or chromosome or a neutral locus thereon may be compared to the intensity of the target locus. The intensity of the label from the sample may also be compared to that of a known standard or control sample. Estimating the amount of a detectable target in a sample is helpful, for instance, in a variety of diagnostic tests, and the estimate may be used to plan a course of treatment for a suspected disease or condition. Several commercial densitometry software programs and related instruments are available to quantitate the intensity of a stained target in a sample, such as those available from Fuji Film, Applied Biosystems, and Molecular Dynamics.

[0097] Kits

[0098] Also described herein are kits comprising one or more tissue sample compartmentalization modules as described herein. For example, the kit may contain modules with various compartment arrays such as 4 × 4 (16 compartments), 5 × 4 (20 compartments), 6 × 6 (36 compartments), 8 × 8 (64 compartments) or lesser or greater numbers of apertures/compartments to accommodate various sizes of tissue sample. The kit may optionally comprise one or more affinity molecules; one or more signaling reagents; optionally, one or more reagents for performing the methods described herein, for instance: deparaffinization reagents for removal of paraffin from FFPE slides, enzyme activation buffers (e.g., Tris buffer + peroxide co-factor to activate HRP), antigen retrieval, sample dilution, reagent dilution, blocking of non-specific binding, or blocking of endogenous enzyme activity. The kit may optionally comprise one or more containers, packaging, instructions for use, MSDS sheets, or reference or control tissue samples and/or targets.

[0099] It will be apparent to one of ordinary skill in the relevant art that suitable modifications and adaptations to the compositions, formulations, methods, processes, and applications described herein can be made without departing from the scope of any embodiments or aspects thereof. The compositions and methods provided are exemplary and are not intended to limit the scope of any of the specified embodiments. Each of the various embodiments, aspects, and options

disclosed herein can be combined in any variations or iterations. The scope of the compositions, formulations, methods, and processes described herein include all actual or potential combinations of embodiments, aspects, options, examples, and preferences herein described. The exemplary compositions and formulations described herein may omit any component, substitute any component disclosed herein, or include any component disclosed elsewhere herein. The ratios of the mass of any component of any of the compositions or formulations disclosed herein to the mass of any other component in the formulation or to the total mass of the other components in the formulation are hereby disclosed as if they were expressly disclosed. Should the meaning of any terms in any of the patents or publications incorporated by reference conflict with the meaning of the terms used in this disclosure, the meanings of the terms or phrases in this disclosure are controlling. Furthermore, the description discloses and describes merely exemplary embodiments. All patents and publications cited herein are incorporated by reference herein for the specific teachings thereof.

[0100] EXAMPLES

[0101] Example 1

[0102] Standard Tissue Immunohistochemistry.

[0103] Immunohistochemical (IHC) staining is used for detecting specific antigens in tissues. Typically, tissue samples are formalin fixed to preserve the integrity of the tissue. A tissue section that was embedded in paraffin must be deparaffinized (i.e., dewaxed) and then rehydrated before applying the primary antibody. A tissue section that was frozen and embedded in optimal cutting temperature compound (OCT) must be rehydrated before applying the primary antibody. Enzyme-conjugated secondary antibodies are then applied, and the specific staining can be visualized after adding the enzyme-specific substrate. Occasionally, when weak or no staining is observed, an antigen may be unmasked or the signal enhanced by means described herein, such as by enzyme digestion or microwave antigen retrieval.

[0104] The sample sections on a substrate, such as a glass slide, are permeabilized for about 30 min at room temperature (RT, i.e., from about 20–25 °C). Then, the permeabilized samples are incubated with a blocking agent such as from about 2% to about 10% normal serum from the same species as the host of the secondary antibody for about 60 min at RT prior to the primary antibody reaction. The blocking agent may decrease background staining. Optimal dilution and incubation times are determined for each primary antibody prior to use. The slides are drained, and excess fluid is removed from each slide around the sample sections. The primary antibody or negative control reagent are diluted to its optimal dilution in diluent. The primary antibody may be directly conjugated to a fluorophore. The diluent alone may be used as a negative control. A

positive control (i.e., a tissue known to contain the target antigen) are also to be included. An appropriate amount of a diluted primary antibody solution is applied to the appropriate slides, covering the tissue sections, and incubated for at least 60 min or an appropriate time at RT or 4 °C overnight in a humidified chamber. After incubation, the slides are rinsed gently with PBS and the slides may be incubated in a PBS wash bath for about 1 minute to about 5 min.

