(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 December 2015 (30.12.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/199976 A1

(51) International Patent Classification:
G01N 1/28 (2006.01) *G01N 1/08* (2006.01)
G01N 1/30 (2006.01)

(21) International Application Number:
PCT/US2015/034930

(22) International Filing Date:
9 June 2015 (09.06.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/016,571 24 June 2014 (24.06.2014) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

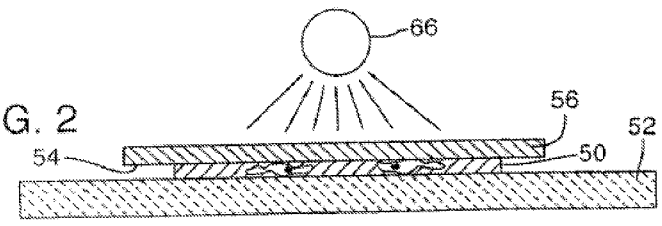
Published:

— *with international search report (Art. 21(3))*

WO 2015/199976 A1

(54) Title: TARGET ACTIVATED MICRODISSECTION

FIG. 2



(57) Abstract: A method of removing a target from a biological sample, comprising: providing a substrate-mounted biological sample with a reagent that selectively acts on the target within the biological sample, wherein the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent; placing a flexible transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample; applying a uniform air pressure gradient across the flexible transfer film to maintain thermal contact between the lower surface of the transfer film and the biological sample; exposing the biological sample to the light of the wavelength to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering the target to the transfer film; and selectively removing the target from the biological sample by removing the transfer film with the adhered target from the biological sample.

TARGET ACTIVATED MICRODISSECTION

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/016,571, filed June 24, 2014, which is herein incorporated by reference in its entirety.

BACKGROUND

A variety of techniques have been used to microdissect specific cells or cell populations from a histological sample under direct microscopic visualization. Original microdissection techniques involved painstaking (and sometimes clumsy) manual dissection using needles or other micro-manipulation devices to isolate individual cells based on visible, histological characteristics.

More recent techniques have been developed to more efficiently separate biological components, such as particular subsets of cells, from a whole tissue sample. For example, Emmert-Buck et al. described the use of laser-based microdissection techniques to rapidly procure microscopic, histopathologically defined cell populations. Examples of such laser capture microdissection (LCM) are shown in U.S. Patent Nos. 5,843,657; 5,843,644; 5,859,699; 5,598,085, and 6,010,888, as well as WO 97/13838; WO 98/35216; WO 00/06992; and WO 00/49410.

SUMMARY

Disclosed herein in one embodiment is a method of removing a target from a biological sample, comprising:

providing a substrate-mounted biological sample with a reagent that selectively acts on the target within the biological sample, wherein the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent;

placing a flexible transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample;

applying a uniform air pressure gradient across the flexible transfer film to maintain thermal contact between the lower surface of the transfer film and the biological sample;

exposing the biological sample to the light of the wavelength to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering the target to the transfer film; and

selectively removing the target from the biological sample by removing the transfer film with the adhered target from the biological sample.

In certain embodiments, applying the uniform air pressure gradient across the flexible transfer film comprises applying a vacuum from at least a portion of a peripheral edge of the substrate so as to generate a negative pressure between the lower surface of the transfer film and the biological specimen.

Disclosed herein in a further embodiment is a method of removing a target from a biological sample, comprising:

providing a substrate-mounted biological sample with a reagent that selectively acts on a cell component within the biological sample, wherein the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent;

placing a flexible transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample, wherein the transfer film comprises a first polymer support layer and a second polymer disposed on the first polymer support layer such that second polymer forms the lower surface of the transfer film, the second polymer having a layer thickness of less than 30 μm;

applying a vacuum from at least a portion of a peripheral edge of the substrate so as to generate a negative pressure between the lower surface of the transfer film and the biological specimen to maintain thermal contact between the lower surface of the transfer film and the biological sample;

exposing the biological sample to 0.2 to 4 J/cm^2 of visible light by pulsing a flashlamp one to ten times, with a pulse length of 100 to 300 μsecs each at 0.2 to 1.0 Joules/pulse-cm^2 to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering both the reagent-stained cell component and any desired unstained adjacent cellular component from the specifically stained cell to the transfer film; and

selectively removing the reagent-stained cell component and the desired unstained adjacent cellular component from the biological sample by removing the transfer film with the adhered reagent-stained cell component and the desired unstained adjacent cellular component from the biological sample.

In certain embodiments, the stained cell component is a cell membrane component and the desired unstained adjacent cellular component is a cell nuclei. In certain embodiments, the method involves selectively removing a large number of desired targets.

Additionally disclosed herein in another embodiment is a method of removing a target from a biological sample, comprising:

placing a slide-mounted biological sample into a slide holder, wherein the biological sample includes a reagent that selectively acts on the target within the biological sample, the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent, and the slide holder includes a base member having a recessed portion having a sufficient dimension to hold the slide-mounted biological sample, wherein the base member includes at least one conduit having at least one first opening into the recessed portion and a second opening located at an outer edge of the base member;

placing a transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample;

applying a vacuum from at least a portion of a peripheral edge of the slide via the at least one conduit of the base member so as to generate a negative pressure between the lower surface of the transfer film and the biological specimen to maintain thermal contact between the lower surface of the transfer film and the biological sample;

exposing the biological sample to the light of the wavelength to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering the target to the transfer film; and

selectively removing the target from the biological sample by removing the transfer film and the adhered target from the biological sample.

In certain embodiments, the reagent selectively acts on large numbers of specific, microscopic targets and the method selectively removes the multiple targets.

Also disclosed herein is an article comprising:

a base member having a recessed portion having a sufficient dimension to hold a slide-mounted biological sample, wherein the base member includes at least one conduit having at least one first port into the recessed portion and a second port located at an outer surface or edge of the base member;

a lid coupled to the base member, wherein the lid includes a window extending there through that is aligned with the recessed portion of the base member; and

sealing members located on at least one of the base member or the lid.

Further disclosed herein is a system comprising:

(a) an irradiation module, comprising

a flashtube;

a first reflector positioned around the flashtube and defining a longitudinal axis and having an opening centered on the longitudinal axis; and

a light output window, wherein the light output window has an inner side facing to the opening of the first reflector, and the light output window is also centered on the longitudinal axis; and

(b) a slide holder adjacent to the outer side of the light output window, the slide holder comprising:

a base member having a recessed portion having a sufficient dimension to hold a slide-disposed biological sample, wherein the base member includes at least one channel having a first opening into the recessed portion and a second opening located at an outer edge of the base member;

a lid coupled to the base member, wherein the lid is positioned adjacent to the light output window of the irradiation module, and wherein the lid includes a window extending there through that is aligned with the recessed portion of the base member and the light output window of the irradiation module; and

sealing members located on at least one of the base member or the lid.

The foregoing will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A, 1B and 1C are a series of cross sectional schematic enlarged views illustrating one embodiment of target-activated transfer, in which a reagent in the target region of a tissue section focally activates adhesion of the target to a transfer surface.

Fig. 2 illustrates an embodiment of the method shown in FIG. 1, wherein the reagent in the target is activated by electromagnetic radiation, such as diffuse light from a flash lamp, which briefly illuminates a large region of the slide including both specific targets and unwanted (unlabeled) cells.

Figs. 3-5 are isometric views of a vacuum substrate holder disclosed herein.

Fig. 6 is an isometric view of a base member of a vacuum substrate holder disclosed herein.

Fig. 7 is a cross-sectional view of a flashlamp system disclosed herein.

Fig. 8 is an isometric view of a flashlamp system disclosed herein.

Fig. 9 is a flowchart showing an embodiment of creating a region-of-interest (ROI) mask for use in the target-activated microtransfer methods disclosed herein.

Fig. 10 is an isometric view of a vacuum substrate holder coupled to a flashlamp system as disclosed herein.

Fig. 11 is a cross-sectional view of a vacuum substrate holder as disclosed herein.

Fig. 12 is flowchart demonstrating integration of the target-activated system and process disclosed herein into upstream preparation/identification/imaging processes and downstream analysis processes.

DETAILED DESCRIPTION

Terms

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided.

Activating moiety: A moiety that participates in altering a transfer surface. The activating moiety can act directly on the transfer surface, for example by releasing heat to partially melt the surface. Alternatively, the activating moiety can act in association with another activating agent, such as light that is absorbed by the activating moiety and emitted as heat. In other examples, the activating moiety acts in association with intermediary agents, such as a substrate for a catalytic activating moiety.

Adherence: A physical association of two surfaces held together by interfacial forces. An example of adherence is the force provided by an adhesive, such as a thermoplastic material that melts to physically attach to another object.

Amplification: Techniques that increase the number of copies of a nucleic acid molecule in a sample or specimen. An example of amplification is the polymerase chain reaction, in which a biological sample collected from a subject is contacted with a pair of oligonucleotide primers, under conditions that allow for the hybridization of the primers to nucleic acid template in the sample. The primers are extended under suitable conditions, dissociated from the template, and then re-annealed, extended, and dissociated to amplify the number of copies of the nucleic acid. The product of *in vitro* amplification may be characterized by electrophoresis, restriction endonuclease cleavage patterns, oligonucleotide hybridization or ligation, and/or nucleic acid sequencing, using standard techniques. Other examples of *in vitro* amplification techniques include strand displacement amplification (see U.S. Patent No. 5,744,311); transcription-free isothermal

amplification (see U.S. Patent No. 6,033,881); repair chain reaction amplification (see WO 90/01069); ligase chain reaction amplification (see EP-A-320 308); gap filling ligase chain reaction amplification (see U.S. Patent No. 5,427,930); coupled ligase detection and PCR (see U.S. Patent No. 6,027,889); and NASBA™ RNA transcription-free amplification (see U.S. Patent No. 6,025,134).

Antibody: Immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen.

A naturally occurring antibody (*e.g.*, IgG, IgM, IgD) includes four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. However, it has been shown that the antigen-binding function of an antibody can be performed by fragments of a naturally occurring antibody. Antibody fragments that perform the antigen-binding function of an antibody are within the scope of the disclosure.

Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture (*e.g., see* U.S. Patent No. 4,745,055; U.S. Patent No. 4,444,487; WO 88/03565; EP 256,654; EP 120,694; EP 125,023; Faoulkner *et al.*, *Nature* 298:286, 1982; Morrison, *J. Immunol.* 123:793, 1979; Morrison *et al.*, *Ann Rev. Immunol* 2:239, 1984).

Antibody-enzyme fusion: A chimeric fusion molecule that includes an antibody, or the variable binding domain of an antibody fused to an enzyme moiety.

Binding partner: Any molecule or compound capable of recognizing and binding to a specific structural aspect of a corresponding molecule or compound. Examples of such binding partners and corresponding molecule or compound include: antigen/antibody, hapten/antibody, nucleic acid probe/complementary nucleic acid sequence, lectin/carbohydrate, apoprotein/cofactor and biotin/(strept)avidin.

Direct Cell Target Microtransfer (DCTM) molecule: A molecule having at least two functional moieties: a targeting moiety (also called a localizing moiety, because it localizes the DCTM molecule to a target cell or site within a sample), that targets specific components (such as cells or structures within or upon those cells); and an activating moiety, that facilitates the adherence of the transfer substrate to the targeted components. In specific embodiments, these two moieties are attached directly to each other. In others, they are attached through a linker. DCTM molecules are more fully described in PCT/US03/12734, which disclosure is incorporated by reference.

Electromagnetic radiation, and activation of reagent: Electromagnetic radiation is a form of energy in which an oscillatory electric field and an oscillatory magnetic field are perpendicular to

one another, and to the direction of propagation of the direction of propagation of the radiation. Light is the name often given to the visible spectrum of electromagnetic radiation. All of these forms of radiation transfer energy, and are capable of activating light responsive reagents, such as chromophores and heat-generating catalysts.

Fusion protein: Proteins that have at least two domains or moieties fused together, each portion of the protein comprising a region capable of independent structural or functional activity (for instance forming a specific complex with a target molecule, or carrying out a biochemical reaction). In some embodiments, the two domains are either genetically fused together (for example nucleic acid molecules that encode each protein domain are functionally linked together) or chemically fused together (for example covalently bonded). By way of example, a linker oligonucleotide may be produced such that it encodes both the targeting and activating moieties within a single polynucleotide molecule. The translated product of such a fusion-encoding polynucleotide is the fusion protein. In other embodiments, chemical linkers may be used to join targeting and activating moieties to form fusion proteins.

Immunocytochemistry: The identification of antigens in a biological sample (for example tissue section, smears, cell culture) using specific immunological (antibody-antigen) interactions culminating in the attachment of a visible marker to the antigen. Examples of visual markers used in immunocytochemistry are fluorescent dyes, colloidal metals, haptens, radioactive markers, and enzymes that convert an externally supplied substrate into a visible indicator molecule, such as an absorbing, fluorescent or luminescent molecule. Immunocytochemistry techniques are known to those of skill in the art, and examples of such techniques are those included in Ausubel *et al.* (In *Current Protocols in Molecular Biology*, Greene Publ. Assoc. and Wiley-Intersciences, 1998).

Isolated: An "isolated" biological component (such as a cell, cell type, nucleic acid molecule, protein or organelle) has been substantially separated or purified away from other biological components in the environment in which the component naturally occurs, *i.e.*, other cells, other chromosomal and extra-chromosomal DNA and RNA, and other proteins and organelles. By way of example, an organelle such as the nucleus can be isolated from a cell or a biochemical reaction mixture, or specific cells can be isolated from a complex tissue.

Linker: A linker is a "chemical arm" between two moieties or domains in a molecule. Linkers may be used to join otherwise separate molecular moieties. The term "linker" also refers to the part of a molecule between two moieties.

Microtransfer: A process of isolating an element, or many specific elements, from a sample, often a tissue sample, at a microscopic level through facilitating adherence of that element, or those elements, to a transfer substrate, or selectively allowing migration of the element(s)

through a focal region on the substrate, to physically separate the element(s) from the remaining components. In some examples, the microtransfer is used to separate cells or cellular components from a sample based upon a distinguishing characteristic (such as expression of a cell antigen or receptor) or pattern of expression (such as expression of a protein, nucleic acid, or other molecule that is the target of the targeting moiety) within the sample.

Moiety: A part or portion of a molecule having a characteristic chemical, biochemical, structural and/or pharmacological property or function. As used herein, the term moiety refers to a subpart of a molecule that retains an independent biochemical or structural activity from the remainder of the molecule, for instance the ability to generate heat or fluoresce, or to bind or associate with a target or to carry out an enzymatic reaction. A single molecule may have multiple moieties, for instance an antigen-binding moiety and a fluorescing moiety, each having a different function.

Operably linked: A first molecule (*e.g.*, nucleic acid sequence, protein, linker, etc.) is operably linked with a second molecule when the first molecule is placed in a functional relationship with the second molecule. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Polymer: Polymers are substances (*e.g.*, protein, nucleic acid sequences, transfer substrates) consisting of large molecules that are made of many small, repeating units called monomers. The number of repeating units in one large molecule is called the degree of polymerization.

The class of polymers includes thermoplastic or electroactive polymers. Examples of thermoplastic or electroactive polymers that may be used in conjunction with the methods disclosed herein include ethylene-vinyl acetate copolymer (EVAc), polyethylene (PE), and polyaniline (PANi) films (see Kamalesh *et al.*, *J. Biomed. Mater. Res.* 52(3): 467-478, 2000) and those set forth in U.S. Patent No. 6,242,503 to Kozma *et al.*, which also describes methods of making such polymers.

Particular thermoplastic polymers may be applied in a powder or liquid form, and caused to form adhesive films (*e.g.*, by melting the powder and resolidifying the liquid polymer on cooling, or by dissolving the powder in a suitable solvent and casting films). Examples of commercial-grade thermoplastic polymers include DuPont ELVAX 410, an ethylene-vinyl acetate block copolymer.

Probes: An isolated nucleic acid attached to a detectable label or other reporter molecule. Typical labels include radioactive isotopes, enzyme substrates, co-factors, ligands, chemiluminescent

or fluorescent agents, haptens, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are known, *e.g.*, Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 1989) and Ausubel *et al.* (In *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1998).

Reagent: Any substance used for the purpose of detecting, measuring, examining, or analyzing other substances. This term includes both organic and inorganic reagents. An immunoreagent is a reagent that includes an antibody or antibody fragment for targeting the reagent to a target.

Separate(d)/Separation: To spatially dissociate components, such as biomolecules, cells, or cell clusters, from their surrounding natural environment, for instance by physically removing the components (for example by causing adherence of selected components to a transfer substrate). Separation may be employed to isolate selected components for subsequent analysis of the characteristics of the separated components.

Separation can be effected on various scales, for instance large-scale physical dissection wherein the operator visualizes the process, and small-scale microtransfer or microdissection wherein the operator uses a visual aid (such as a microscope) to accomplish the separation.

Separation is not an absolute term (in that separation need not be perfect or "complete" for components to be "separated").

Specific binding agent: An agent that binds substantially only to a defined target. Thus a protein-specific binding agent binds substantially only to the defined protein, or a peptide region within a protein. As used herein, the term "specific binding agent," refers to a specific protein or peptide, including antibodies (and functional fragments thereof) and other agents (such as soluble receptors or ligands for receptors) that bind substantially only to target proteins or nucleic acids. In some embodiments, these target proteins or nucleic acids are within target cells of interest.

Antibodies may be produced using standard procedures described in a number of texts, including Harlow and Lane (*Antibodies, A Laboratory Manual*, CSHL, New York, 1988). The determination that a particular agent binds substantially only within the target cells may readily be made by using or adapting routine procedures. One suitable *in vitro* assay makes use of the Western blotting procedure (described in many standard texts, including Harlow and Lane, *Antibodies, A Laboratory Manual*, CSHL, New York, 1988). Western blotting may be used to determine that a given protein binding agent binds substantially only to the specified protein.

Shorter fragments of antibodies can also serve as specific binding agents. For instance, FAbs, Fvs, and single-chain Fvs (SCFvs) that bind to a protein or peptide within a target cells would be target cell-specific binding agents. These antibody fragments are defined as follows: (1)

FAb, the fragment that contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) FAb', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two FAb' fragments are obtained per antibody molecule; (3) $(FAb')_2$, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; (4) F(Ab')2, a dimer of two FAb' fragments held together by two disulfide bonds; (5) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (6) single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Methods of making these fragments are routine.

Target: A sub-portion of a biological sample that is desired to be removed from the biological sample. The sub-portion can include a cell, cellular organelle or subcellular element, or a biomolecule (such as a protein or nucleic acid). Particular non-limiting examples are a portion of a histological specimen (such as a nest of malignant cells within the epithelium of a tissue section of a prostate); a particular type of cell (such as a leukocyte, or even a CD4+ cell); a cell that expresses a particular protein (such as a tumor promoter protein); a cell that carries a particular receptor on its surface (such as an estrogen receptor); a particular molecule (such as an m-RNA molecule associated with the expression of a biomolecule of interest), or a portion of the biological sample that has a common chemical characteristic (such as the property of being stained by a specific histological stain such as eosin). The target is sometimes (although not always) spread throughout the biological specimen, but is characterized by a common physiochemical or biological property (such as recognition by a specific binding agent) that allows the target to be specifically recognized in the biological sample.

The target may also include non-specific material, and need not be purely a cell, cellular component or biomolecule of interest. For example, the target may include some incidental adherent material that is removed with a cell or biomolecule of interest.

Target Activated Microtransfer (TAM): Removal of a target, or many microscopic targets, from a biological sample by alteration of a transfer substrate, wherein the target(s) alters the transfer substrate to help effect the removal.

Targeting Moiety: A moiety that selectively binds to or otherwise localizes in or adjacent a target. Non-limiting examples include an antibody, receptor, ligand or probe.

Thermoplastic: A material that softens when exposed to heat and returns to it original condition when cooled to room temperature. For example, ethylene vinyl acetate copolymer resins form a family of thermoplastics that (depending on molecular weight and chemical composition) can be extrudable, foamable, injection moldable, blow moldable and suitable for compounding with other olefinic resins and rubbers to form hot glues. Specific formulations of EVA's can be extruded into strong, thin, transparent transfer films which can then be focally activated to effect a thermoplastic adhesive bond to specific targets.

Trigger event: "Trigger event" refers to an event that affects the reagent, such that the reagent alters the transfer surface. In a non-limiting example, the trigger event changes the reagent, which in turn changes the transfer surface. This change in the transfer surface permits selective removal of the target from the biological sample. In yet another example, the trigger event changes the target, which makes the target susceptible to selection (for example by selective heating of the target).