[0105] If the primary antibody is not conjugated to a fluorophore, a secondary antibody conjugated to a fluorophore, or an enzyme is to be used. The secondary antibody is diluted to its optimal dilution in diluent. An appropriate amount of a secondary antibody solution is applied to the appropriate slides, covering the tissue sections, and incubated for at least 30 min or an appropriate time at room temperature. The samples are protected from light. After incubation, the slides are washed 3 times for 5 to 15 min each in a wash buffer.

[0106] If the secondary antibody is not conjugated to a fluorophore but an enzyme instead (e.g., horse radish peroxidase, "HRP" or alkaline phosphatase, "AP"), a tertiary detection substrate such as a fluorescent tyramide is to be used. The fluorescent tyramide is diluted to its optimal dilution in a diluent consisting of HRP reaction buffer and required co-factors such as hydrogen peroxide. An appropriate amount of reactive tyramide is applied to the appropriate slides, covering the tissue sections, and incubator for 2 to 10 min at RT. The samples are protected from light. After incubation, HRP enzymatic activity may be quenched by use of a reaction stop reagent, and the slides are washed 3 times for 5 to 15 min each in a wash buffer. The slides may be mounted with an anti-fade mounting media. The slides are visualized using a fluorescence microscope and the fluorescence data is analyzed and unmixed using software.

[0107] Example 2

[0108] Tissue Immunohistochemistry using a Tissue Sample Compartmentalization Module.

[0109] Typically, tissue samples are formalin fixed to preserve the integrity of the tissue. If the tissue section was embedded in paraffin, it must be deparaffinized (i.e., dewaxed) and then rehydrated before applying the primary antibody. If a tissue section was frozen and embedded in OCT, it must be rehydrated before applying the primary antibody. An antigen may be unmasked, or the signal enhanced by means described herein, such as by enzyme digestion or microwave antigen retrieval.

[0110] The tissue sample sections on a substrate ("tissue sample/substate"), such as a glass slide, are permeabilized for about 30 min at RT. The permeabilized tissue samples are incubated with a blocking agent such as from about 2–10% BSA or normal serum from the same species as the host of the secondary antibody for about 60 min at RT prior to the primary antibody reaction. The blocking agent may decrease background staining.

[0111] The sample is placed in the compartmentalization module after dewaxing/antigen retrieval, permeabilization and blocking and the module is removed after antibody staining for washing. Removing liquid from apertures by pipetting can damage tissue so all steps that can be performed for the whole sample should be done without the module. However, if e.g., secondary antibodies are to be used and apertures need to be washed individually, one can immerse the whole microscope slide in washing solution with the apparatus in place.

[0112] The tissue sample/substate is placed onto a first member of the tissue sample compartmentalization module described herein and the tissue sample/substrate is aligned with the first member. In one aspect, the plurality of first apertures defined by the first member is aligned to overlap as much of the tissue sample with as many apertures as possible to provide multiple sample compartments. An insert is placed or positioned on the tissue sample/substate to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member. Then, second member is aligned on the tissue sample and with first member, sandwiching the substrate and tissue sample between the first member and the second member. The second member is pivoted relative to the first member to sandwich the insert and the tissue sample/substrate between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures. The first member and the second member are fastening together by a locking arm, thereby sealing the insert to the tissue sample/substrate and forming a plurality of individual sample compartments.

[0113] Optimal dilution and incubation times are determined for each primary antibody prior to use. The slides are drained, and excess fluid is removed. The primary antibody or negative control reagent is diluted to its optimal dilution in diluent. The primary antibody may be directly conjugated to a fluorophore. The diluent alone may be used as a negative control. A positive control (i.e., a tissue known to contain the target antigen) can also be included. An appropriate amount of a primary antibody solution is applied to each sample compartment, covering the tissue section segments in each sample compartment and incubated for at least 60 min or an appropriate time at 37 °C in a humidified chamber. After incubation, the sample compartments are rinsed gently with PBS and the sample compartment may be incubated in a PBS wash bath for about 1 minute to about 5 min.

[0114] If the primary antibody is not conjugated to a fluorophore, a secondary antibody conjugated to a fluorophore is used. The secondary antibody is diluted to its optimal dilution in diluent. An appropriate amount of a secondary antibody solution is applied to the appropriate sample compartment, covering the tissue section segments in each sample compartment and incubated for at least 30 min or an appropriate time at room temperature. The samples are

protected from light. After incubation, the sample compartments are washed 3 times for 5 to 15 min each in a wash buffer.

[0115] If the secondary antibody is not conjugated to a fluorophore but an enzyme such as horse radish peroxidase (HRP), a tertiary detection substrate such as a fluorescent tyramide is to be used. The fluorescent tyramide is diluted to its optimal dilution in a diluent consisting of HRP reaction buffer and required co-factors such as hydrogen peroxide. An appropriate amount of reactive tyramide is applied to the appropriate slides, covering the tissue sections, and incubator for 2 to 10 min at RT. The samples are protected from light. After incubation, HRP enzymatic activity may be quenched by use of a reaction stop reagent, and the slides are washed 3 times for 5 to 15 min each in a wash buffer. The tissue sample compartmentalization module may be removed, and the slide may be mounted with an anti-fade mounting media. The slide is visualized using a fluorescence microscope.

[0116] Example 3

[0117] Fluorophore Spillover Experiment using a Tissue Sample Compartmentalization Module.

[0118] The goal of this experiment was to demonstrate that the design of a 3D-printed tissue sample compartmentalization module prevents fluorophore spillover from one subcompartment to the other and that the tissue divided by the subcompartment can be stained independently from other surrounding regions. Each subcompartment was stained with only one primary antibody conjugated to a fluorophore. Normalized average spectral intensities from compartmentalized samples were compared to extracted spectral profiles from control tissue samples stained without compartmentalization where spillover cannot occur.

[0119] Three normal human tonsil formalin-fixed paraffin-embedded (FFPE) tissue samples (Zyagen) were dewaxed using Biogenex EZ-AR2 Elegance solution (Biogenex) in a Biogenex EZ-retriever microwave-based antigen retrieval system (Biogenex) using two thermal cycles: 95 °C for 5 min, and 107 °C for 5 min. Samples were permeabilized for 30 min at RT with 0.1% *t*-octylphenoxypolyethoxyethanol (Triton™ X-100) in phospate buffered saline (PBS) to improve antibody penetration in the tissue. The permeabilized tissues were blocked for 60 min using 3% bovine serum albumin (BSA) in PBS at RT to prevent non-specific binding. Non-specific binding of the dye-conjugated antibody was also reduced by treating the samples for 30 min at RT with Image-IT FX Signal Enhancer (Invitrogen).

[0120] The samples were stained with primary antibodies conjugated with fluorophores. Two control samples were stained with single antibody-fluorophore conjugates (CD20 Monoclonal Antibody (L26), Alexa Fluor™ 488, eBioscience™ (Invitrogen) and Vimentin Monoclonal Antibody (V9), Alexa Fluor™ 555 (Invitrogen). One tissue sample was divided in subcompartments using

a tissue compartmentalization module equipped with a silicone gasket (i.e., an apparatus as described herein). The subcompartments were stained with the same antibody conjugates that were used to stain the control samples.

[0121] Images were acquired with 25 spectral channels for each sample. Characteristic dye profiles were obtained from all images by taking the average intensity of the entire image for each acquisition channel.