Transfer substrate: A transfer substrate is a material (such as a thermoplastic polymer) that serves as a substrate for selective adhesion or transmission of the target, for selective removal of the target from the biological sample.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

Target-Activated Microtransfer

The adoption of conventional microscope-based laser microdissection to applications in clinical molecular diagnostics and in high-throughput research has been limited by instrument complexity, dissection time, and the dependence on user targeting decisions. In contrast, target-activated microdissection (TAM) instrumentation can be simple, high speed, and amenable to automated operation. As such, TAM is well-suited for integration with existing, highly developed practices, including upstream processes such as automated immunostaining and digital imaging, already used in high throughput clinical diagnostics, as well as downstream molecular analysis, currently performed on whole tissue scrapes. Existing molecular-labeling microscopy has proven clinically important in the development of molecular medicines and their appropriate use in patient therapy. In parallel, complex *in vitro* molecular assays have been developed that can comprehensively analyze hundreds or thousands of genes and gene products in order to improve diagnosis and better predict the patient's response to different molecular therapies. Efficient,

accurate, and automated microdissection could be a critical link between these two highly developed methods by providing a rapid and robust means to isolate specific phenotypic tissue components, for example a clonal population of carcinoma cells from a surgical specimen that contains a complex mixture of normal and abnormal epithelium, , stromal cells, etc.. For the clinical diagnostics and high-throughput research applications that would be improved by routine microdissection, efficient and reliable integration of TAM with existing upstream automated immunostaining/digital microscopy and downstream molecular analysis is critical. The TAM devices and methods disclosed here aim to create a system capable of providing specific dissection of immunostained tissue for a large range of immunostains, specific targets, and tissue microstructure , with the specificity and efficiency of capture chosen to optimize the desired molecular analysis.

Disclosed herein in certain embodiments are methods, systems and devices for analyzing a biological sample, such as a cellular or tissue specimen. In one aspect of TAM, a proximal activating event comes from within the biological sample, focally altering an adjacent transfer film surface for selective removal of a target from within the sample. *In situ* activation from within the sample avoids the necessity of actively selecting each target and then altering the transfer surface by application of external energy, as in laser capture microdissection. The method of *in situ* or target-activated alteration allows for more automatic identification and removal of the target, allows large numbers of individual scattered cells to be collected quickly for molecular profiling, and improves collection of cells when histological identification of targets is challenging or impossible. The self-identification and selective adherence of the targets to the transfer surface permits a significant increase in the efficiency of transfer microdissection, and enables high-throughput methods of analysis.

Another aspect of TAM is a method of removing a target from a biological sample by contacting the biological sample with a reagent that selectively acts on the target within the biological sample. A transfer surface (such as a thermoplastic film) is placed adjacent the biological sample, and the reagent produces a change that selectively affects the transfer surface. As a non-limiting example, this change could result in focal adhesion of the target to the transfer surface such that the target can be selectively removed from the biological sample by removing the transfer surface and the adhered target from the biological sample. Alternatively, the reagent could focally increase the surface microstructure and/or permeability of the transfer surface to the targets, such that the targets and the transfer surface can more efficiently come into direct contact.

Target activated microtransfer offers several advantages over established commercial laser microdissection methods, including:

- Increased dissection rate (orders of magnitude)
- Increased dissection precision (subcellular)
- Removal of variance among individual operators, permitting standardization of the dissection process
- Elimination of targeting difficulties due to poor image quality of noncover-slipped (nonindex-matched) histology sections
- Capture on clear thin transfer film maintains spatial relationships (morphology) of targeted tissue on transfer film and allows image documentation of specific material analyzed
- Improved downstream molecular analysis from thin film (microvolume extraction, in situ or microfluidics based)

In general, high-throughput systems might require image analysis of a coverslipped, batch–immunostained serial section to guide selection of an appropriate transfer film and illumination pulse train. Efficient, high-throughput microdissection of desired targets could then be achieved by developing specifically tuned protocols that utilize optimized transfer film and pulse sequences based on similar stain localization and desired targets. Transfer films optimized to efficiently and specifically capture a wide range of immunostained targets can greatly improve throughput while reducing complexity of the automated system.

For example, disclosed herein is a systematic logic for combining adjustable TAM parameters is needed to optimize capture of the desired targets, either 1) confined precisely to the specific stain localization or 2) including adjacent unstained elements that may be required for a specific molecular analysis (e.g., nuclear or mitochondrial DNA, nuclear regulatory factors, mRNA, organelle proteomics). Disclosed herein are specific TAM design parameters that can be combined to create a versatile logic that can allow either capture of only the stained subcellular targets or alternatively capture of the whole cell even in cells with absorptive stains highly localized to specific subcellular compartments. This flexibility allows a large variety of immunostains with previously validated specificity for phenotypic cells to be used to capture the components critical to the desired downstream molecular analysis, independent of the subcellular target distribution. For example, a nuclear stain may be used to specifically capture the nucleus, or a cell membrane stain might be used to capture the whole cell including the unstained nucleus. It is also possible that a sample could be enriched for cytoplasmic components, including mitochondria, with a sequential capture process, in which an initial high-efficiency capture of stained nuclei is then followed by capture of the enucleated cells. There are a number of parameters, covered in more detail herein, which can be adjusted to optimize the capture of particular cellular components for a given

combination of stained subcellular targets and tissue type. For example, the relative strength of the polymer-tissue and internal tissue bonds is largely a function of the mechanics of the tissue, but can be altered by tissue section protocols which could partially degrade and weaken specific structural proteins, allowing for more specific capture. The lateral flow of the melted polymer can be increased by using high melt-index polymers, thicker polymer layers, greater mechanical pressure at the polymer-tissue interface, and/or longer light pulses, allowing for more efficient capture of whole cells even when the absorptive stain is localized to a specific subcellular component. Multiple stains could also be used in combination to provide additive heating in doubly-stained regions, or as part of a sequential capture process in which specifically stained, unwanted material is first removed. The use of interchangeable color filters to restrict the spectrum of the flashlamp could further enhance these additive or subtractive processes.

The specificity in TAM is achieved through spatial localization of an optically absorptive stain to the desired targets. As a result, TAM is useful in rapidly and efficiently isolating previously unpurified tissue components down to individual cells or their labeled subcellular components. Given the large number of existing tissue staining protocols that can be used to identify specific cell types or specific disease states, the possibilities for target selection are enormous. However, frequently the most specific and biologically meaningful markers yield staining with spatial localization that excludes subcellular components critical for analysis. For example, there is considerable interest in molecular analyses of cells overexpressing particular cell membrane receptors such as EGFR1 and EGFR2, but the staining protocol targeting such receptors will likely result in stain localized on the cell membrane surface. In this case, one TAM parameter domain (short pulse, ultrathin EVA layer, threshold light fluence) might yield efficient capture of the cell membrane and attached cytoskeletal elements without the cell nucleus. Alternatively a second TAM parameter domain (thicker, high melt index EVA, with a supra-threshold, longer light-pulse) applied to an identical slide-mounted tissue section might significantly increase the yield of cell nuclei from the stained cells, enabling detailed analysis of changes in DNA and nuclear regulatory factors for only EGFR+ cells.

The use of multiple, spectrally distinct stains could further expand the microdissection logic capabilities. As an example, enriched samples of nuclei stained with hematoxylin can generally be captured when a sufficient (orange-red) light dose is used. Using hematoxylin in combination with a spectrally distinct and membrane-localized stain (e.g., a DAB immunostain targeted at EGFR), with appropriate corresponding light doses (e.g., a supra-threshold blue light dose for the EGFR and a sub-threshold orange light dose for the hematoxylin), might significantly increase capture of EGFR+ cell nuclei relative to the use of DAB-EGFR alone. In another case, one might be able to

isolate a population of cells labeled with "Biomarker A" but not "Biomarker B", by using two immunostains with spectrally distinct chromagens and a two-step process in which a first microdissection, spectrally targeted at Biomarker B, is followed by a second microdissection, on a separate capture film, targeted at Biomarker A. Using these sorts of methods could extend the capability of TAM to allow isolation of targets based on a multiparameter biomarker logic, similar to the multiparameter separations used in a flow cytometer. In a laser scanning TAM system, spectral selection would be accomplished by combining multiple laser sources. In flashlamp TAM systems, colored filters can be used to modify the light dose among stain chromagens of different colors.

Disclosed herein are sets of procedures and methods for TAM parameter selection based on the stain color and localization as well as the identity of the microdissection targets desired for downstream molecular analysis. It starts with the spectral analysis of a digital RGB image of the tissue section slide-mounted for microdissection, or alternately, for greater precision, an image of an equivalently stained serial section that has been optimally prepared for imaging (e.g., index-matched and coverslipped). This digital image could be obtained from a slide spectral imaging system, but, ideally a high-quality digital imaging system could be used to obtain a simple RGB transmission image, provided that the illumination is spectrally structured to separate each chromagen's absorbance into a different RGB component. After appropriate calibration, the image is used to identify the range of optical densities of the different chromagens in the desired set of targets, as well as their spatial overlap. This information can then be converted to a 2D (or higher dimension) histogram from which different clusters can be identified by spectral signature, size, and proximity to other stained structures. TAM logic can be applied to determine the appropriate spectral and temporal light dose and transfer film to be used to specifically capture the desired elements. Computerized algorithms could be used to predict bonding and generate an image of the captured elements expected for a given film and spectral light dose. These software tools could be used by a pathologist during an initial pathology microscope exam, when ordering TAM capture and downstream molecular analysis to be performed in a core lab using an equivalently stained serial section. For example, a simple case would be to use a coverslipped slide DAB immunostained for EGFR1 and counterstained for hematoxylin. A TAM logic analysis might optimize the light dose for specific capture of cell nuclei within EGFR1 positive cells for analysis of alterations in DNA and nuclear regulatory factors, by using a blue-filtered Xenon flash so as to combine strong EVA melting where in contact with the stained cell membranes and lateral flow into the modestly heated hematoxylin stained nuclei. In cells without strong EGFR staining, the modest nuclear hematoxylin heating generated by the blue filtered light would be insufficient to

melt the transfer film polymer (e.g., EVA). This logic might also select among a smooth or rough EVA surface, with thick or thin layers of low or high melt index EVA in order to optimize transfer. The development of such logic-based algorithms for optimizing TAM will improve the ease of use and reliability, allowing faster optimization of specific microdissections based on stain profiles. A software implementation of this sort of TAM logic-based algorithm, using digital slide images as the input for analysis, could be a critical element in automation and integration with CLIA-approved clinical molecular diagnostics. In the case where tissue staining is not sufficiently uniform, the microdissection prediction software tool could also suggest regions of interest more likely to give the desired logic-based capture. These ROIs would then be used to generate illumination masks used in the TAM procedure.

TAM provides a specific advantage over existing microdissection techniques by replacing microscopy-based targeting decisions with automatic capture of specifically stained targets in the whole slide or a selected region of interest. The actual specificity and yield is a complex interaction of stain localization, light source fluence and pulse length of a light source, mechanical pressures at the transfer substrate polymer-tissue interface, and thermoplastic polymer composition and layer geometry. These adjustable TAM parameters combine with mechanical strength of the stained and adjacent unstained tissue elements to determine what material is captured.

An important feature of TAM capture is that upon melting, the polymer flows into the desiccated tissue filling the microscale voids left when water was removed from the tissue section (or subsequently, in the case of paraffin-embedded tissues, when the paraffin was removed by an organic solvent [typically xylene]). To some depth this thermoplastic polymer flow creates a polymer tissue composite that is inherently stronger that the surrounding desiccated tissue and the bond of the tissue to the underlying glass slide. After TAM bond formation, removal of the transfer film (e.g., a quick peel) from the tissue section shears the tissue at mechanically weaker points outside the edges of this composite. Exactly where this fracture occurs laterally depends on the 3D geometry of this composite and the strengths of unstained tissue elements at the bond edges. For the 5-10 micron-thick tissue sections, the depth of capture below the bond edges is strongly dependent on the strength of the tissue section adherence to the glass slide (i.e., surface on which the tissue section is mounted). For paraffin-embedded tissue sections, this glass-tissue adhesion is quite uniform, which increases the reproducibility of target capture. As a result, the precise localization of capture to stained subcellular organelles is enhanced when the tissue mechanics is associated with a strength discontinuity at the stained-unstained tissue borders. For example, while polymer flow into immunostained cell membranes (e.g., breast cancer cells immunostained for overexpressed her2neu receptor protein) can create

sufficient composite strength to capture the unstained cell nuclei within the annular stain when capture parameters are appropriately chosen, the stroma surrounding these annular bonds is much less likely to be captured due to the bond 3D geometry and to the extended lateral strength of the stroma compared to the epithelial glands (or invasive carcinoma) within it. The mechanical properties of the tissue are therefore often particularly important in determining specificity, ease, and efficiency of the microdissection of different targets. Standard tissue section protocols can be modified to partially degrade and weaken the specific structural proteins that provide the bulk tissue and cellular mechanical strengths. This would shift the balance towards more specific capture of the stained cellular components alone, particularly when the lateral polymer flow is minimal, as in short pulse TAM. Pulse lengths shorter than the thermal relaxation time of the absorbing targets (i.e., size dependent radial thermal diffusion) can increase the efficiency and spatial specificity of thermoplastic bonds to polymer surface. Alternatively, longer pulses or pulse trains combined with high melt-index thermoplastic polymers can be used to induce additional lateral flow to capture adjacent unstained elements, for example, nuclei within cells with specifically stained cellular membranes.

The thickness, surface, and thermoplastic properties of the microbonding polymer (e.g., EVA) layer on the transfer tape also creates a parameter space to designate different disposables for different stained targets and desired degree of adjacent capture. Shorter pulse length (<0.2msec), thinner EVA layer (~1 micron), and lower melt index (e.g., 30) of thermoplastic polymer all combine in optimal TAM designs for higher precision and restriction to only small stained targets, whereas combining increased pulse length (>1 msec), a smooth surface, thicker EVA layer (≥5microns), and higher melt index (e.g. 500) can increase the ability to capture the entire cell even when the stain is localized to a specific subcellular compartment. This dense parameter space allows a variety of combinations that might be tailored to already validated immunostains for clinical diagnostics that require capture of specific cells from specimens stained for specific cellular components. In some cases, immunostains will result in extremely localized stained targets (1 um or less); the spatial distribution of stain within the subcellular compartment and desired specificity of capture will be important considerations in choosing optimized capture parameters, including an increase in the required dose as the size of the stained component decreases).

TAM capture logic also includes the use of spectrally specific counterstains. In the above example, all cell nuclei might be stained lightly with hematoxylin and exposed to a light dose insufficient to capture the nuclei in the absence of surrounding immunostained membranes but the lower level direct nuclear light absorption and heating enhances the lateral flow of polymer into nuclei surrounded by strongly stained cell membranes. The use of spectrally distinct stains, such as

two immunostains with different enzyme-chromagen pairs, also provides a series of logic options. In the case where threshold doses can be accurately determined (e.g., by image analysis of an adjacent serial section identically stained and coverslipped), both additive and subtractive logic can be applied when the spectral composition of the light pulses can be suitably adjusted. Two laser wavelengths with different target fluences (laser-scanning TAM) or colored filtering of the white xenon flash (fTAM) can be selected to combine or restrict the heating of the two chromagens. For example, one can selectively heat and capture one chromagen creating a captured sample expressing one biomarker independent of the level of the second. The resulting depleted tissue section could then be treated with a second spectrally tuned light pulse to capture those targets that express the second biomarker but not the first (previously removed). By using multiple distinct chromagens a single tissue section differently stained for different biomarkers can be serially processed to compare different target populations. For example, to analyze clinical carcinoma samples, a specific cancer immunostain with distinct chromagen might be used to isolate cancerous cells for direct comparison with normal epithelial cells, identified by a different biomarker in the same slide after the cancerous cells have been removed.

Practical applications of TAM logic may also include flexibility with respect to the trade-offs between efficiency (yield) vs. specificity of desired targets. For example, if DNA is to be analyzed from specific cells only, using a cell-specific cytoplasmic stain can allow capture of a high percentage of their nuclear DNA and mitochondrial DNA whereas using a specific nuclear stain would reduce mitochondrial DNA contamination but also restrict capture to only those stained nuclei (i.e., transected) in direct contact with the polymer surface.

The methods and systems disclosed herein permit simpler integration of target-activated microdissection with clinical pathology and optimal target region selection in research studies of specific components in the complex molecular biology of tissues.

Disclosed herein are methods and devices for removing a target (such as a subset of cells) from a biological sample (such as a tissue section or cellular preparation) by using an *in situ* activating event from within the biological sample to alter a transfer surface for subsequent removal of the target from the sample. In some particular examples, the activating event focally adheres the target to a transfer surface that is in contact with the sample.

The activating event that alters the transfer surface can be provided by a reagent that specifically binds to the target and performs the activating event that alters the transfer surface. In one example, the reagent includes a chromophore that can be stimulated with light to emit heat. After the biological specimen is exposed to the reagent to selectively bind it to the target and concentrate the reagent within the target, a transfer surface (such as a thermoplastic film) is placed

in contact with the biological sample. Exposing the biological sample to light stimulates the chromophore to produce a change in or adjacent the target that selectively affects the transfer surface. Heat emitted from the chromophore focally heats the transfer surface to selectively adhere the target to the transfer surface, such that removal of the transfer surface from the biological sample also selectively removes the target from the sample. In this case, "selective removal" is not restricted to the cases in which only stained targets are removed from the sample, but rather may also include the additional capture of both stained and desired unstained, microscopically contiguous structures. In some instances where efficiency of capture is more important that absolute purity, the selectively removed target will include biological material other than that specifically desired, but the level of non-desired material will have been substantially reduced from that seen in the original biological sample.

In most instances, multiple targets that share a similar characteristic will be present within the biological sample, and many of these targets will specifically bind the reagent and focally alter the transfer surface, such that the multiple targets within the sample can be efficiently and quickly removed, with a minimum of selection by an operator. This feature of target "self-selection" based on physical, chemical or biological characteristics of the target allows efficient and rapid removal of the large numbers of microscopically distinct targets.

The target activated transfer method differs from standard laser capture microdissection in that the transfer surface is altered from within the biological sample, instead of being changed from outside the sample by the focal application of external laser energy to a uniformly absorbing transfer layer. Providing an internal (sample based) event, instead of an external force, to alter the transfer surface provides an accurate focal alteration of the transfer surface with increased precision and greatly reduces the light energy density required for effective microbonding compared to laser capture microdissection. Molecular targeting of the reagent to individual scattered cells (for example using an antibody or probe) allows target cells to be collected, even when histological or microscope-based identification of the cells is challenging or impossible. Moreover, by targeting the reagent to specific molecules or structures of interest within the sample, this method is readily suitable to automation. Furthermore, due to the specificity of the method for target cells or cell components of interest, this method reduces the number of cells and numbers of tissue sections needed to obtain molecular profiling information in comparison to existing molecular profiling systems. Furthermore, the higher sample purity obtained with target-activated microdissection reduces uncertainties in the subsequent molecular analyses performed.

Replacing the "human dissector" with an "antibody/probe dissector" or "stain dissector" allows the technique to become operator-independent. This change allows certain embodiments of

the method to become several orders of magnitude faster than traditional laser capture microdissection (LCM), in which the investigator needs to tediously dissect each cell/gland one at a time. LCM often requires 10,000-100,000 individually-executed laser shots for each histology slide used in a study. In such cases, a standard LCM dissection may require an hour or more to procure the cells from each slide, and days/weeks to complete an entire study. In contrast, target activated transfer methods disclosed herein can often procure substantially all of the targeted cells in each slide in just a few seconds using rapid, spatially uniform wide-field light pulses.

In a simplified schematic example shown in FIG. 1A, a tissue section 10 has a bottom surface adhered to a glass support slide 12 with polylysine. A top surface of tissue section 10 is in contact with an overlying transfer surface 14 of a planar transfer member 16, which in this example is a thin film with a thermoplastic surface. Tissue section 10 contains cellular structures 18 (such as prostate epithelial tissue) that include a target 20 (such as an area of highly atypical cells). The thermoplastic film is capable of being focally melted in locations where the surface contacts the targets and reagent. In this example, transfer member 16 is placed on top of and in contact with tissue section 10, such that transfer surface 14 is in substantially contiguous contact with the biological specimen. Such contiguous contact can be achieved, for example, by pressing transfer member 16 against tissue section 10. The biological specimen contains the reagent (not shown), which localizes in targets 20 and focally heats thermoplastic surface 14 of member 16. Localization of the reagent in the targets is achieved, for example, with a specific binding agent that specifically binds the reagent to the target.