[0122] The normalized average spectral intensities were acquired from the compartmentalized sample and the control samples were nearly identical. *See* FIG. 8A–B. These experiments demonstrate that the tissue compartmentalization module enables independent staining in subcompartments and the fluorophores from subcompartments do not have overlapping spectral profiles.

[0123] Example 4

[0124] Cell-based Application for Tissue Compartmentalization Module.

[0125] If using standard uncoated glass microscope slides the slides need to be pretreated to allow for cell attachment. As cells do not readily adhere to glass, the slide surface has to be first coated with cell adherence facilitating substance such as surface polarity modifying poly-amino acids (e.g., poly-L-lysine, poly-D-lysine, poly-ornithine), non-specific proteinous coating (e.g., Gelatine), specific extracellular matrix components (e.g., purified Collagen I, IV, Fibronectin, Laminin, Vitronectin, Osteopontin), specific partial sequences of extracellular matrix proteins (e.g., RGD-Motivs, Fibronectin domains), or specific mixtures of extracellular matrix proteins (e.g., solubilized basement membrane preparations, Corning Matrigel®; Thermo Fisher Geltrex™). Coating can be performed for the whole slide surface area or only for the area covered by the module aperture array if the module has been assembled on the slide prior to the coating. Once the slide surface has been modified for cell adherence, the module is assembled on the slide (if not already) and cell suspension is pipetted in apertures in cell-specific cell culture medium. Cell number per aperture depends on the experiment, cell type used and the size of the aperture. The module is sealed to prevent contamination but allowing for gas exchange and the slide-module complex is placed in incubator to let the cells adhere. The incubation time and environmental conditions depend on the cell type used. After the cells have adhered and reached the desired confluency, the user can further commence to experimental protocols. The number of individual experimental environments per slide depends on number of apertures per module and the number of modules per slide. For example, by using two 5 × 4 tissue compartmentalization modules a user can have 40 unique experimental environments on one single slide. The experiments can be e.g., drug treatments, genetic modifications, toxicological assays, or cell differentiation assays.

After exposing the cells to experimental environments, the cells can imaged live using a microscope or further processed e.g., by fixation and staining with immunohistochemical assays. The sample can be preserved by disassembling the module and covering the cells with mounting medium and cover slip. Preserved coverslip covered slides can be inspected further by high-power microscopy.

CLAIMS

1. A sample compartmentalization module comprising:
a first member defining a first plurality of apertures; and
a second member defining a second plurality of apertures, the second member pivotably connected to the first member,
wherein the second member is configured to engage with the first member to form a closed configuration or pivot relative to the first member between an open configuration and the closed configuration, wherein in the closed configuration the first plurality of apertures and the second plurality of apertures are aligned.

2. The sample compartmentalization module of claim 1, further comprising a locking arm coupled to the second member, the locking arm configured to rotate relative to the second member, wherein in the closed configuration the locking arm is configured to engage a portion of the first member to fasten the first and second members together.

3. The sample compartmentalization module of claim 2, wherein the locking arm defines a central channel, wherein in the closed configuration the central channel of the locking arm is configured to receive a portion of the first member.

4. The sample compartmentalization module of claim 1, further comprising an insert defining a plurality of third apertures, wherein in the closed configuration the insert is positioned between the first member and the second member and the third plurality of apertures are aligned with the first plurality of apertures and the second plurality of apertures.

5. A method for labeling one or more analytes in a tissue sample, the method comprising:
(a) placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module of claim 1;
(b) aligning the tissue sample with the first member;
(c) placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member;
(d) engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures;

(e) fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample; and

(f) incubating a solution comprising an affinity molecule in an aligned second and third aperture, wherein the affinity molecule labels an analyte in the tissue sample.

6. The method of claim 5, wherein the sample is from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof.

7. The method of claim 5, wherein the analyte comprises one or more of metabolites, proteins, nucleic acids, carbohydrates, or lipids.

8. The method of claim 5, further comprising labeling two or more distinct analytes, wherein each aligned second and third aperture comprises a solution comprising a distinct affinity molecule for each analyte.