FIG. 1B schematically illustrates changes that occur in transfer member 16 after the reagent heats the adjacent surface of the transfer member to adhere it to the targets. As the thermoplastic surface 14 adjacent the targets heats and melts, the thermoplastic material focally flows down and perhaps around targets 20 to form a physical connection 22 that adheres the targets to transfer member 16. As shown in FIG. 1C, transfer member 16 is then lifted away from tissue section 10, which selectively removes targets 20 from the tissue section, while substantially leaving non-targeted material in tissue section 10. The precision of removal of each target can vary depending on a variety of circumstances, such as the size and shape of the target, and the histological architecture of surrounding tissue. Moreover, the specificity of removal of only target tissue from surrounding tissue need not be 100%, but can vary depending on the end use to which the target tissue is to be put. It is sufficient, in many embodiments, if the specificity of removal of target tissue is small (for example the desired target is only 5% or less of the removed material). However, the flexibility and precision of this method can also permit specificity of removal of at least 10%, 30%, 50%, 75%, 90%, 95% or more (that is, at least that percentage of the removed

tissue is the desired target). Variation of parameters that affect the precision of target capture are known to those of skill in the art, and include varying the thickness of the tissue section or other characteristics of the biological sample.

A particular advantage of some embodiments of this technology is that the reagent can be activated by an external stimulus or trigger event, even though the proximate event that alters the transfer surface comes from within the biological sample. FIG. 2 illustrates an embodiment in which the reagent is activatable by electromagnetic energy, such as light energy from a Xenon flashlamp. For example, the reagent is an immunostain that carries an absorbing dye (such as a chromophore) that emits heat when exposed to light of a specified activating wavelength or wavelengths. In particular examples, the activating wavelengths are the broad visible spectrum of a Xenon flash (400nm to 600nm) that are specifically absorbed by the desired oxidized-diaminobenzamine (DAB)-immunostained targets within the tissue section.

FIG. 2 illustrates an embodiment in which a tissue section 50 is mounted on a support slide 52, and covered by a thermoplastic film 56 having a transfer surface 54. A light source 66 is provided in a position (such as above transfer film 56) that uniformly illuminates the entire surface of tissue section 50, to supply energy to the chromophore that is concentrated at the targets. The electromagnetic energy supplied by the light is then converted into heat by the reagent, as shown in FIG. 3, to fuse targets 60 to transfer surface 54. Preferably, thermoplastic film 56 is transparent and absorbs little if any of the incident light, to minimize non-specific adherence of tissue section 50 to transfer film 56. In contrast, LCM thermoplastic films strongly absorb the focused laser beam used in LCM.

The reagent may include both a targeting moiety to localize the reagent in the target, and an activating moiety to activate the change that adheres the target to the transfer surface, or focally alters the transfer surface so that the target can that selectively move through the transfer surface. The targeting moiety and activating moiety are directly attached to one another in one embodiment. A simple reagent is any absorptive histochemical stain with appropriate target specific absorption, e.g., hematoxylin which selectively binds to all cell nuclei and strongly absorbs orange light. In another highly developed process specifically useful for many TAM applications, the reagent is a standard immunohistochemistry kit consisting of a primary antibody, a secondary enzyme-linked (e.g., horse radish peroxidase) antibody and an chromophore (e.g., diaminobenzamine [DAB]) on which the secondary antibody acts to form strong, visible-light absorption within the tissue section wherever the antigen (e.g., target-specific biomarker molecule) to the primary antibody is present.

In other examples, the biomarker-specific targeting moiety is a nucleic acid molecule that binds a complementary nucleic acid sequence specific to the desired targets (such as an mRNA

sequence that is selectively expressed in malignant cells). This specific tissue targeting moiety is coupled to an enzyme can create a localized tissue deposition of a stain with high optical absorption. Alternatively, the targeting moiety (the primary antibody in the immunohistochemistry kit) specifically binds to a cell surface antigen associated with a particular type of tumor, for example the prostate specific antigen (PSA). In another example, the targeting moiety is an antibody for an estrogen receptor that is expressed on the surface of certain types of estrogen receptor positive (ER+) breast tumor cells. In most current examples of molecularly specific targeting, the absorptive stain contrast that determines localized TAM transfer requires binding a secondary enzymatic moiety to the primary biomarker-specific moiety, followed by supplying small molecule chromophores on which the enzyme acts to create an absorptive stain, localized to the particular biomarkers of interest. This is an effective class of methods for providing localized microbonding in target-activated microdissection.

The biological sample on which the method is performed can include a broad variety of biological materials, such as a preparation of cells, biopsy material, a tissue section, a cell culture preparation, or a cytology preparation. The sample can either be a coherent tissue specimen with recognizable histological architecture, or a processed or liquid specimen that has been derived from a tissue or other biological specimen, such as a cell suspension or cell culture. In particularly disclosed examples, the biological sample is a standard tissue section, such as a paraffin section that has undergone routine formalin fixation. The specimen may or may not have been stained (for example with eosin) to visualize cellular components of the specimen.

After the target is selectively removed from the biological sample, it may be subjected to a biological analysis of the target. The target is first exposed to targeting moiety in the form of a primary antibody that selectively binds to the target, and then exposed to a secondary antibody that carries the activating moiety. The activating moiety is then activated (for example by supplying a substrate for an enzymatic activating moiety, or irradiating the slide), adherence to the transfer surface is accomplished, and the transfer surface (with adhered target) is removed from the biological sample and introduced into a container, such as an analysis tube. The analysis (or analyses) carried out can include determining whether biomolecules of interest are present or absent, quantifying a biomolecule in the target, and/or amplifying the target (for example using the polymerase chain reaction). In certain examples, the analysis can include screening for the presence of a protein, or a nucleic acid encoding the protein, wherein the presence or absence of the protein in the target is indicative of a disease in the subject. For example, the test could screen for the presence of an oncogene or an expressed protein associated with malignant tumors (or associated with tumors having particularly good or poor clinical prognoses).

In contrast to laser capture microdissction, the irradiation passes through the transfer layer without absorption or direct effect but rather is absorbed by the stain within the tissue. Only where the local stain density absorbs light pulse fluence is there a proportional localized tissue heating. Only where that tissue heating is efficiently coupled to the thermoplastic transfer film surface and activates it, is a biomarker-specific bond of the tissue and film surfaces created. Minimizing absorption of light energy by the transfer layer reduces non-specific heating of the tissue surface, and thus reduces non-specific adherence of the transfer layer to unstained regions of the biological sample. By only melting the surface of the thermoplastic polymer proximal to the biological sample (rather than the full film thickness as in LCM), the total energy required to form a tissue-target-specific bond is reduced by an order of magnitude, and the local specificity of the bond formed is increased, as compared to LCM. In addition, there is no need for microscopic visualization in the process.

The transfer substrates can be made of a variety of materials that are suitable for selective removal of targets from biological samples. Generally the transfer film materials are transparent or translucent to allow efficient illumination through the transfer film of the stained tissue targets at the tissue-film interface. Where the tissue is to be illuminated through the glass slide directly, a high turbidity (without absorption) of the polymer film may increase the light fluence at the tissue transfer film interface. Film transparency also simplifies visualization (such as microscopic examination) of the tissue components captured on the transfer substrate after the film is removed from the tissue. Cells or organelles adherent to the transfer substrate retain their morphologic features for subsequent visual inspection and quality control of capture.

A critical requirement for the transfer film is that it makes sufficient physical contact with the stained tissue section surface to allow efficient transfer of thermal energy into the film from the stain during its transient heating due to the absorption of the light pulse. This requires a uniform applied pressure gradient across the film-tissue interface surface and good physical contact. This generally requires that either the transfer film or the tissue section be flexible. In cutting laser microdissection, tissue sections are frequently placed on very thin flexible polymer films (e.g., 1 micron thick polyethylene nitile). In such cases the flexible tissue section could be pressed by uniform air pressure against a thermoplastic layer on a rigid substrate. A more practical option for compatibility with routine tissue processing onto standard glass slides is to use a thin flexible polymer tape with a low-melting temperature thermoplastic adhesive layer (e.g., 1 to 20um thick EVA) coated onto a high-strength, high melting temperature, flexible thin polymer layer (e.g., 12 micron-thick polyethylene terephthalate [PET]). The surface of the EVA coating can be finely microscopically textured which can increase focal random

microscopic contact of the tissue surface, particularly when applying a vacuum to the film-tissue slide interface.

Transfer films suitable for use in target activated transfer may be made of a wide variety of thermally activatable materials, such as ethylene vinyl acetate (EVA), polyurethanes, polyvinyl acetates, and the like. Specific other selectively activatable materials found useful in the methods of the disclosure are: thermal sensitive adhesives and waxes (mixtures of EVAs or related polymers); or thermally activated emulsions that undergo chemical or physical changes to create surface bonds between the tissue surface and the flexible transfer backing layer (e.g., thermally activated adhesive emulsions coated onto a PET backing). The backing layer provides physical support for the adhesive surface, and thus can be integrated physically into the activatable adhesive surface.

The transfer substrate is initially positioned over a cellular material sample which can be a microtome section or cell smear that is supported on a support member, and is most typically a histological section mounted on a microscopic slide. Paraffin embedded, formalin-fixed tissue samples mounted on a glass slide and immunohistochemically stained in a standardized, automated process are particularly well-suited for target-activated microdissection. The transfer film is brought into contact with the cellular material sample by an applied air pressure gradient, either positive pressure applied to the outer backing surface of the transfer film or a negative (vacuum) pressure applied through the porous tissue section to the thermally-activated adhesive layer. Although a large region of the tissue sample is uniformly illuminated, the localized tissue heating creates bonds to the transfer surface only where it is in contract with a microscopic stained tissue element. This localized bond can range in size from an area smaller than a single cell (less than 10 microns), to single cell or contiguous cluster of specifically stained cells. All such bonded elements within the large illuminated portion of the tissue section (e.g., ~1cm^2) can be selectively transferred to the transfer film as it is peeled (e.g., separated) off the tissue section. As shown, the removed transfer substrate carries with it only the precise cellular material from the region of interest, which is pulled away from the remaining cellular material sample.

The reagents used in the target activated transfer methods may be constructed to combine at least two functional domains, or "moieties" in a single molecule or reagent. The simplest examples are small molecule histochemical stains with high spectral absorptivity that bind specifically to specific cell types or organelles (e.g., hematoxylin to cell nuclei, Congo red to amyloid, and Nile red to neural cell nuclei). Greater molecular specificity of targeting is readily achieved by using standard double-antibody enzyme linked immune-histochemical staining. Here the staining is accomplished by an enzyme-linked immunoreagent bound to tissue biomarker molecules and that

acts to deposit locally a freely diffusible small molecule with a high spectral absorptivity as a chemically modified absorptive precipitate (e.g., oxidized diaminobenzamine created locally by a peroxidase-antibody fusion product bound to tissue biomarkers) selectively within the cells or cellular components of interest.

Targeting moieties are selected in response to the nature of the target. For example, essentially any cell type is suitable for targeting, provided a molecule is known or can be identified that is suitable as a cell-specific target molecule to which the targeting moiety can be directed. A targeting molecule can be applied to various types of samples, for instance to a tissue section mounted on a standard glass histopathology slide, tissue arrays, or to cells otherwise cultured on an amenable substrate.

The antigen that is the target of the targeting moiety is either solely expressed by the cells of interest, or is expressed at a higher level in the cells of interest, such that the expressing cells can be sufficiently differentiated from non-expressing cells by the density of the absorptive precipitate. For example, cells expressing antigens or sufficiently higher numbers of antigens than background cells will have an optical density (OD) of at least about 0.2- 0.6, such that the precipitate will efficiently absorb the energy of the stimulus (such as a flash lamp), causing sufficient local heating of the target to cause it to adhere to a transfer film. The enzyme-linked reaction that deposits the precipitate is continued until these local optical density values are achieved at the desired target sites.

In some embodiments, the targeting moiety is a hapten, a lectin, a carbohydrate, a cofactor, a receptor ligand, or a protein with high specificity for a binding partner, such as the biotin/(strept)avidin binding pair, or protein A or G.

In other embodiments, a probe or short DNA sequence serves as the targeting moiety, and binds to a complementary molecule or sequence (such as expressed transcription factor or DNA regulatory sequence) within the target cells. In certain embodiments, the complementary sequence is present as a result of ongoing transcription within the cell. These embodiments may be used to profile the response of target cells to internal or external conditions (such as treatments with pharmaceuticals, onset of disease, etc.).

Indicators may also be included, either in the transfer substrate or in a separate layer or layers, to define the location of adhesion of cells or cellular components. Such indicators include thermochromic dyes, dye precursors that combine upon melting to form a color for visible or instrumental identification, and dyes that are converted to color by other effects of optical absorption. Suitable indicators also include other physical effects, such as the appearance or disappearance of translucency or opacity upon optical exposure or upon heating.

A variety of biological samples can be analyzed using the methods disclosed herein, such as biopsy material, tissue sections, cell culture preparations, cell smears and cytology preparations. Particular examples of such samples include cytology preparations or freshly ethanol-, methanol-, or acetone- fixed tissue sections.

In one example, histopathology slides are produced using traditional techniques (*see e.g.*, Shin *et al.*, *Lab. Invest.* 64(5): 693-702, 1991). Following fixation of tissue samples to the slides, the samples are subjected to automated enzyme-linked immunosorbant assay (ELISA)–like techniques (*see* Erdile *et al., J. Immunol. Methods*, 258(1-2): 47-53, 2001) to introduce the reagent into the tissue and allow binding of the targeting moiety (for example an antibody) to its target.

To increase the volume of thermoplastic polymer melted for a given stain level (OD), more flash lamp energy may be applied. Providing a single pulse of greater energy may be adequate to locally melt sufficient thermoplastic polymer to cause the adherence of cells containing a sufficient density of reagent to the transfer substrate in some instances. However multiple pulses, such as a series of flash lamp pulses of lower-intensity, can help reduce the lateral flow and corresponding enlargment of the adhered region that may result from application of a single strong stimulus. Each stimulus in the series is applied in an intensity sufficient only to cause adherence to target cells containing the reagent, thereby substantially avoiding adherence of nonstained cells to transfer substrate. Subsequent pulses improve the strength of the adherence by allowing melted thermoplastic polymer to expand and flow into/onto the targeted cells, which also allows the thermoplastic polymer to adhere more strongly to the transfer substrate.

Following removal of the target from the biological sample, the target may be subjected to further analysis. For example, after a thermoplastic transfer layer is focally adhered to the targets and then removed from the sample, the transfer layer is separated from the sample and the adhered components are reserved for subsequent analysis. In one example, the transfer substrate itself is introduced directly into a tube that contains buffer. Alternatively, the targets may be flash-frozen on the transfer substrate, or the targets may be fixed to a separate stable medium (such as a glass slide). In other embodiments, the targets are physically cut from the transfer substrate, focally dissolved from the transfer substrate, or excised with a hot wire knife (which is self-sterilizing to eliminate contamination between specimens). As a final example, the transfer substrate, with the attached tissue can be bonded to a substrate with channels and microscale reaction chambers, for extraction of the molecular components into a minimal volume. This microfluidic substrate could be compatible with automated flow instrumentation.

The biological material in the targets, such as cells or cellular components, are amenable to methods of analysis known in the art, such as those provided in Sambrook *et al.* (In *Molecular*

Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989), and Ausubel *et al.* (In *Current Protocols in Molecular Biology*, Greene Publ. Assoc. and Wiley-Intersciences, 1992). Specific methods of analysis of the isolated components include resolution of proteins through electrophoresis (*e.g.*, SDS-PAGE), followed by visualization using an appropriate method (*e.g.*, blot staining with Ponceau S, autoradiography, etc.), and those in which the isolated components are sequenced or amplified, for instance using the polymerase chain reaction, and subsequently visualized on a gel, or in an array, etc.

Another type of assay that can be used to analyze the extracted components is two-dimensional polyacrylamide gel electrophoresis (2D PAGE). By running proteins extracted from cells of a control sample and proteins extracted from cells of an experimental sample (such as a tumor sample) and comparing the blots, differential protein expression can be seen. In particular, by scanning the stained gels into a computer, and using image comparison software, the location of proteins that are present in one cell type and absent in the other can be determined. Furthermore, these altered proteins can be isolated from the gel where they are present, and mass spectroscopy MS-MS sequencing can be used to identify the protein, if the sequence exists in a database. In this way, the protein differences between control and experimental cells can be more fully understood. Additionally, proteins of interest isolated from a 2-D gel may be used in binding studies, where the protein is functionally tested for an alteration in the ability to bind with a putative or known ligand. Finally, this comparative analysis can be performed on samples from various stages of progression of a disease (*i.e.,* isolated stages of a tumor), where the different stages exhibit sufficient differences to allow separate isolation of populations using the methods disclosed herein.

For messenger RNA analysis, the tissue section may be pretreated to irreversibly inactivate RNase and/or preferentially bind RNA to polycationic nanoparticles or to polycationic surfaces within the thermoplastic transfer film for greater RNA stability and sensitivity of downstream analytical methods such as next generation sequencing (RNA Seq). The mRNA can be extracted using an oligo dT column (Micro-Fast track MRNA Isolation Kit, Invitrogen Co.). Recovered mRNA can alternatively be amplified and investigated using polymerase chain reaction (PCR) technology, such as, for example, by RT-PCR as known to those skilled in the art.

To analyze DNA from cells isolated by the present method, the isolated cells are extracted (*e.g.*, placed in a single step extraction buffer solution of 50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20, and 0.2 mg/ml proteinase K, incubated for four hours at about 37° C, followed by ten minutes incubation at about 95° C). Recovered DNA can also be amplified and analyzed using PCR technology in combination with analysis techniques, such as blotting, sequencing, etc., known in the art. If native DNA is required for DNA fingerprinting analysis, the proteinase K can be

added after DNase in the fingerprinting protocol. For DNA analysis of paraffin embedded tissue, the isolated cells are extracted, (*e.g.*, placed in a single step extraction buffer solution of 50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20, and 0.2 mg/ml proteinase K), and incubated (*e.g.*, depending on sample size, from two to twenty-four hours at about 37° C, followed by a ten minute incubation at about 95° C).

The ability of the targets to "self-identify" once they have been exposed to the reagent, and then select themselves for adherence to (or movement through) the transfer layer, makes this method ideally suited to automation. For example, current automated methods process hundreds or thousands of human clinical tissue sections with disease-specific immunohistochemical stains, then automatically scan these sections with a digital imaging system. The resulting images are sent to the computer screen of a clinical pathologist, who makes a molecular pathology diagnosis of the disease and its suitability for different molecular therapies. This pathologist could routinely select those tissue sections in which immunohistochemistry stain localization is specific to the tissue targets for which more comprehensive molecular analysis would be most informative. For example, when looking at a cancer patient's tissue section with high levels of epidermal growth factor receptor [EGFR], as identified by EGFR-immunohistochemistry, the pathologist might order that adjacent serial sections be stained identically, but not coverslipped, and submitted for target-activated microdissection. Automated quantitative image analysis of the original slide image viewed by the pathologist would determine the size, number of target cells in the slide, and the spectral absorbance of the stained targets to determine the optimal light dose and transfer film properties to isolate the stain targets. The software could also be used to predict the amount of nucleic acid that will be collected from an adjacent serial section by automated TAM with the selected parameters. Based on the amount of recovered DNA required for the downstream analysis, the appropriate number of sections and transfers would be ordered for that particular surgical specimen. Those tissue sections, with appropriate tracking labels, would be loaded in to the automated TAM device. The transfer films would be imaged after dissection and prior to extraction as an initial quality control step. After passing QC with a refined estimate of amount of nuclei acid recoverable from the films, the extracted nucleic acids would be extracted and purified (e.g., DNA and RNA separation) and submitted for downstream molecular analysis (e.g., exome sequencing or RNA Seq analysis). This is an example of one method in which high throughput standardized TAM could be integrated into existing upstream molecular diagnostics that rely on immunohoistochemical digital imaging by pathologists, and used to routinely supply high quality nucleic acid samples from specifically microdissected tissue for further analysis. As an example, these samples could be used in any of the evolving methods for comprehensive biomolecular

characterization of an individual patient's disease states and improved patient-specific selection of new molecular therapies. Such automation increases the efficiency of producing and analyzing samples, reduces operator error, and increases consistency of results.

In one particular automated embodiment, multiple tissue section slides from a patient's surgical specimens are fixed to individual supporting media (*e.g.*, glass slides), and the samples are subjected to automated histochemical and immunohistochemical staining for a variety of relevant targets (*e.g.*, Benchmark XT, Ventana Medical, Tucson AZ). After processing by the Benchmark XT system, the tissue slides are automatically scanned by the iScan HT digital slide imager (Ventana Medical, Tucson AZ) and the images transmitted to a board certified cancer pathologist who makes a cancer diagnosis and identifies those stained slides appropriate for TAM and downstream nucleic acid sequence analysis. Real-time image analysis tools would provide the pathologist with an image of the targets that would be estimated to be recovered as a function of optimized TAM parameter selection, providing better guidance for the selection of slides to be ordered for microdissection and downstream sequence analysis.

For the target-activated microdissection process to be efficiently automated and integrated into the above clinical work flow, the tissue and film handling for microdissection and subsequent molecular extraction off the thermoplastic transfer film need to be automated. In addition, the requirements for robustness of microdissection and the downstream molecular analyses require automated process parameter selection (e.g., selection of specific transfer film, pulse energy and pulse sequence to be used, number of films and/or tissue sections required). The quantity of purified targets (and thus the number of films) required to support the desired comprehensive molecular analyses will depend on what is ordered: such for example, qPCR, qRT-PCR, whole exome sequencing, RNA-Seq, MS proteomic analysis, and 2-D gel proteomic analysis require significantly different amounts of purified biomolecules, each with different concentrations in tissue.

Parameters which control the specificity and efficiency of microdissection for a particular stain localization, stain density, and target identity include: properties of the specific thermoplastic polymer used in the microbonding layer, including e.g., melting temperature, thickness, and melt-index (or change in fluidity with increasing temperature about the melting point); the absorptivity, size, and number of specific stained targets and of any adjacent desired unstained subcellular elements; the light pulse dose (pulse energy density, pulse duration, and number of light pulses in a pulse sequence); and the pressure applied to the transfer film-tissue interface. Critically, all these parameters can be integrated in an automated parameter selection process, based on quantitative image analysis of a coverslipped serial tissue section viewed by the pathologist, and controlled by

the design of a target-activated microdissection system (standardized transfer films, pulse light sources, and film-tissue cassette used in the microdissection and downstream molecular extraction off the film). A specific transfer film can then be automatically applied to the tissue slide, pressure applied, a light pulse sequence selected, the transfer film removed, the captured material imaged, and the sample transferred to a system for subsequent thin-layer fluid extraction and purification of molecular species, followed by the desired comprehensive molecular analysis. In examples of such embodiments, each slide might typically contain many thousands of target cells, and the automated microdissection, imaging and extraction might be completed in a few minutes and reliably supply enough target specific nucleic acids for downstream comprehensive genomic analysis. Since all processes downstream of the initial pathologist review of the tissue slides and digital images are automated and easily parallelized, very high clinical throughputs are feasible with this system, but are unattainable using traditional operator-dependent, laser microdissection techniques.

Using the methods disclosed herein, high throughput functional genomics and proteomics can be brought down to the level of specific diseased cell populations in a tissue. Following separation of targets from the biological sample, the analysis of protein, mRNA, and DNA from isolated specific cells and tissue structures helps determine whether and to what extent genes are operative in normal as compared to diseased cells. Isolation of specific cells using these methods makes it possible to detect, for example, somatic mutations in cellular DNA that result in malignancy. The methods can be used to follow changes in gene expression that accompany cell maturation, tumorigenesis, and cell apoptosis (by targeting the reagent to cells having these characteristics). Furthermore, the identification and isolation of specific protein products specifically produced by diseased cells provides information that can be used to develop new diagnostic methods to scan for the presence of such proteins. Each of these areas can be advanced by the methods disclosed herein, which enable selective isolation of specific cells or cellular components (*e.g.*, DNA, RNA, proteins, and mRNA) from target tissue samples having target characteristics (such as atypia, viral infection, or aggressive infiltrative tumor cells).

Subjecting purified populations of cells to high-throughput genomic or proteomic analysis can also be used to correlate disease prognosis or therapeutic outcome with molecular characteristics, such as structural changes in genes or proteins, copy number or expression alterations of genes. Genomic and proteomic analysis can also identify novel targets for gene prevention, early diagnosis, disease classification, or prognosis; and to identify therapeutic agents.

Examples of high-throughput technologies that can be used for these and other purposes include cDNA and genomic Next Gen sequencing, serial analysis of gene expression (SAGE), representational difference analysis (RDA), differential display and related PCR-based

technologies, hybridization-based sequencing, subtractive cDNA or genomic hybridizations, cDNA arrays, Comparative Genomic Hybridization (CGH) arrays, electrophoretic, mass spectrometric, or other separation and identification methods for DNA or proteins, yeast two-hybrid technology or related techniques of molecular biology.

Purified cell and tissue structure samples obtained by target activated transfer may also be used in combination with array techniques, and can provide information about the frequency of a multitude of genetic alterations or gene expression patterns (including normal gene expression patterns) in a variety of tissue types (such as different types or grades of tumors), and in tissue of a particular histological type (such as a tumor of a specific type, such as intraductal breast cancer), as well as the tissue distribution of molecular markers tested.

Differential gene expression can be analyzed by detecting different levels of proteins or RNA using semi-quantitative RT-PCR on the substantially pure cell populations obtained by the targeted transfer techniques described herein. Once the differential expression is determined using this approach, the different levels of expression can be used for diagnostic or therapeutic purposes. For example, overexpression or underexpression of particular proteins can be associated with particularly benign or malignant tumors, which provides prognostic information about the likely clinical course of a tumor. This information in turn helps determine whether particularly aggressive anti-tumor chemotherapy must be undertaken to obtain a greater likelihood of response or remission. Similarly, information about differential protein expression in particular types of disease (such as tumors of a particular type) can be used to target treatment. Hence if up-regulation of a protein is found in a particular type of tumor cell, therapies aimed at disruption of that up-regulation can be administered.

Establishing reliable physical and thermal contact of the biological sample surface with the transfer film polymer surface is critical for successful target-activated microdissection. One critical component is the application of a suitable air-pressure gradient to force the flexible transfer film into conformal contact with the sample surface, which can be accomplished with either the vacuum chamber "suction" approach described herein or an air-bearing pressure applied to the top surface of the transfer film described in U.S. Patent No. 8,460,744.

The use of a textured film to provide vacuum transmission between the surface of the substrate on which the sample is mounted and the transfer film interface is a particularly attractive approach for achieving the desired contact. Practical implementations of this approach include ultrathin release coatings on the transfer film surface, radial or random microchannels defined in the surface of the thermoplastic adhesive layer, or surface microchannels formed or deposited on the surface of the glass substrate.

Transmission of vacuum from the peripheral edges of a support substrate (e.g., an impermeable glass microscope slide) to the biological sample/transfer film interface is significantly enhanced via microchannels between the transfer film surface and the adjacent surface of the biological sample and/or support substrate surface. In one embodiment, the transfer film's surface may define integral microchannels. These microchannels can be deliberately engineered, for example through embossing of the transfer film with channels with lateral dimensions of a few to a few hundred microns, for example, 1 to 300, 2 to 200, 1 to 10, 2 to 5, 5 to 100, or 100 to 300 microns, or can be present on the transfer film surface as a result of the microtexture created during the manufacturing process of the film. Randomly textured surfaces with feature heights from 0.1 to 1microns, for example, 0.1 to 0.3, 0.3 to 0.5, or 0.5 to 1.0, microns, effectively provide many parallel paths, or microchannels of varying lengths, for vacuum transmission when the film comes into contact with the glass slide on which the sample is mounted. The resistance to gas flow through these microchannels determines the time needed to achieve uniform thermal contact between the biological sample surface and the thermoplastic surface of the transfer film; the vacuum pressure determines the limiting force. In addition, in certain embodiments the surface texture or embossed microchannels extend from the edge of the specimen support (i.e., the glass slide) to some portion of the edge of the tissue section or biological sample. At this point, certain desiccated samples used in TAM are sufficiently porous to enable vacuum transmission through the sample itself, thus providing conformal contact with the film across the tissue section or biological sample.

Another embodiment is imposition of a thin wire beneath the transfer film, which creates a channel for vacuum transmission even when a smooth transfer film surface is used to maximize the fraction of the tissue surface that contacts the transfer film during light pulse microbonding.

More complicated transfer film designs could incorporate a specific capture area, optimized for microbonding, placed over the region of interest on the tissue section, with embossed microchannels and/or surface treatment of the surrounding transfer film for rapid transmission of vacuum. Such a design may also help to minimize inadvertent and nonspecific transfer of tissue elements outside this desired region.

Another option is a hybrid film, incorporating a small area (approximately the size of the biological sample) of flexible EVA coated film mounted on an flexible, inert polymer layer with a release coating (for example, a polyethylene (PE) release liner such as the type commonly used in dry film photoresist). The release coating prevents adhesion between the plastic and the glass slide outside the biological sample, and permits vacuum transmission to the sample edge. It is further possible that a similar treatment with a release agent could be applied directly to the EVA layer, in

order to reduce unwanted nonspecific adhesion before melting. If the release agent layer were sufficiently thin, there might be minimal interference with capture of tissue elements upon melting and flow of the EVA into the biological sample.

In one embodiment the transfer film includes a low-melting temperature thermoplastic polymer, such as ethylene vinyl acetate (EVA), disposed onto a strong, transparent, and flexible high-melting temperature polymer, such as but not limited to polyethylene terephthalate (PET) or polyethylene naphthalate (PEN). The thickness, surface properties, melting point and decrease in viscosity above the melt temperature (melt index) of the thermoplastic layer can be optimized for bonding the desired targets, with possible adjustments based on the stain localization. Successful bonding has been achieved with adhesive layers ranging from one to several hundred micrometers in thickness, with melt indices (measured at 190° C/2.16 kg) ranging from 30 to 500 g/10 min. Melting points from perhaps twenty degrees Celsius above ambient temperature and up to approximately 100 degrees Celsius would be acceptable, but a melt temperature in the range of 50 to 80 degrees Celsius is probably ideal. Although most efforts have used ethylene vinyl acetate as the adhesive, successful bonding has also been achieved with a terpolymer (Elvax 4310) that includes an organic acid as well as poly(ethylene) and poly(vinyl acetate) segments. For example, a one μm thick or thinner layer of EVA on PET limits the depth of polymer penetration into the tissue surface over a wide range of thermal transients. These ultrathin adhesives can therefore increase the robustness of the specific capture of stained subcellular targets, such as cell nuclei, from the surrounding unstained tissue. On the other hand, using thicker adhesive thermoplastic polymer layers (10 um or more) with high melt indices (200-500) would provide greater bond penetration and thus be better suited for robust capture of unstained subcellular elements within cells stained for a localized biomarker (e.g., capture of cell nuclei in cancer cells expressing a particular cell membrane receptor).

A vacuum chamber is used to create and sustain sufficient contact between the transfer film and the biological sample (e.g., desiccated biological tissue section). Typically, the vacuum is initiated prior to illumination and microdissection. The vacuum chamber includes a region for holding a support substrate upon which a biological sample is mounted. Illustrative embodiments of vacuum chambers are shown in Figs 3 to 6 and 11.

Figs. 3-6 show a vacuum chamber 101 that includes a base member 102 and a lid 103 coupled to the base member 102. In certain embodiments, the lid 103 is movably attached to the base member 102. For example, the lid 103 may be fastened to the base member via a hinge 104 and clip 105 arrangement. In this manner, the lid 103 may be opened, but still fastened to the base member 102.

The base member 102 includes an area 106 of appropriate design to hold and align a substrate-mounted biological sample along with a transfer film. Within area 106, a recessed portion 113 of appropriate depth, width, and length (typically recessed portion 113 is rectangular) defines a floor and sides that provide a compartment for holding a single laboratory slide 203. For example, the recessed portion 113 may have a depth of 1 to 2 mm, a width of 25 to 27 mm, and a length of 75 to 78 mm for a standard 1" x 3" slide. Indexing the glass slide to a specific corner of portion 113 provides positional accuracy required when illumination masks are used or tissue target regions of interest are desired. The width and length dimensions of the recessed portion 113 may be adjustable to accommodate support substrates of various sizes or variations in manufacturer-specific slide actual dimensions. For example, adjustable shims may be inserted in slots 127 provided at the periphery of the recessed portion 113. The base member 102 defines a first surface 107 facing the lid 103, and an opposing second surface 108. The first surface 107 may include raised ridges 126. The recessed portion 113 opens at the first surface 107. The base member also includes edges 109 abutting the first surface 107 and second surface 108. In certain embodiments, when lid 103 is closed against base member 102, the raised ridges or ribs 126 provide pressure points against a sealing member 125 (e.g., a gasket) on lid surface 116; the transfer film is sandwiched between the raised ridges and the sealing member to establish a vacuum-sealed closed volume between the sample substrate and transfer film.

The base member 102 also includes within the recessed portion 113 at least one integral conduit 110 connected via another conduit 111 to at least one port 112 located at at least one of the outer edge(s) 109, first surface 107, or second surface 108 of the base member. In certain embodiments, the integral conduits 110 and 111 are disposed within the cross-section of the base member 102. In certain embodiments the conduits 110 and 111 can consist of a network of passages that fluidly communicate between more than one port 112. In certain embodiments, the conduit 110 could be of a different profile and/or path while still ensuring fluid communication to the glass slide edge perimeter.

In certain embodiments, an overall vacuum fluid communication path 204 connects port 112 to the volume between the sample substrate and transfer film. As shown in Fig. 11, this fluid communication path 204 is comprised of port 112 to conduit 111, to conduit 110, to sample substrate 203 perimeter edge, and then to volume between the sample substrate and transfer film. The direction of the application of the vacuum is shown by arrows in fluid communication path 204. Between the sample substrate and transfer film surfaces, vacuum transmission continues via voids in the transfer film surface (e.g., texture, channels) and voids in the desiccated tissue to bring

the film in thermal contact with the tissue. Overall sealing of the closed vacuum volume is achieved by sandwiching the transfer film between the raised ridges 126 and lid sealing member 125.

Port 112 is configured for operably connecting to a vacuum-generating source (not shown). For instance, tubing 114 connected to a vacuum-generating source may be inserted into or attached to the port 112.

Port 112 may each have a dimension sufficient to transmit a vacuum.

In certain embodiments, the lid 103 is dimensioned so as to cover a substantial portion of the first surface 107 of the base member 102 when the lid 103 is closed. The lid 103 includes a window 115 extending there through that is aligned with the recessed portion 113 of the base member 102 when the lid 103 is closed. A longitudinal axis 121 extends through the window 115 and into the recessed portion 106. In certain embodiments, the window 115 and the recessed portion 113 are axially centered along longitudinal axis 121. The window 115 may have peripheral dimensions smaller than, equal to, or larger than, the peripheral dimensions of the recessed portion 106. The window 115 is typically rectangular. In certain embodiments, window 115 could be an open void, or an optically transparent material, both enabling illumination from the lid 103 side of the sample substrate. In certain embodiments, a second window of transparent material could be in base member 102, axially centered along longitudinal axis 121 for illumination from the bottom member 102 side of the sample substrate The lid 103 defines a first surface 116 facing the first surface 107 of the base member 102, and an opposing second surface 117.

A sealing member 125 is included to provide a seal between the transfer film and the base member 102 of the holder, such that the space between the top of the sample substrate 203 and the transfer film can be evacuated. In certain embodiments, the recessed portion 113 is provided with ribs 126 to help hold the transfer substrate in place and to assure a good seal. In certain embodiments, the sealing member 125 could be on base member 102 surface 107 with raised ridges 126 on lid surface 116; or opposing sealing members (e.g., gasketing) could be utilized on both base member 102 and lid 103 to achieve sealing without the need for hard raised ridges.

A mask holder 118 may be coupled to the lid 103. The mask holder 118 defines a first surface 122 facing the second surface 117 of the lid 103, and an opposed second surface 123. The mask holder 118 includes an opening 119 extending there through, wherein the opening 119 is aligned with the window 115 of the lid 103 and the recessed portion 113 of the base member 102. A region-of-interest (ROI) mask 120 is affixed to the surface 122 of the mask holder 118 in alignment with the opening 119. The mask holder 118 and the attached ROI mask 120 may then be inserted in the opening 115 of the lid 103, such that the ROI mask is in contact or near contact with the transfer substrate on top of the sample. Alternatively, the ROI mask120 may be inserted into

the opening 119 directly. In certain embodiments, the ROI mask 120, the opening 119, the window 116, and the recessed portion 106 are all axially centered along longitudinal axis 121. The mask holder 118 may be coupled to the lid 103 in any manner. For example, the mask holder 118 may be removably inserted into the window 115 of the lid 103. Alternatively, non-permanent fasteners (e.g., aligned and attracting magnets 201 and 202) may be provided on the mask holder 118 and the lid 103 or recessed portion 113 to secure the mask holder 118, the lid 103 and the base member 102 together.

The base member 102/lid 103/optional mask holder 118 assembly is adjustable to provide reproducible and predictable lateral positioning (relative to each other and relative to the irradiation source). For example, the ROI mask can be well aligned with biological specimen undergoing dissection by using fiducial marks on the corner of the mask holder 118 to position the ROI mask 120, with the relative positions of the mask holder 118 in the opening 115 and the relative positions of the sample substrate in the recessed area 113 likewise determined by the mechanical position of a reference corner on each piece. The position of the assembly 102/103/118 can be freely positionable relative to the irradiation source, but reproducible alignment can be achieved by the use of fiducial marks or mechanical stops as needed.

The vacuum chamber disclosed herein may be used with any light irradiation source (e.g, a flashlamp or a laser). In one embodiment, shown in Fig. 10, the system is a flashlamp targeted activated microtransfer (fTAM) system that includes the vacuum chamber and a flashlamp system as described herein.

An illustrative flashlamp system 150 is shown in Figs. 7 and 8. The system includes a flashtube 151, a first reflector 152, an optional second reflector 153, and a light output window 154. The flashtube 151, first reflector 152, second reflector 153, and light output window 154, are all aligned and centered along the same longitudinal axis 155 so as to provide uniform irradiation across the light output window 154. Light generated from the flashtube 151 is collected by the reflectors 152 and 153 to exit the system 150 via the light output window 154. The flashtube 151, first reflector 152, second reflector 153, and light output window 154 may be housed within an enclosure 156 having an illumination surface 157. In certain embodiments, an array of flashtubes could be used to achieve illumination intensity and homogeneity at light output window 154. The use of multiple flashtubes in close proximity to the light output window could reduce the need for relatively large or complex reflectors, as well as enable larger area output windows.

The first reflector 152 surrounds the flashtube 151. In certain embodiments, the first reflector 152 is ellipsoidal or parabolic with an opening 158 facing in the direction of the illumination surface 157. The opening 158 is centrally aligned along longitudinal axis 155 with the

flashtube so as to provide uniform irradiation across the opening 158. The first reflector 152 focuses and/or collimates light emitted from the flashtube 151.

The reflector 152 (e.g., Edmund Optics 90-969, f1=22mm, f2=145mm, hole size=27mm, height=43mm, diameter=1 05mm) directs as much light as possible to light output window 154. An ellipsoidal reflector focuses light generated at the first focal point (located within the reflector) to a second focal point some distance from the reflector. The amount of light falling on the tissue section, as well as the general spot size, can be adjusted by changing the distance of the output window from the reflector. The flashtube is positioned so that it is centered on the first focal point. Through experimentation, the optimal tissue section position from the reflector is determined by evaluating delivered power and homogeneity at various heights above the reflector. Once the optimal position is found, the lid height is fixed in place to ensure that the tissue section sits at the desired position when the vacuum slide/film holder is used.

The flashtube 151 may be any type of flashtube that can generate irradiation appropriate to perform target activated microtransfer as described herein. In certain embodiments, multiple flashtubes could be utilized to achieve desired illumination intensity and/or homogeneity. The flashtube 151 may be secured to the enclosure 156 via any means such as, for example, a flashtube clamp 159. Associated with the flashtube 151 are flash capacitors 160 and charging and flash trigger electronics 161. In certain embodiments, the flashtube 151/flashtube clamp 159, the first reflector 152, the flash capacitors 160, and the charging and flash trigger electronics 161 are mounted on a platform 162 that is secured inside the enclosure 156. A wiring terminal block 163, which may optionally be mounted on platform 162, is provided for electrically connecting the flashtube 151, the flash capacitors 160, and the charging and flash trigger electronics 161.

The large photo-flash capacitors provide the charge for the xenon flash. Certain embodiments may use two 1500 μF capacitors charged to 300V, which provides 135J of electrical energy to the xenon bulb. The bulb is an Amglo (Bensenville IL) linear xenon bulb, Model AQL-1210. A cerium-doped version of the bulb may be preferred to reduce UV light output. If a non-doped version of the bulb is used, a UV blocking filter (e.g., Schott GG-400, Edmund Optics, Barrington, NJ) can be added at the light output window to prevent UV light from reaching the tissue section sample. The flash duration is approximately 150 μs with the 3000 μF capacitance. There are various circuit additions that can be used to control the flash duration. For instance, an Insulated Gate Bipolar Transistor (IGBT) can be used to shorten the pulse, or inductance could be added to the tube path to lengthen the pulse. To accommodate the possibility that additional light energy might be required for the TAM process (e.g., if spectral shaping of the light pulse is desired,

the stain contrast is low, or if the stained targets are very small), the system may include a third photo-flash capacitor.