9. The method of claim 5, wherein the affinity molecule is an antibody, a portion of an antibody, an antibody-like molecule, a ligand receptor, a ligand for a receptor, one member of a coupling pair, an aptamer, or an antigen.

10. The method of claim 5, wherein the affinity molecule is conjugated to a fluorescent molecule, conjugated to an enzyme, bound by another affinity molecule that is conjugated to a fluorescent molecule, or bound by another affinity molecule that is conjugated to an enzyme.

11. The method of claim 5, wherein the substrate is a microscope slide.

12. A method for dividing a sample into compartments for labeling two or more analytes in the tissue sample comprising:

(a) placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module of claim 1;

(b) aligning the tissue sample with the first member;

(c) placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member;

(d) engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures; and

(e) fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample.

13. The method of claim 12, wherein the sample is a tissue sample from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof.

14. The method of claim 12, wherein the substrate is a microscope slide.

15. The method of claim 12, wherein step (c) further comprises overlapping as many of the plurality of first apertures defined by the first member; as possible with the tissue sample.

16. A method of analyzing a tissue sample for a plurality of analytes comprising:

(a) placing a substrate comprising the tissue sample onto the first member of the tissue sample compartmentalization module of claim 1;

(b) aligning the tissue sample with the first member;

(c) placing an insert on the tissue sample to align a plurality of third apertures defined by the insert with the plurality of first apertures defined by the first member;

(d) engaging or pivoting the second member relative to the first member to sandwich the insert and the tissue sample between the first member and the second member, and to align the plurality of second apertures with the plurality of third apertures;

(e) fastening the first member and the second member together by a locking arm, sealing insert to the tissue sample; and

(f) incubating a solution comprising an affinity molecule in an aligned second and third aperture, wherein the affinity molecule labels an analyte in the tissue sample;

(g) detecting a signal from each affinity molecule that is bound to the plurality of analytes, thereby detecting the presence or amount of each analyte in the plurality of analytes.

17. The method of claim 16, wherein the detecting is performed using a light or fluorescence microscope, a charge coupled device (CCD) camera or imager, a phosphorimager, or a combination thereof.

18. The method of claim 16, wherein the tissue sample is from a subject selected from humans, non-human primates, rats, mice, guinea pigs, rabbits, pigs, cows, sheep, goats, horses, dogs, cats, fish, birds, reptiles, amphibians, insects, plants, fungi, bacteria, or combinations thereof.

19. The method of claim 16, wherein the plurality of analytes comprises one or more of metabolites, proteins, nucleic acids, carbohydrates, or lipids.

20. The method of claim 16, wherein the affinity molecule is an antibody, a portion of an antibody, an antibody-like molecule, a ligand receptor, a ligand for a receptor, one member of a coupling pair, an aptamer, or an antigen.

21. The method of claim 16, wherein the affinity molecule is conjugated to a fluorescent molecule, conjugated to an enzyme, bound by another affinity molecule that is conjugated to a fluorescent molecule, or bound by another affinity molecule that is conjugated to an enzyme.

22. The method of claim 16, wherein the affinity molecule is conjugated to an enzyme.

23. The method of claim 22, wherein the enzyme is horse radish peroxidase.

24. The method of claim 23, wherein the signal detected is from a tyramide conjugate.

25. The method of claim 24, wherein the tyramide conjugate comprises on or more of Alexa Fluor 350, 488, 546, 588, 594, 647, or 750 tyramide reagents or Biotin-XX tyramide reagent.

26. A kit comprising:

one or more tissue sample compartmentalization modules of claim 1;

one or more affinity molecules;

one or more signaling reagents;

optionally, one or more reagents comprising deparaffinization reagents, enzyme activation reagents, antigen retrieval reagents, sample dilution reagents, reagent dilution buffers, blocking reagents, or endogenous enzyme activity blocking reagents; and

optionally, one or more containers, packaging, instructions for use, MSDS sheets, reference or control tissue samples, or reference or control targets.