The high-voltage charging circuit is based on the CHARGE800V capacitor charger from Information Unlimited (Amherst, NH). The charging circuit is powered by 12VDC, and takes approximately 30-35 seconds to charge the 3000uF capacitance to 300V. The CHARGE800V circuit was modified to remove the charge status indicator circuitry on the output, and a printed circuit board was designed and fabricated. In another embodiment, a charger circuit is based on the Linear Technology (Milpitas, CA) LT3751 High Voltage Capacitor Charger Controller. Specifically, the L T3751 evaluation board may be incorporated in the system. The high-voltage charging circuit reduces the charge time to approximately 4 seconds, which facilitates the TAM process when multiple flashes are required.

The flashtube trigger circuitry is based on a design described in the Linear Technology Application Note 95 (March 2004), and uses the Amglo VE-11 trigger transformer. A 0.15 μF ceramic capacitor is charged to 300V by bleeding a small fraction of the current from the main photo-flash capacitor circuit. An IXYS (Milpitas, CA) IXGN320N60A3 Insulated-Gate Bipolar Transistor (IGBT) is used to quickly drain the ceramic capacitor charge through the input coil of the trigger transformer. On the output coil of the transformer, this generates the high-voltage necessary to ionize the gas in the bulb, thereby reducing the bulb resistance and initiating a flash.

The support circuitry includes an IGBT gate driver, debouncing circuitry for the manual switch used to initiate a flash by the user, and LED indicators to notify the user of charging and ready states. In the CHARGE800V charger version of the system, the support circuitry also includes a comparator circuit to disable charging once the photo-flash capacitors reach 300V, as well as to maintain the full charge if there are long intervals (minutes) between flashes, as some charge is lost during periods of inactivity. In the LT3751 version of the charger, the LT3751 chip maintains the voltages through on-chip circuitry. A small cooling fan is used to vent heat generated by the charging circuit and flash.

An optional second reflector 153 may be located adjacent to the opening 158 of the first reflector 152. The second reflector 153 has a first opening 164 adjacent to the opening 158 of the first reflector 152 and an opposing second opening 165. The second reflector 153 may have any shape, but typically is conical with the first opening 164 forming the larger diameter of the cone. Other reflector shapes are possible, such as ellipsoidal or parabolic. The second reflector 153 can increase the light intensity at the light output window 154 for a given flashtube 151 selection. While the first reflector 152 directs a large amount of light to the flashlamp system output window, there is still a significant amount of light that lands outside the output window (i.e., scattered and

absorbed internal to the flashlamp system enclosure). To increase light output and system efficiency, the second reflector 153 may be incorporated to help direct more of this otherwise lost light to the output window.

The light output window 154 is centrally axially aligned with the first reflector opening 158 (and the optional second reflector openings 164 and 165, if present). The light output window 154 may be located at the enclosure illuminating surface 157. The light output window 154 may be integrally inserted into the surface 157 wall or it may be secured to the surface 157 wall via a fastener such as, for example, an output window mount 166. Typically, the light output window 154 is circular with a diameter of 10 to 25 mm, but other window shapes with lateral dimensions up to 50 mm may be used. The light output window 154 may be void, but more typically a transparent member, either glass or a UV blocking material such as Schott GG400, will be disposed within the window to provide mechanical protection for the flashtube.

An optional light filter member 167 may be interposed along the light path running parallel to the longitudinal axis 155. The light filter member 167 includes at least one filter that attenuates at least one light wavelength. For example, the light filter member 167 may include at least one color filter, at least one neutral density filter, or a combination of at least one color filter and at least one neutral density filter. The neutral density filter, for instance, may be a graduated neutral density filter, a neutral density filter-wheel, or a variable neutral density filter wheel. If more than one type of filter is included in the light filter member 167, the filters may be interchangeably arrayed for alternating the specific filter interposed in the light path. For example, the light filter member 167 may consist of a circular filter array (e.g., a filter wheel as shown in Fig. 8 that includes a circular array of individual filters 168), a rectilinear filter array, a stacked filter array, or similar arrays.

For example, user-installed color filters (e.g., rectangular pieces of colored glasses or polycarbonate films corresponding to tissue target stain color and desired microdissection function) may be inserted into slots along the perimeter of a filter mounting wheel. In place of color filters, neutral density filters could be used as a simple way to reduce the amount of light energy delivered to the tissue section during the TAM process. This reduction could also be accomplished by modification of the charging circuitry. It is likely that different samples will require different light doses for optimal dissection, making on-site user adjustability a convenient feature. Color filters and neutral density filters could be combined depending on the microdissection application and relative stain densities. The filter mounting wheel is mechanically indexed and labeled along the thumbwheel adjustment protruding from the side of the system enclosure. Parameters of the TAM process can be altered by adjusting the filter wheel, as well as varying the total number of flashes.

An alternative to using a filter wheel for changing the TAM illumination color or intensity could be to use an indexed linear sliding mechanism to move the filters into the light path.

In certain embodiments, all the components of the flashlamp system are mounted in a small-footprint (e.g., 9.5" W x 9.5" L) benchtop enclosure powered by a standard AC/DC power adaptor.

If desired, an embedded microprocessor, either with an interface screen or a connection to a laptop or tablet could provide additional functionality, including automatic multiple flashes, experiment archiving, and system tools such as flashtube monitoring and total flash count, while not adding significantly to the overall cost of the TAM flash lamp system.

The target activated transfer system may also optionally include a scanner and a computer or tablet monitor. Imaging of the samples for targeting is not required in this direct transfer process unlike other forms of tissue microdissection which use microscopes. This is because the distribution of stain in the specimen determines precisely where the light is absorbed (all tissue whether stained or not is exposed to a uniform light dose) and where the polymer melts and binds to the specifically stained regions. However a flat-bed or slide scanner may be useful for archiving images of the tissue and transfer film before and after transfer and for more sophisticated forms or automated dosimetry and process control. In order to determine dosimetry and to identify regions to be selectively transferred, the stained tissue section may be imaged before transfer in the scanner. The scanned reference image may be displayed on the computer monitor. The scanner may be a low cost flat bed color scanner with sufficient resolution and contrast sensitivity (e.g. 48-bit 3 color images) to allow automatic determination of OD variations within the tissue after appropriate calibration. However, any type of imaging system can be used to make the scanned reference image. Illustrative imaging systems include digital imaging systems such as CCD camera systems, a photomultiplier or an automated color film scanner. An image analysis system (for example, using image analysis software in conjunction with a dosimetry database to characterize the sample or reference image) or a manual observation of the images may be used to determine the type of staining and the variability of the staining with and between tissue regions or individual cells. The staining intensity can be measured qualitatively, semi-quantitatively (scale 0 to 4, for example) or by performing a full quantitative analysis of the sample image (gray scale values, for example from 1-256, possibly using different spectral channels (RGB) to separate contributions of different chromophores or tissue background in the specimens). The imaging system could separately determine the stain intensity in different parts of the spot, such as separately in stromal or carcinoma components. A computer may be used to compare automatically the staining results between adjacent sections of the same tissue section with same or different antibodies to form ratios of molecular intensities. Another example of a scanning system that could be employed in the

target activated transfer system is described in U.S. Patent Publication No. 2003-0215936-A1, which is incorporated herein by reference.

A scanner permits unattended automatic scanning of prepared tissue sections for the detection of candidate objects of interest, such as particular cells which may contain marker identifying reagents, and evaluation of the amount of the reagent that is present. For instance, the scanner may automatically locate candidate objects of interest present in a biological specimen on the basis of color, size and shape characteristics.

An imaging system not only acquires images from a microscope slide, but may also archive, display, and analyze the images and incorporate data in a database. For example, the imaging system can display consecutive images on a computer screen for an observer to analyze, interpret and store. Alternatively, the program can pre-process images to display areas of positive staining, and present images of selected slides or specific regions of slides to the observer for approval. Completely automated image acquisition and analysis and microtransfer is possible.

In general, during the TAM process, the tissue slide and transfer film are secured inside the vacuum slide/film holder, and the assembly is then placed on top of the flashlamp system with the vacuum slide/holder illumination window facing downward. A grid overlay on the flashlamp system surface 157 enables the user to align the tissue over the flashlamp system output window 154. A front-panel momentary toggle switch on the flashlamp system enclosure 156 allows the user to initiate a TAM flash (exposure). Also on the front panel, LED indicators inform the user when the system is charging and when it is ready to initiate another flash.

During a dissection sequence, the vacuum is applied, the flashlamp is appropriately pulsed, the vacuum released, and the transfer substrate (optionally labeled with the slide barcode) peeled off the tissue. Following that, the film with captured tissue can be translated to an imaging and extraction station while the next substrate-mounted tissue is presented to the vacuum chamber.

More specifically, a vacuum is initiated by the vacuum-generated source that is applied to the vacuum chamber via port 112. In certain embodiments, the overall vacuum fluid communication path 204 connects port 112 to the volume between the sample substrate and transfer film. As shown in Fig. 11, this fluid communication path 204 is comprised of port 112 to conduit 111, to conduit 110, to sample substrate 203 perimeter edge, and then to volume between the sample substrate and transfer film. Between the sample substrate and transfer film surfaces, vacuum transmission continues via voids in the transfer film surface (e.g., texture, channels) and voids in the desiccated tissue to bring the film in thermal contact with the tissue. Overall sealing of the closed vacuum volume is achieved by sandwiching the transfer film between the raised ridges 126 and lid sealing member 125.

The flashlamp (or other irradiation source) is pulsed to irradiate the transfer substrate/biological specimen through the lid window 115 (and initially through the ROI mask 120 and mask holder opening 120, if present). If the flashlamp system 150 is used, the vacuum chamber module 101 is placed adjacent the light output window 154 of the system 150 so that the recessed portion 106 and lid window 115 face the light output window 154. The flashtube 151 generates a pulse that passes sequentially along a light path (parallel to the longitudinal axis 155) through the first reflector 152, the second reflector 153 (if present), the light filter member 167 (if present), and the light output window 154.

In an automated system, a continuous roll of transfer film could be used with a slide sorter, supplying a film segment for each barcoded slide, which would then be loaded into a vacuum-application station centered on the flashlamp illumination window 154.

When a short (sub-millisecond) light pulse is used to initiate microbonding, specific capture of stained targets with subcellular resolution can be achieved. The short pulse results in highly localized heating at the stain location, as the subsequent thermal diffusion into the bulk of the transfer film is sufficiently rapid to prevent polymer from melting over adjacent unstained regions. Submillisecond microbonding of stained targets over the entire area of a tissue section (typically < 6 cm^2 in area) can readily be achieved using a high power, high intensity xenon flash tube (e.g., Amglo AQL1210) to uniformly illuminate the tissue section – transfer film interface with ~ 0.1 to 1 J/cm^2 of visible light. The full visible light spectrum can be used, or spectrally selective color filters can be chosen to match the specific stain absorption but exclude wavelengths absorbed by counterstains. This flashlamp-based system is simple, fast, and precise, and should permit ready integration of the microcapture process into high-throughput tissue molecular diagnostics. More elaborate flashlamp circuit designs could allow independent adjustment of flashlamp pulse energy and duration in order to further optimize capture of different sizes of subcellular components, either specifically stained or unstained, which reside within specifically stained target cells.

Using the design shown in Figs. 3-11, a fixed pulse length of 50 to 1000, more particularly 100 to 300, microseconds is sufficient to achieve extremely high intrinsic resolution and efficiency, as demonstrated by specific capture of subcellular elements from the full illuminated area, using ultrathin (~1-25μm), high melt-index (> 200 dg/min) polymer coated transfer tapes. If adjacent unstained cellular components (e.g., cell nuclei in cells with membrane biomarker staining) are desired, multiple pulses and transfer tapes with thicker polymer layers can be used to allow lateral expansion of the tissue bonds beyond the edges of the stain absorption.

An additional embodiment uses rapid scanning of a laser beam (e.g., with either a programmable General Scanning Model E00-XY0507S galvo-mirror scanner or a Texas

Instruments DLP digital light mirror), with the laser wavelengths chosen to match the absorption peaks of the tissue stains. While the laser scanning format offers greater flexibility in adjusting capture parameters for specific tissue targets within the tissue section, this flexibility comes at the cost of increased instrument complexity and time required for microdissection.

The whole tissue is scanned with a uniform intensity/duration of light energy density (fluence), but only those regions that are strongly stained absorb sufficient energy to melt the thermoplastic film surface where it contacts the tissue, thereby automatically bonding only the tissue locations exceeding some threshold stain density. As no microscopic targeting decisions must be made by a viewer or computer analysis (as required in present laser microdisection techniques), the system can routinely bond more than 5000 specifically labelled cells per sec, which represents an increase of at least 1000-fold over conventional laser capture microdissection.

The measured local light transmission or optical density of the tissue slide pre-microtransfer image can then be related to microtransfer dosimetry (dependent on factors including stain absorption spectrum, wavelength used, tissue section thickness, and thermoplastic polymer used) in order to predict what regions will be transferred for any given dosimetry. In one aspect, a computer database may store dosimetry information for correlation with the scanned reference image. This feature is particularly useful when stain density among different sections varies or if the sample is to be selectively mined by first transferring only the most highly stained regions and then after removal of these targets, placing another film on the partially harvested tissue section and transferring less heavily stained cells (e.g., those with slightly lower targeted molecule expression levels). Similarly, it will be possible to use spectrally separated specific stains of different molecules and use two light wavelengths (different color filters used with a flashlamp, or different laser wavelengths) to collect either two different cell populations or in a combined mode only those cells whose combined expression of two molecules exceeds a certain level. This method could allow mulitparameter cell isolation similar to the ability of a fluorescence activated cell sorter to isolate cells from single cell suspensions on the basis of the coincidence of multiple parameter windows (e.g co-expression of two marker macromolecules).

A roadmap image of the entire specimen can be rapidly generated at ~10 um resolution with a standard commercial flat bed scanner. However, as explained above, such scanning with an imaging system is not required to optimize TAM parameter selection and mask positioning, and thus the region to be targeted for microdissection. For example, the location of specific regions of cells in an original high-resolution image (e.g., iSCAN HT, Ventana Medical, Tucson, AZ) of an identically imunostained adjacent serial section created by an automated immunostainer can be substantially equivalent to that of the section submitted for target-activated microdissection (for

which the optical constrast is compromised by its desiccation and lack of index-matching coverslipping). The imaging system described herein is useful for modifying or optimizing the dosimetry and/or for image archiving.

Along these lines, further disclosed herein are methods for limiting flashlamp illumination, and hence fTAM capture, to a specific ROI determined by examination of the sample slide or a serial section prepared for optical microscopy (e.g., coverslipped). This method allows TAM implementation to be readily integrated with current practices of clinical pathology that use microscope-based imaging of index-matched, coverslipped tissue sections.

The goal is to permit selection of specific larger regions of the tissue to be selectively microdissected - for example a whole prostate section might be imaged, but only the regions in which invasive carcinoma appears might be transferred onto one film, with regions of normal or premalignant lesions transferred onto a second film in a subsequent slide dissection for comparison. Although this requires some general targeting decisions, the microscopic targeting would be automatically afforded by the cell specific absorptive stain. A similar example of this regional selection would be to limit the illuminated area to specific regions of the brain that are readily recognized by gross morphology (e.g., hypothalamus or a substructure like the supra-optic nucleus). Again, specific stains would allow specific cell types or organelles to be collected from within the macroscopic regions selected with the ROI mask. Other methods of selecting regional samples based on morphology in addition to the molecularly specific stains include rapid automatic microdissection of the whole slide, followed by cutting out specific regions from the transferred film based on the intact morphological structure preserved in the direct transfer process. Using affinity particle purification within the transfer film could allow in situ microscopic examination of the distribution of retained and subsequently labeled macromolecules.

In one embodiment, the method uses digital images of two complete slides with a fixed slide reference position (e.g., corner or other fiducials). One image is of the high-optical quality indexmatched, coverslipped slide in which specific regions of interest (e.g., thought to contain a clonal population of specifically stained cancer cells) have been previously identified by a pathologist. The second image is of a suitably congruent slide-mounted serial section as prepared for fTAM (uncoverslipped, desiccated and stained with one or more spectrally separable absorptive stains specific to the desired targets, such as particular cancer biomarkers). In a semi-automated process, the region of interest defined in the first slide is mapped onto the equivalent region on the second slide using custom software, and the resulting digital map used to direct the creation of a mask with the mapped ROI contours and indexed to the corners of the second slide. In this first

embodiment, the mask is made of a reflective tape cut with a programmable razor cutter such that a hole is cut to match the contours of the selected ROI and such that the mask corner can be aligned with an insert which sits at a fixed position in the vacuum chamber (i.e., slide/film holder) disclosed herein. A second embodiment of ROI mask fabrication involves printing the digital map, with non-ROI regions in black (absorptive high OD, blocking illumination), on a transparent sheet (e.g., tape) secured and aligned in a similar way with the vacuum slide/film holder. Because the flash lamp light pulse is very short (-150us), the thickness of the tape and capture film backing, or any other means for maintaining a minimum controlled spacing (e.g., <:5-10um) between the absorptive mask and activatable layer component of the transfer film, provides sufficient thermal insulation to prevent activatable layer bonding to tissue under the dark regions (i.e., non-ROI regions) of the mask. Yet a third embodiment of making a mask could further involve reflective.or non-reflective masks with colored openings (i.e., ROI) corresponding to a specific color stains.

A similar process of registering the ROI from a reference slide to the tissue section used for fTAM can also be applied to other methods for spatially restricting the illumination during TAM, including the direction and automated linear modulation or shuttering (i.e., binary) of a scanned laser beam, or the use of a digital light micromirror array (DLP) to project light onto the desired ROI. With a sufficiently intense broadband light source, colored filters could be used to match the transmitted light to the absorption of different specific stains; in this way targets for different biomarkers could be sequentially captured from within the same ROI or different ROIs.

The method disclosed also can be used to integrate or combine information from multiple differentially stained high quality slides into registered image stacks, allowing the user to select ROIs based on multiple biomarkers in multiple serial sections. Using the high quality stack of images, each showing a different stain, the ROI selection process can be facilitated by using custom software visualization and interpretation tools providing registered image overlays, actual or pseudo-color stain rendering, and other image processing functions such as adding two (e.g., false colors assigned to different stains) images to enhance visualization of co-localized biomarkers stained on different slides. Similarly, once selected based on the image(s) from the reference slide(s), these ROIs could be mapped onto multiple serial sections prepared for TAM (i.e., stained with an optically absorbing stain specific to the same or different biomarkers but left desiccated and accessible to the EVA transfer film surface).

In certain embodiments, the method may include the use of 1) automated tissue processing/immunostaining systems for uniform and reproducible staining of serial sections; 2) CLIA certified specific biomarker stains to generate the reference slides; and 3) digital imaging systems for histopathology and ROI selection by a pathologist. By restricting illumination in the

TAM system to the selected ROI (e.g., presumed clonal population of cancer cells), appropriately mapped on to the serial section prepared for TAM (i.e., immunostained to label the specific cells of interest within the selected ROI, desiccated and uncoverslipped), greater selectivity is achieved. The resulting specifically dissected targets on the TAM transfer film would be subjected to molecular extraction and downstream molecular analyses, for example to give a more definitive molecular diagnosis by providing an assessment of tumor molecular heterogeneity among different selected ROIs or by providing a comparison of normal and pathological cells within the same patient tissue sample.

TAM and fTAM allow rapid microdissection of specific cell populations with subcellular resolution, but it would be useful in certain circumstances to limit capture to a specific region of the tissue section (a few hundred μm (e.g., 100 -300 μM) to a few mm (e.g., 1 to 3 mm) in size). Without such a limitation, the stained material from the entire tissue section is uniformly captured in the microdissection step. In many cases, this may not be desirable. For example, the absorptive stain could be localized to an epithelial marker, but it might be important to capture only cells from within a tumor, or even just the cells from a specific region of a tumor thought to contain a clonal population of cells. Ideally, the cells or subcellular organelles from a specific ROI selected by a pathologist could be routinely isolated for downstream molecular analysis. However, a complicating issue is that the slides prepared optimally for TAM are not amenable to high-quality optical microscopy, which is the standard technique used by a pathologist to examine patient tissue samples (biopsies, pleural effusions, etc.). The method disclosed herein enables ROIs marked on slides prepared for optical microscopy to be reliably transferred to stained serial sections for TAM in a semi-automated manner. The specifically stained targets within the identified ROIs can then be captured for downstream comprehensive molecular diagnostics. A further potential advantage of this technique is that the ROI selection capability could limit the need to develop highly specific stains for the TAM process; as long as the appropriate ROIs can be identified by standard histopathology, a more universal chemical stain (e.g., hematoxylin) can be used to capture the desired cells or organelles.

The methods described herein can be implemented by software as described below and as shown in the flowchart of Fig. 9. Using a high-resolution image scanner, digital images of the two tissue section slides with a fixed corner reference or other fiducial marks are acquired. The first slide is a high-optical quality, index-matched, coverslipped slide containing targets identified by a pathologist as significant (HQ slide). The second slide is a suitably congruent serial section, stained with a spectrally absorptive stain, desiccated, and left without a coverslip (TAM slide). The custom software reads in the two digital images (HQ image and TAM image) and displays them to the user.

A Mask image (requiring user input for ROI selection) is also generated and displayed with a corner reference (i.e., mask origin) that has a known offset relative to the corner reference of the TAM image. All three images are displayed to the user.

A semi-automated process is used to register (including translation and rotation) the HQ image and TAM image. The user selects three points in the HQ image that have surrounding features facilitating easy identification of the same three corresponding points in the TAM image. Once the two sets of three points are identified and stored, the software will then register the images using a rigid-body registration algorithm, More advanced registration algorithms (e.g., non-rigid body registration, algorithm-assisted point selection, localized ROI registration as opposed to whole slide registration) and processes (e.g., tissue fold identification, ROI border safety margin) could be used to improve ROI mask accuracy and functionality.

The user identifies and marks regions of interest (ROIs) on the HQ image that contain the tissue targets of interest. The registration parameters (rotation angle, x-coordinate offset, and y-coordinate offset) are automatically used to transform the ROIs in the HQ image to their tissue-equivalent locations in the TAM image and Mask image. Once the user has selected all of the ROIs desired and verified that the ROIs in the TAM image mark the same congruent regions, the Mask image is saved to a file. The Mask image is then used, in conjunction with an automated cutter or printer, to create a thin-film mask. In the first embodiment of this technique, the mask is made of a reflective tape with holes cut out to match the contours of the ROIs as displayed in the Mask image. Another method of mask making involves printing the Mask image onto a transparent sheet where the non-ROI areas are black using a COTS thermal-transfer printer. The black areas have a high OD and are effective at blocking illumination of tissue outside the ROIs.

After the mask is generated, preparation for the fTAM capture can begin. The TAM slide is placed in the vacuum slide/film holder, positioned based on the fixed corner reference, and the transfer film is placed on top. The lid is then closed on the vacuum slide/film holder and a vacuum is applied. The mask is positioned on the mask holder, based on the Mask image fixed corner reference (i.e., mask origin and angular alignment), and secured in place. The mask holder is then placed, mask side down, in the illumination window on the vacuum slide/film holder. The assembled vacuum slide/film holder, containing the tissue slide, capture film, and aligned ROI mask, is then placed into position for fTAM illumination and capture.

For example, the fTAM tissue slide is secured in the vacuum slide/film holder ensuring a slide corner is aligned with the recessed corner of the vacuum slide/film holder. The mask is adhered to the mask holder ensuring the mask corner is aligned with the corner of the inset frame (i.e., window) of the mask holder. The mask holder fits snugly inside the illumination window on

the vacuum slide holder, ensuring reproducible positioning. The known fixed mechanical offset between the recessed corner and the mask/illumination window corners enables the custom software to generate a properly registered fTAM ROI mask for the slide to be dissected, based on the ROIs selected using the high-optical quality index-matched coverslipped slide image. Magnets sitting outside the inset frame of the mask holder are used to pull the mask holder in close proximity to the transfer film and tissue slide.

In both mask implementations (i.e., reflective sheet with cut holes, and transparent sheet with optically absorptive print), the adhesive side of the mask is used to secure the mask to the mask holder. When using an absorptive mask, thermal isolation is necessary to prevent the transfer film from bonding in non-ROI regions, but a minimal thickness (5-10 μm of a typical thermoplastic polymer) is sufficient to achieve this isolation for the short flashlamp pulses (e.g., -200 μsec) used in the flashlamp system. Given this, sufficient thermal isolation can also be achieved with either a thermally stable substrate for the transfer film or by establishing a small air gap spacing between the mask and transfer film.

Vacuum is applied to establish thermal contact between the tissue and the transfer film. The mask holder with mask is close-fit inserted into the vacuum slide/film holder illumination window to ensure the mask location relative to the tissue slide corners or other fudicials used in the ROI mapping process. The mask is pulled in close proximity to the transfer film surface and tissue slide via magnets inset in both the mask holder and vacuum slide/film holder base. fTAM light energy illuminates the tissue through the mask ROIs (e.g., cut holes in reflective mask, or transparent areas on printed tape).

Examples

Example 1: The use of an immunostain localized at the cell membrane to capture nuclei from specific cells.

In many cases, specific cells are most easily identified by the presence of particular cell membrane receptors; furthermore, when these receptor-targeted reagents are already CLIA-approved, it is highly desirable to be able to use them for targeting dissection. Typically, genetic analysis is desired, thus requiring the transfer of the cell nuclei to the capture film even when the nucleus itself is unstained and the stained cell membrane ~5-10 microns away. In one embodiment, the method has the following parameters:

Film choice	High Melt Index EVA layer >10microns thick
Light intensity	~0.3 J/cm^2
Pulse train	Four to six 200microsec pulses at ≤0.2pps

Example 2: The use of a region of interest (ROI) mask for dissection and analysis of clonal populations in a tumor.

The heterogeneity of tumors is increasingly recognized as a factor in determining clinical outcomes. The use of a region of interest mask to isolate different regions of a solid tumor for independent genetic analysis could be a useful tool in identifying clonal subpopulations. In cases where one or more immunostain discriminates between clonal subpopulations, different colored chromagens can be used and color filters of "white" flashlamp pulse select for the different population. In other cases, biomarker density (hence stain optical density) might discriminate between different populations. In this case, different transfer films can be applied in sequence with increasing light doses.

Film choice	Determined by immunostain cellular localization (as in 1)
Light intensity	Successive films use increasing light dose 0.2 to 0.6J/cm^2
Pulse train	Two pulses at ≤0.2pps

In other cases, the prior microscopy report of the pathologist viewing serial sections may have identified phenotypic differences in morphology of similarly labeled cell populations in macroscopically different locations (>1mm separation). A simple, reflective mask placed or printed on the transfer film support layer allows such different regions to be separately illuminated and captured on different sequential films (with different mask locations)

Film choice	Reflective tape backing except over desired ROI.
Light intensity	Successive films at same light dose but masked for pathologist defined ROIs.
Pulse train	Two pulses at ≤0.2pps

Example 3: Selective dissection of sub-cellular targets.

Successful nuclear capture has been demonstrated previously. More recently, there has been some success using fTAM to capture axon terminations of dopaminergic neurons, with the aim of comparing the mitochondrial DNA damage at axonal terminations in the striatum with the mitochondrial DNA within the surviving cell soma. This is a research application, and as such optimization of capture parameters for each sample is possible. The specificity of the dissection is crucial, and some concurrent loss in the dissection efficiency is tolerable.

Film choice	Ultrathin EVA film layer (1micron or less), High melt-index EVA
Light intensity	Brief, high intensity pulse (100microsec, 0.6J/cm^2)
Pulse train	No more than 2 pulses at ≤0.2pps

Example 4: Tolerance for variation in stain density and semi-automated setting of capture parameters

When using fTAM in a clinical diagnostics workflow, a premium will be placed on a microtransfer process that is efficient and sufficiently specific for a wide range of patient samples. Filter wheels with colored filters and neutral density filters provide the ability to automatically adjust flashlamp intensity to stain density that was determined by automated image analysis of the stained targets, which were previously identified by a pathologist examining an identically stained serial section (coverslipped for microscopy). Unlike example 2, in some cases the molecular

analysis may require pooling cells with natural variations in biomarker stain density. The threshold intensity for target capture necessarily increases when stain density is lower. Consequently a premium is placed on transfer film designs that are efficient while retaining specificity when the biomarker density varies considerably (e.g. 3-5 fold) which requires a higher light dose to bond the weaker stained targets.

Film choice	Thin EVA coating on thermally conductive thicker support tape and/or lower EVA melt index thermoplastic adhesive
Light intensity	0.8J/cm^2
Pulse train	Two to four pulses at <0.2pps

Example 5 - Use of multiple color stains to sort two or more target types

As noted above, the same tissue section can be stained with different colored chromagens labeling different biomarker molecules. In this case sequential transfers using different spectral sources (e.g., different colored filters on the flashlamp or different laser wavelengths in a scanning laser system) provide a logic for separation similar to multiparameter fluorescence activated cell sorting but is this case sequential capture based on density and color of different absorptive biomarker stains.

Example 6 - Region of interest selection for use with non-specific stain

High background stain or localized contaminating cell populations may be avoided by using masks. For example Ki67 stains rapidly replicating cancer cells but also lymphocytic nests with a high density of "contaminating cells". Region of interest masking can exclude such contaminations or alternatively isolate them specifically as a control sample

In view of the many possible embodiments to which the principles of the disclosed systems and methods may be applied, it should be recognized that the illustrated embodiments are only examples of the invention and should not be taken as limiting the scope of the invention.

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What is claimed is:

1. A method of removing a target from a biological sample, comprising:

providing a substrate-mounted biological sample with a reagent that selectively acts on the target within the biological sample, wherein the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent;

placing a flexible transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample;

applying a uniform air pressure gradient across the flexible transfer film to maintain thermal contact between the lower surface of the transfer film and the biological sample;

exposing the biological sample to the light of the wavelength to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering the target to the transfer film; and

selectively removing the target from the biological sample by removing the transfer film with the adhered target from the biological sample.

2. The method of claim 1, wherein applying the uniform air pressure gradient across the flexible transfer film comprises applying a vacuum from at least a portion of a peripheral edge of the substrate so as to generate a negative pressure between the lower surface of the transfer film and the biological specimen.

3. The method of claim 1 or 2, wherein the lower surface of the transfer film includes at least two microchannels, and applying the vacuum comprises applying the vacuum via the microchannels.

4. The method of any one of claims 1 to 3, wherein the biological sample is a desiccated tissue sample mounted on a slide.

5. The method of any one of claims 2 to 4, wherein the vacuum is first initiated, the biological sample then is exposed to the light, the vacuum is subsequently released, and the target then is selectively removed from the biological sample.

6. The method of any one of claims 1 to 5, further comprising placing a mask adjacent to the upper surface of the transfer film for selectively exposing at least one region of interest in the biological sample to the light.

7. The method of any one of claims 1 to 6, wherein the transfer film includes a reflective mask disposed on the upper surface of the transfer film for selectively exposing at least one region of interest in the biological sample to the light.

8. The method of any one of claims 1 to 7, further comprising positioning a wire between the lower surface of the transfer film and the biological specimen.

9. The method of any one of claims 1 to 8, wherein the lower surface of the transfer film defines at least two integral microchannels.

10. The method of any one of claims 1 to 9, wherein a release agent is disposed on the lower surface of the transfer film to provide the microchannels.

11. The method of any one of claims 1 to 10, wherein exposing the biological sample to the light comprises exposing the biological sample to 0.1 to 1 J/cm^2 of visible light by pulsing a flashlamp for a pulse length of 10 to 1000 μsecs.

12. The method of any one of claims 1 to 10, wherein exposing the biological sample to the light comprises exposing the biological sample to a pulse length of less than 0.5 msec, and the transfer film comprises a polymer layer of less than 1 micron and a melt index of 100 or less.

13. The method of any one of claims 1 to 10, wherein exposing the biological sample to the light comprises exposing the biological sample to a pulse length of greater than 0.5 msec, and the transfer film comprises a polymer layer of greater than 5 microns and a melt index of 400 or greater.

14. A method of removing a target from a biological sample, comprising:

providing a substrate-mounted biological sample with a reagent that selectively acts on a cell component within the biological sample, wherein the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent;

placing a flexible transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample, wherein the transfer film comprises a first polymer support layer and a second polymer disposed on the first polymer support layer such that second polymer forms the lower surface of the transfer film, the second polymer having a layer thickness of less than 30 μm;

applying a vacuum from at least a portion of a peripheral edge of the substrate so as to generate a negative pressure between the lower surface of the transfer film and the biological specimen to maintain thermal contact between the lower surface of the transfer film and the biological sample;

exposing the biological sample to 0.2 to 4 J/cm^2 of visible light by pulsing a flashlamp one to ten times, with a pulse length of 100 to 300 μsecs each at 0.2 to 1.0 Joules/pulse-cm^2 to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering both the reagent-stained cell component and any desired unstained adjacent cellular component from the specifically stained cell to the transfer film; and

selectively removing the reagent-stained cell component and the desired unstained adjacent cellular component from the biological sample by removing the transfer film with the adhered reagent-stained cell component and the desired unstained adjacent cellular component from the biological sample.

15. The method of claim 14, wherein the cell membrane component is a cell membrane receptor, and the desired unstained adjacent cellular component is a cell nuclei..

16. A method of removing a target from a biological sample, comprising:

placing a slide-mounted biological sample into a slide holder, wherein the biological sample includes a reagent that selectively acts on the target within the biological sample, the reagent comprises an agent that absorbs light of a wavelength that selectively heats the reagent, and the slide holder includes a base member having a recessed portion having a sufficient dimension to hold the slide-mounted biological sample, wherein the base member includes at least one conduit having at least one first opening into the recessed portion and a second opening located at an outer edge of the base member;

placing a transfer film having a lower surface and an opposing upper surface such that the lower surface of the transfer film is adjacent to the biological sample;

applying a vacuum from at least a portion of a peripheral edge of the slide via the at least one conduit of the base member so as to generate a negative pressure between the lower surface

of the transfer film and the biological specimen to maintain thermal contact between the lower surface of the transfer film and the biological sample;

exposing the biological sample to the light of the wavelength to selectively heat the reagent and produce a change in the transfer film resulting in selectively adhering the target to the transfer film; and

selectively removing the target from the biological sample by removing the transfer film and the adhered target from the biological sample.

17. The method of any one of claims 1 to 16, further comprising obtaining an image of a biological sample treated with more than one reagent, identifying a range of optical densities of the different reagents in a desired set of targets, as well as their spatial overlap, within the biological sample, preparing a two-or-higher dimensional histogram from which different target clusters can be identified by spectral signature, size, and proximity to other stained structures, and selecting a spectral and temporal light dose and transfer film based on the histogram for selectively capturing the desired targets.

18. The method of claim 17, wherein the selection of spectral and temporal light dose and transfer film is governed in part by predetermined specificity and efficiency of capture as required by downstream molecular analysis of the captured material.

19. The method of any one of claims 1 to 18, wherein the method comprises removing multiple discrete targets from the biological sample.

20. An article comprising:

a base member having a recessed portion having a sufficient dimension to hold a slide-mounted biological sample, wherein the base member includes at least one conduit having at least one first port into the recessed portion and a second port located at an outer surface or edge of the base member;

a lid coupled to the base member, wherein the lid includes a window extending there through that is aligned with the recessed portion of the base member; and

sealing members located on at least one of the base member or the lid.

21. The article of claim 20, wherein the second port is configured for operably connecting to a vacuum-generating source.

22. The article of claim 20 or 21, wherein the at least one first port is positioned at the edge of an area for holding the slide-mounted biological sample.

23. The article of any one of claims 20 to 22, wherein the lid is hingeably attached to the base member.

24. The article of any one of claims 20 to 23, further comprising a mask holder coupled to the lid and aligned with the window.

25. A system comprising:

(a) an irradiation module, comprising

a flashtube;

a first reflector positioned around the flashtube and defining a longitudinal axis and having an opening centered on the longitudinal axis; and

a light output window, wherein the light output window has an inner side facing to the opening of the first reflector, and the light output window is also centered on the longitudinal axis; and

(b) a slide holder adjacent to the outer side of the light output window, the slide holder comprising:

a base member having a recessed portion having a sufficient dimension to hold a slide-disposed biological sample, wherein the base member includes at least one channel having a first opening into the recessed portion and a second opening located at an outer edge of the base member;

a lid coupled to the base member, wherein the lid is positioned adjacent to the light output window of the irradiation module, and wherein the lid includes a window extending there through that is aligned with the recessed portion of the base member and the light output window of the irradiation module; and

sealing members located on at least one of the base member or the lid.

26. The system of claim 25, further comprising a vacuum source operably connected to the second opening of the base member.

27. The system of claim 25 or 26, wherein the irradiation module further comprises a second reflector interposed between the first reflector and the light output window, wherein the second reflector is centered on the longitudinal axis.

28. The system of any one of claims 25 to 27, further comprising a filter member disposed adjacent to the light output window and the filter member includes at least one filter that attenuates at least one light wavelength.

29. The system of claim 28, wherein the filter member includes at least two sequentially arranged filters.

30. The system of claim 28, wherein the filter member includes at least two curvilinearly arranged filters.

31. The system of any one of claims 25 to 30, wherein the flashtube is positioned so that light from the flashtube passes through the opening of the first reflector, and the light output window.

32. The system of any one of claims 25 to 31, further comprising a mask holder disposed on a surface of the lid opposing the base member and aligned with the window.

FIG. 1A

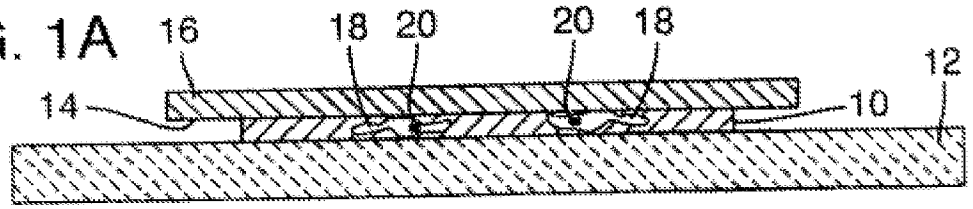


FIG. 1B

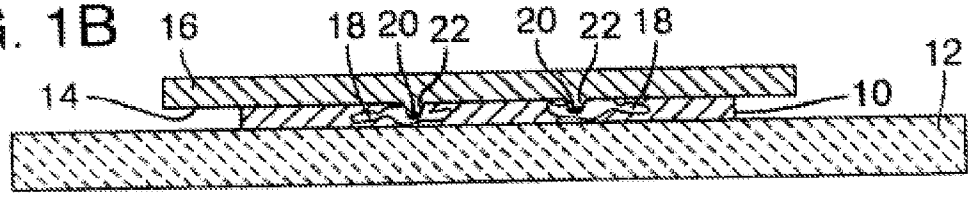


FIG. 1C

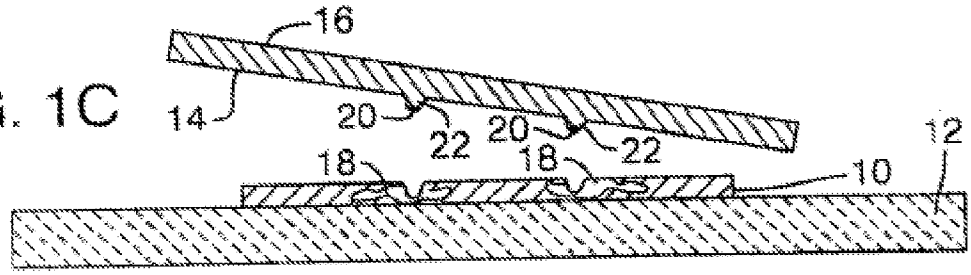
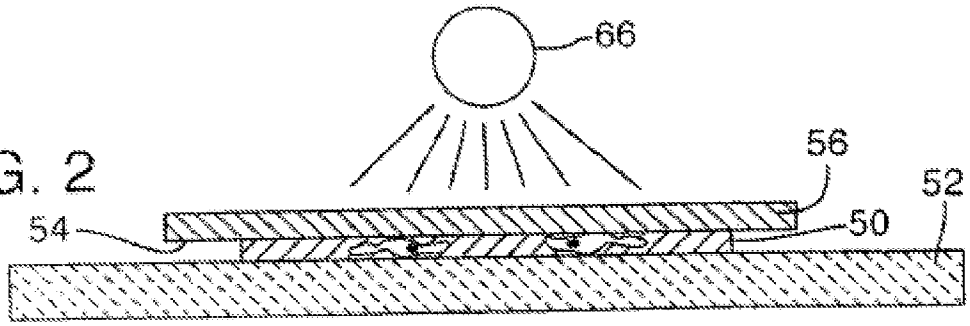


FIG. 2



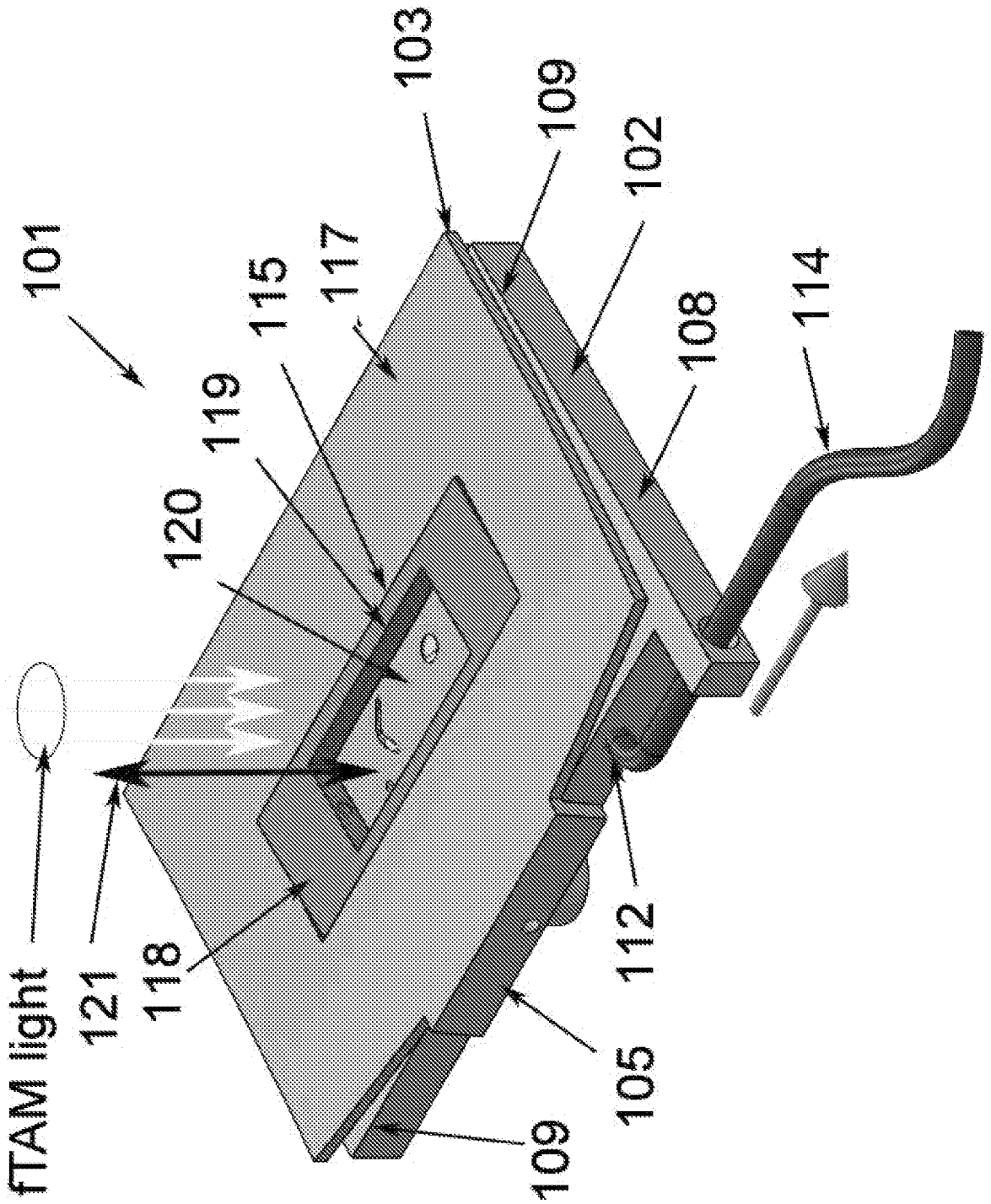

fTAM light
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FIG. 3

FIG. 4

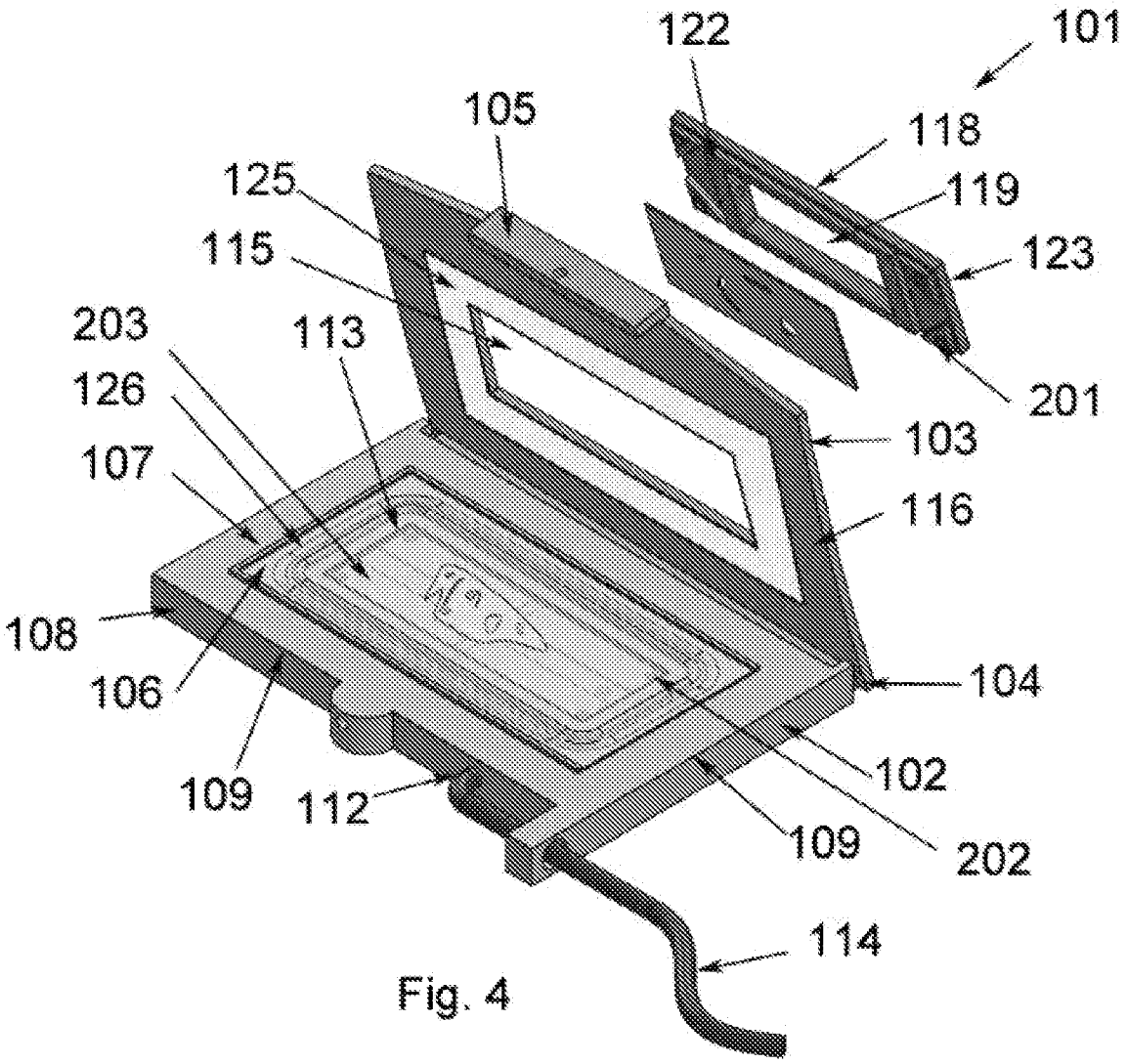
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Fig. 4

FIG. 5

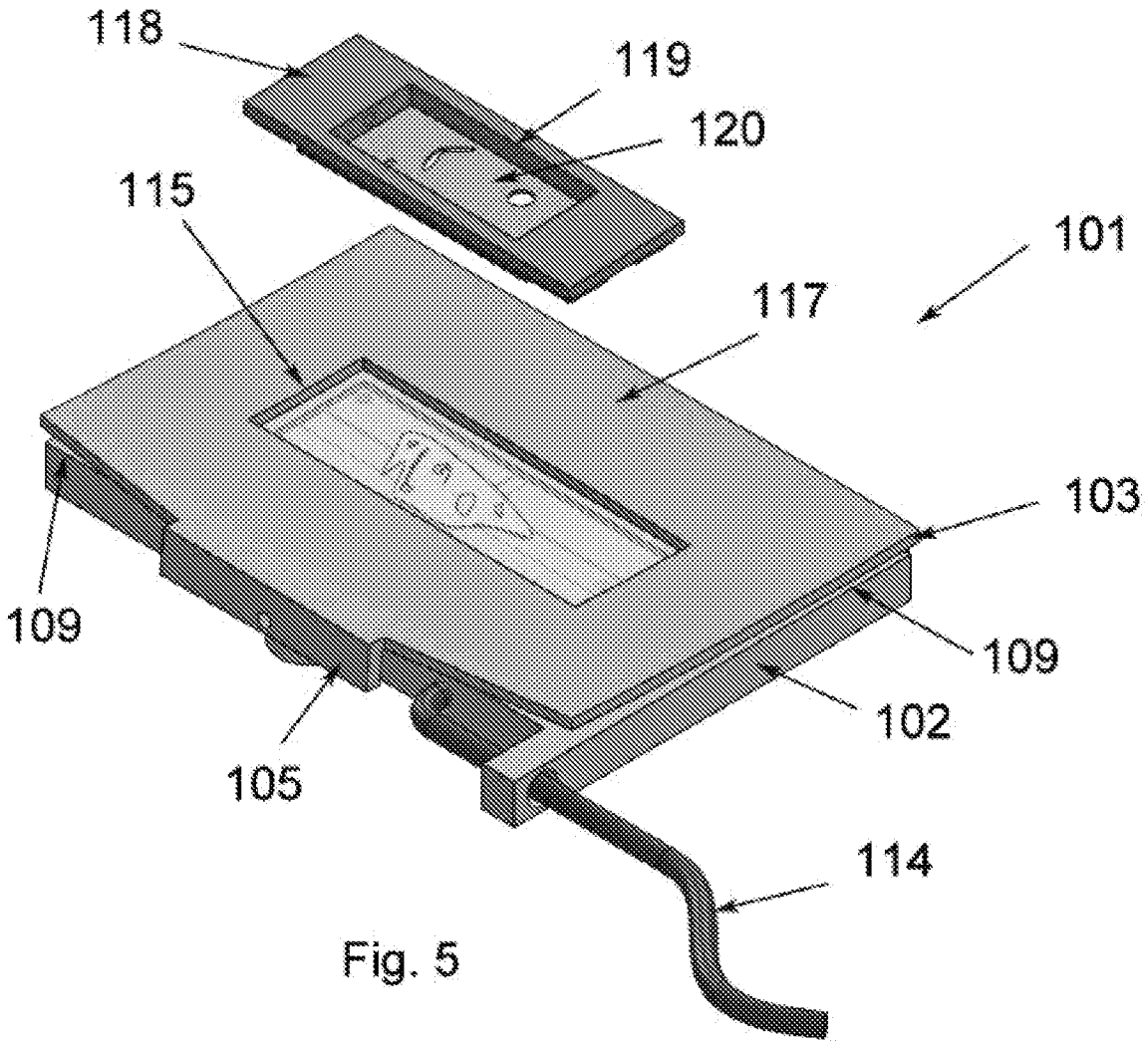

Fig. 5

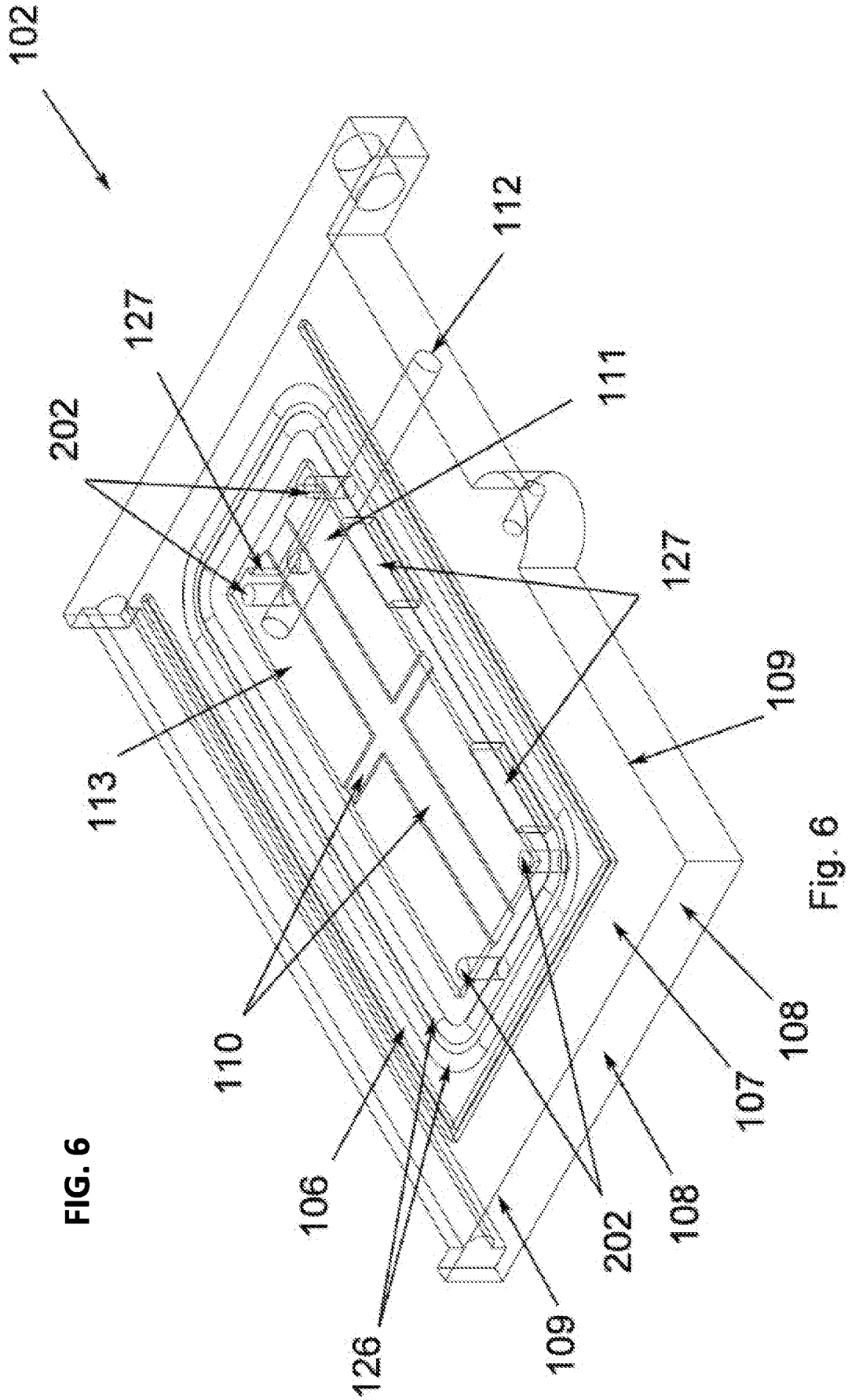

FIG. 6

Fig. 6

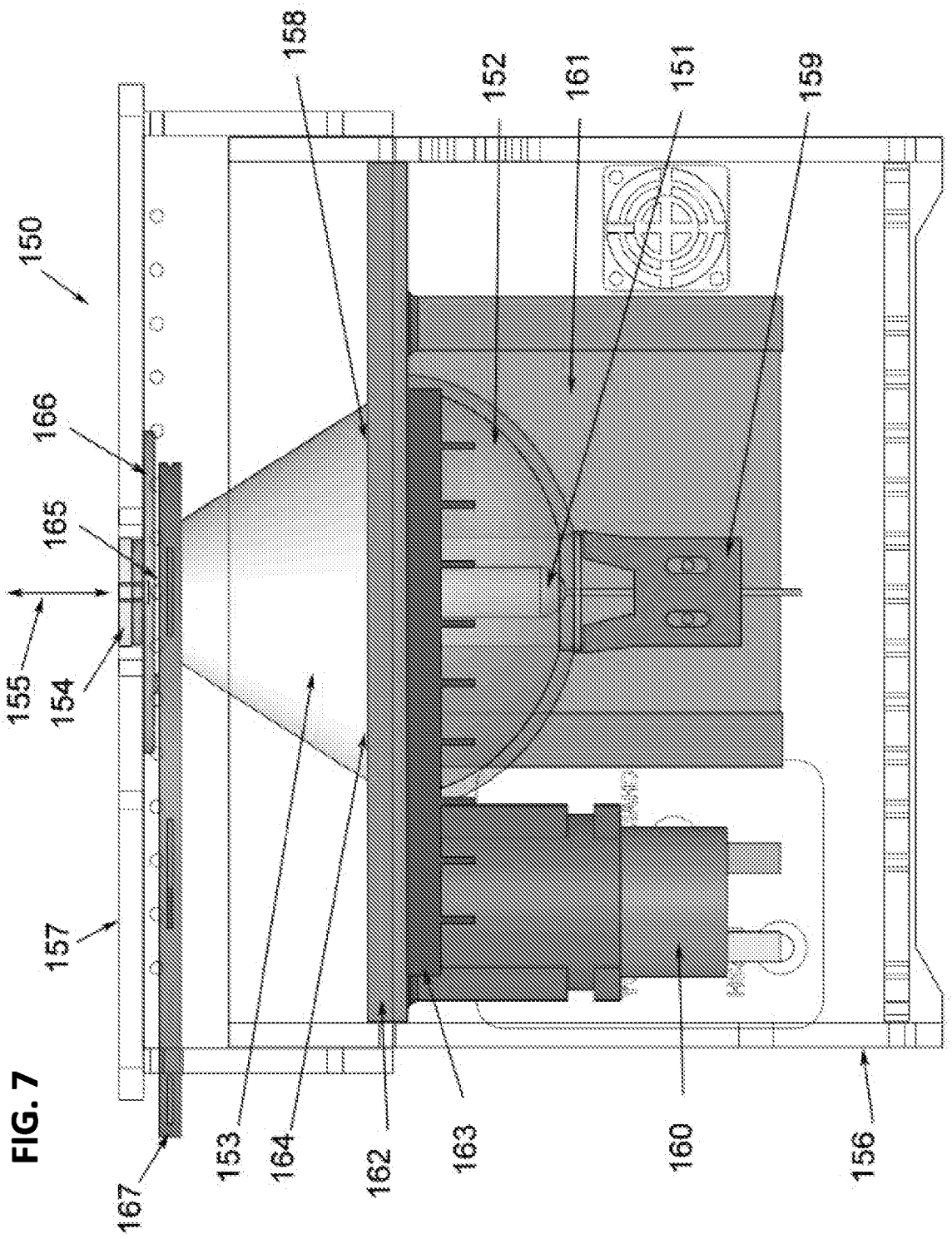
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FIG. 7

FIG. 8

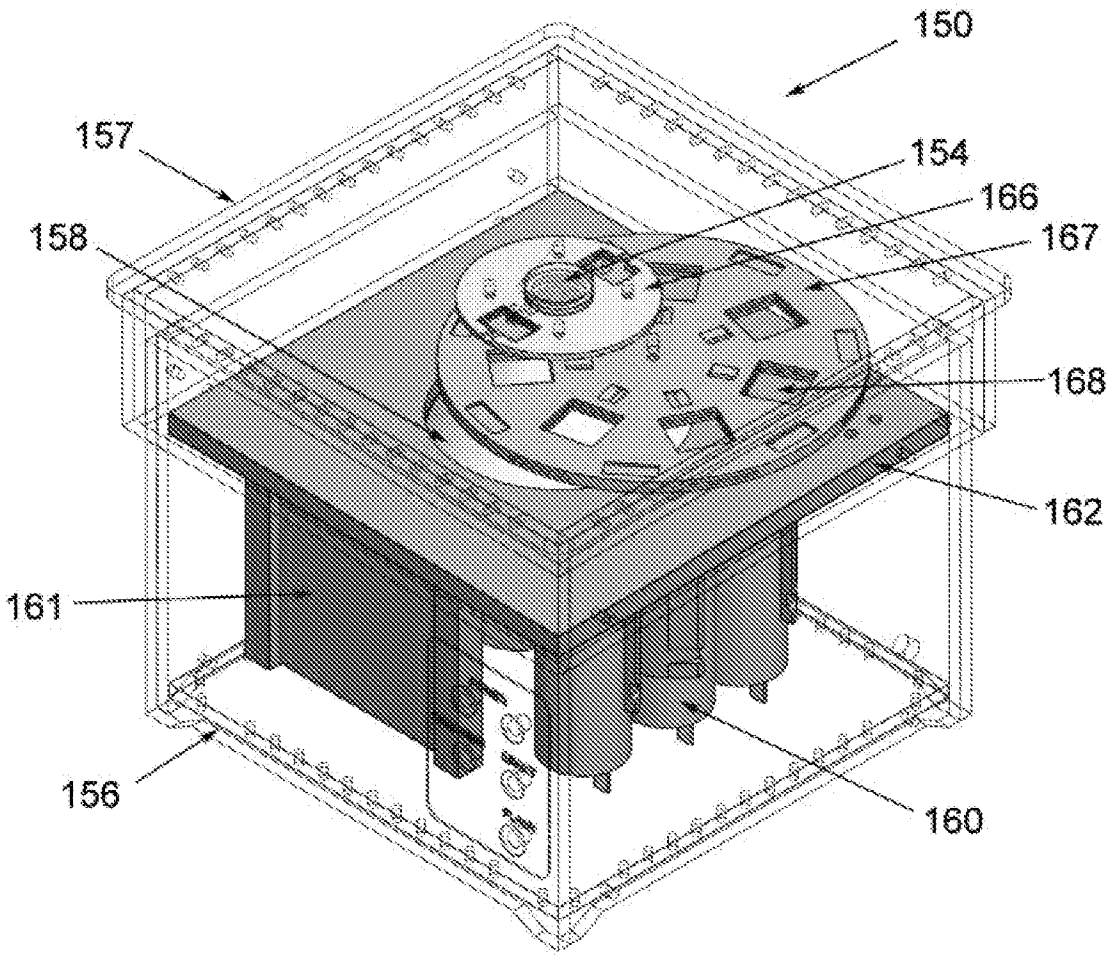


FIG. 9

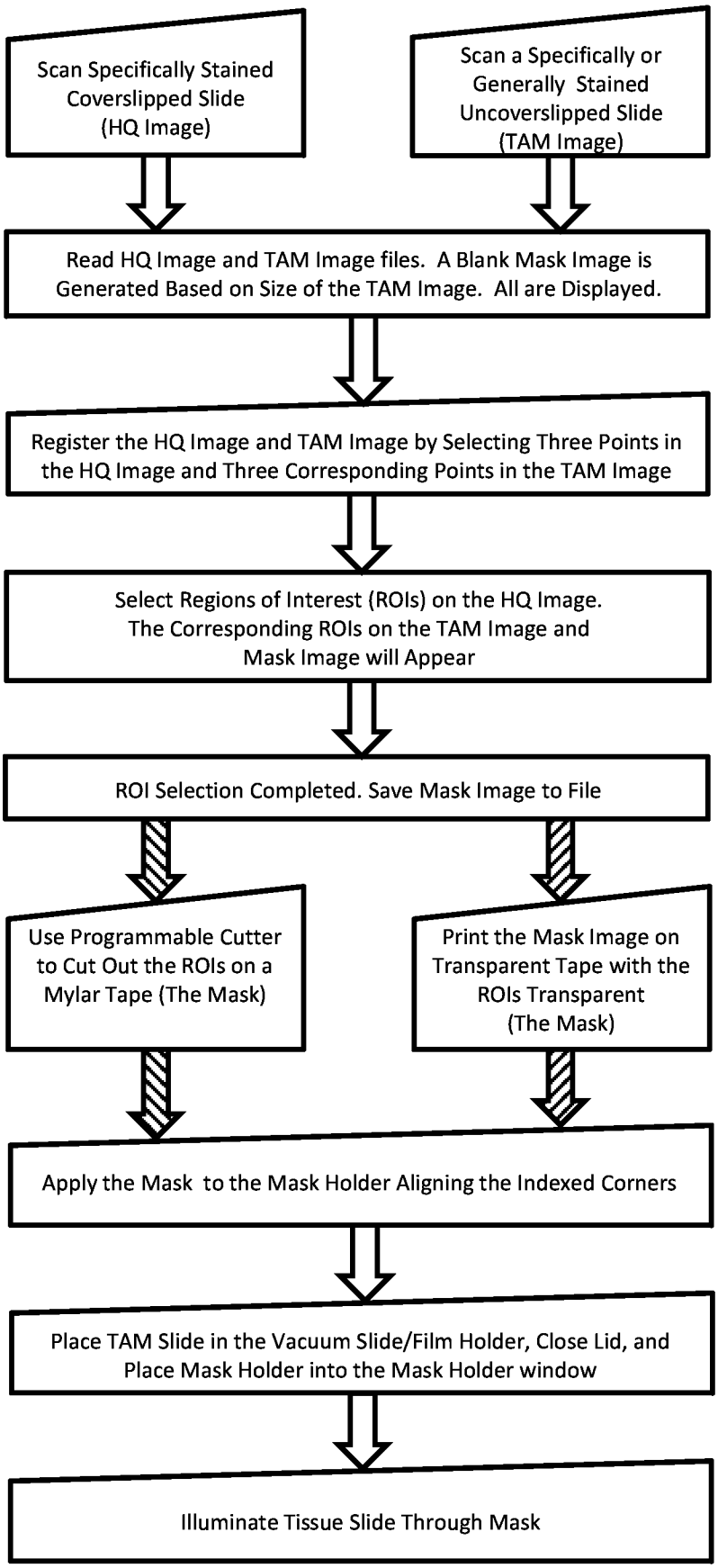
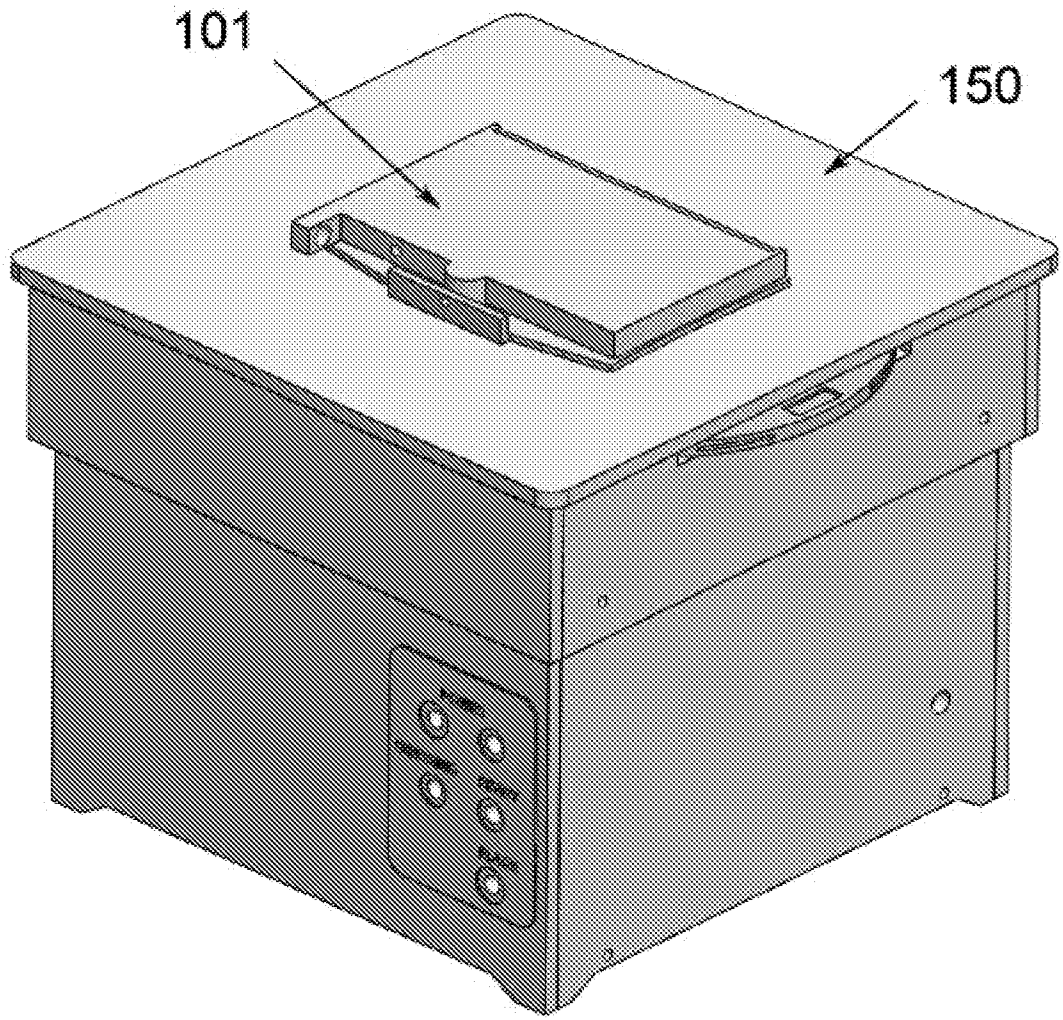

Scan Specifically Stained Coverslipped Slide (HQ Image)
Scan a Specifically or Generally Stained Uncoverslipped Slide (TAM Image)
Read HQ Image and TAM Image files. A Blank Mask Image is Generated Based on Size of the TAM Image. All are Displayed.
Register the HQ Image and TAM Image by Selecting Three Points in the HQ Image and Three Corresponding Points in the TAM Image
Select Regions of Interest (ROIs) on the HQ Image. The Corresponding ROIs on the TAM Image and Mask Image will Appear
ROI Selection Completed. Save Mask Image to File
Use Programmable Cutter to Cut Out the ROIs on a Mylar Tape (The Mask)
Print the Mask Image on Transparent Tape with the ROIs Transparent (The Mask)
Apply the Mask to the Mask Holder Aligning the Indexed Corners
Place TAM Slide in the Vacuum Slide/Film Holder, Close Lid, and Place Mask Holder into the Mask Holder window
Illuminate Tissue Slide Through Mask

FIG. 10



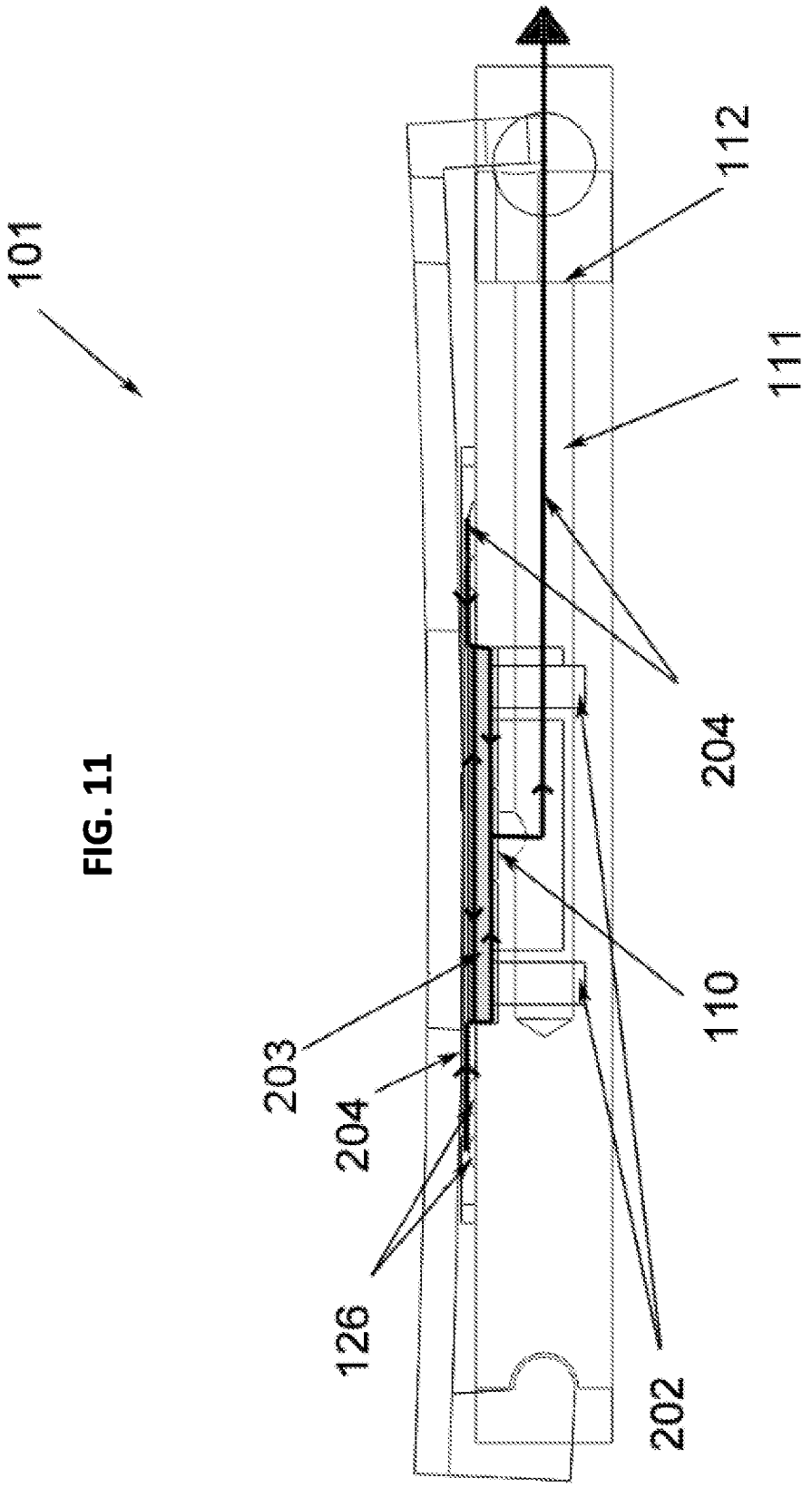


FIG. 11

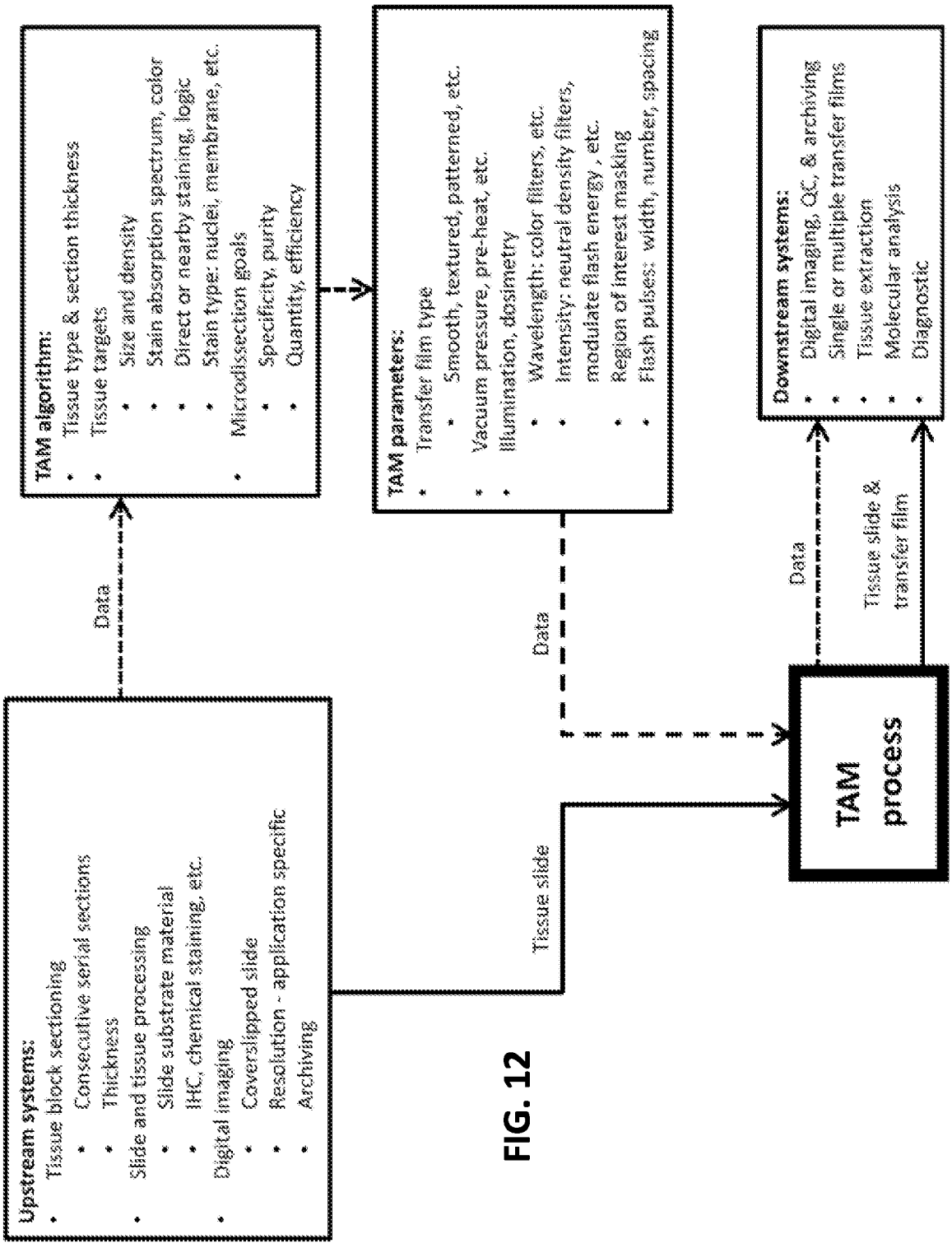

Upstream systems:
Tissue block sectioning
Consecutive serial sections
Thickness
Slide and tissue processing
Slide substrate material
IHC, chemical staining, etc.
Digital imaging
Coverslipped slide
Resolution - application specific
Archiving
Data
TAM algorithm:
Tissue type & section thickness
Tissue targets
Size and density
Stain absorption spectrum, color
Direct or nearby staining, logic
Stain type: nuclei, membrane, etc.
Microdissection goals
Specificity, purity
Quantity, efficiency
Data
TAM parameters:
Transfer film type
Smooth, textured, patterned, etc.
Vacuum pressure, pre-heat, etc.
Illumination, dosimetry
Wavelength: color filters, etc.
Intensity: neutral density filters, modulate flash energy , etc.
Region of interest masking
Flash pulses: width, number, spacing
Tissue slide
Tissue slide & transfer film
TAM process
Data
Downstream systems:
Digital imaging, QC, & archiving
Single or multiple transfer films
Tissue extraction
Molecular analysis
Diagnostic

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/034930

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N1/28 G01N1/30 G01N1/08
ADD.

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)
G01N B01L

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. R. BLACKLER ET AL: "Proteomic Analysis of Nuclei Dissected from Fixed Rat Brain Tissue Using Expression Microdissection", ANALYTICAL CHEMISTRY, vol. 85, no. 15, 6 August 2013 (2013-08-06), pages 7139-7145, XP055210963, ISSN: 0003-2700, DOI: 10.1021/ac400691k abstract; figures 1,3 page 7139, right-hand column, line 3 - line 15 page 7140, left-hand column, paragraph Custom Flashcube page 7140, left-hand column, paragraph Vacuum chamber page 7140, left-hand column, paragraph Thin EVA capture films page 7140, left-hand column, paragraph Flashcube-xMD -/--	1-9, 11-16, 19,25-32

[X] Further documents are listed in the continuation of Box C.

[X] 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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 2015	14/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lefortier, Stéphanie

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	page 7141, right-hand column, paragraph three ----	
X	US 2006/172278 A1 (BONNER ROBERT F [US] ET AL) 3 August 2006 (2006-08-03) figures 1-3 paragraph [0088] - paragraph [0089] paragraphs [0112], [0141], [0183] paragraph [0241] - paragraph [0242] paragraph [0244] - paragraph [0245] paragraph [0252] ----	1-19
X	US 4 856 351 A (SMITH MICHAEL P [US] ET AL) 15 August 1989 (1989-08-15) figure 7 column 7, line 10 - line 59 ----	20-24
A	US 2012/180576 A1 (RAFFERTY DAVID [US] ET AL) 19 July 2012 (2012-07-19) figures 1, 2 paragraph [0026] - paragraph [0028] ----	16,20-32
A	Michael Tangrea: "New In Situ and Microdissection Technologies for Molecular Pathology", Pathology Informatics Summit 2014, 13 May 2014 (2014-05-13), XP055211113, Retrieved from the Internet: URL:http://www.pathologyinformatics.com/sites/default/files/archives/2014/Day1/20140513%201035%20-%20New%20In%20Situ%20and%20Microdissection%20Technologies%20for%20Molecular%20Pathology.pdf [retrieved on 2015-09-03] page 42 - page 46 page 60 - page 62 page 66 ----	1-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/034930

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		US 2010216166 A1	26-08-2010
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US 2012180576 A1	19-07-2012	CA 2824525 A1	19-07-2012
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		EP 2663995 A1	20-11-2013
		JP 2014503825 A	13-02-2014
		US 2012180576 A1	19-07-2012
		WO 2012097058 A1	19-07-2012