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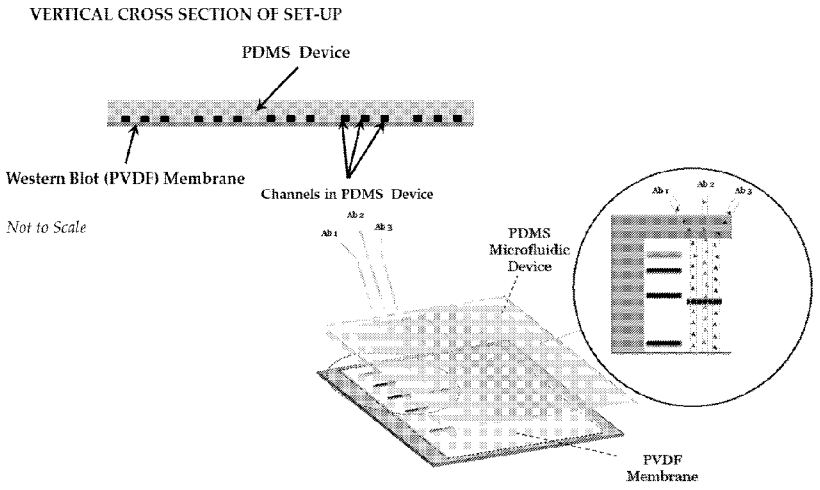
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(54) **Title**: MICROFLUIDIC DEVICE FOR IMMUNOBLOTTING

FIG. 1



(57) **Abstract**: The present invention provides microfluidic devices, systems, and methods for use in immunoblotting applications. In particular, devices and methods provided herein have the advantages of traditional Western blotting with increased throughput and multiplex ability, and decreased time, sample, and reagent requirements.

WO 2014/165185 A1

MICROFLUIDIC DEVICE FOR IMMUNOBLOTTING

This application claims priority to U.S. provisional patent application 61/777,682, filed March 12, 2013, which is incorporated herein by reference in its entirety.

FIELD

The present invention provides microfluidic devices, systems, and methods for use in immunoblotting applications. In particular, devices and methods provided herein have the advantages of traditional Western blotting with increased throughput and multiplexability, and decreased time, sample, and reagent requirements.

BACKGROUND

Traditional Western blot analysis (or immunoblotting) is used to detect a specific protein in a cell, tissue, organ, body fluid, etc. The technique is a mainstay of biochemistry and molecular biology. Proteins in a sample are separated by gel electrophoresis (e.g., SDS-PAGE), and then transferred from the gel to a membrane (e.g., nitrocellulose or PVDF). The membrane is then stained with antibodies specific for a target protein. Although reliably yielding useful information, the technique is: time consuming, requiring of technical skill, reagent intensive, limited throughput, and poorly suited for multiplexing. What is needed is a technique that provides the advantages of traditional Western blotting with the increased throughput, multiplexability, and reduced reagent usage.

SUMMARY OF THE INVENTION

The present invention provides microfluidic devices, systems, and methods for use in immunoblotting applications. In particular, devices and methods provided herein have the advantages of traditional Western blotting with increased throughput and multiplexability, and decreased time, sample, and reagent requirements.

In some embodiments, the present invention provides microfluidic immunoblotting devices comprising a flat membrane-contacting surface and a plurality of non-connected parallel microfluidic channels, wherein the length of said microfluidic channels are open to the membrane-contacting surface. In some embodiments, the microfluidic channels are spatially grouped into two or more

channel sets. In some embodiments, each of the two or more channel sets comprises 2-20 microfluidic channels. In some embodiments, the distance separating microfluidic channels with a channel set is less than the distance between channel sets. In some embodiments, a microfluidic channel (e.g., each of the microfluidic channels) comprises a reservoir at one end of the microfluidic channel. In some embodiments, a microfluidic channel (e.g., each of the microfluidic channels) comprises reservoirs at both ends of the microfluidic channel. In some embodiments, each of the microfluidic channels is 50-300 μm wide. In some embodiments, each of the microfluidic channels is 100-200 μm wide. In some embodiments, each of the microfluidic channels is 140-160 μm wide. In some embodiments, each of the microfluidic channels is 30-200 μm deep. In some embodiments, each of the microfluidic channels is 50-150 μm deep. In some embodiments, each of the microfluidic channels is 90-110 μm deep. In some embodiments, the ratio of width to depth of the microfluidic channels is less than 10:1 (e.g., <9:1, <8:1, <7:1, <6:1, <5:1, <4:1, <3:1, <2:1). In some embodiments, the ratio of width to depth of the microfluidic channels is between 3:1 and 1:3 (e.g., 2:1 to 1:2). In some embodiments, the ratio of width to depth of the microfluidic channels is 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:4. 1:5, 1:6, 1:7, 1:8, or 1:9. In some embodiments, the ratio of width to depth of the microfluidic channels is between 3:1 and 1:3 (e.g., 2:1 to 1:2). In some embodiments, the device comprises polydimethylsiloxane (PDMS). In some embodiments, the device consists essentially of polydimethylsiloxane (PDMS). In some embodiments, the present invention provides methods of immunoblotting comprising applying antibody solutions to a membrane using a device of the present invention.

In some embodiments, the present invention provides systems (e.g., assays) comprising a device of the present invention and a membrane. In some embodiments, an activating solution is located or placed between said device and said membrane. In some embodiments, the activating solution comprises polysorbate-20 (Tween-20) and BSA. In some embodiments, the activating solution comprises 0.01-0.5% Tween-20 and 0.01-0.5% BSA. In some embodiments, the activating solution comprises 0.05-0.15% Tween-20 and 0.05-0.15% BSA. In some embodiments, the activating solution comprises 0.1% Tween-20 and 0.1% BSA. In some embodiments, the membrane comprises membrane nitrocellulose or polyvinylidene fluoride (PVDF). In some embodiments, the membrane comprises peptides, polypeptides, or proteins on or

within it. In some embodiments, systems further comprise one or more antibody-containing solutions within the microfluidic channels. In some embodiments, each microfluidic channel of a channel set comprises a different antibody-containing solution. In some embodiments, each channel set comprises the same combination of antibody-containing solutions. In some embodiments, each microfluidic channel comprises a different antibody-containing solution. In some embodiments, the present invention provides methods of immunoblotting comprising applying antibody solutions to a membrane using system of the present invention.

In some embodiments, the present invention provides methods of immunoblotting comprising: (a) transferring proteins from a sample onto a membrane; (b) placing a membrane-contacting face of a microfluidic immunoblotting device onto the membrane; (c) introducing (e.g., injecting) an activating buffer into microfluidic channels of said device; (d) introducing (e.g., injecting) primary antibody solutions into the microfluidic channels of said device; and (e) detecting binding of antibodies with said antibody solutions to said proteins.

BREIF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of a microfluidic immunoblotting device and an interface with a membrane. Three microfluidic channels overlie each sample lane that can be used to probe for three different proteins per lane.

Figure 2 shows antibody dilutions in clinical PBMC samples. (A) Traditional immunoblotting with primary antibody against RelA/p65 using 1:500 and 1:5000 antibody dilutions. (B) Microfluidic immunoblot probing for RelA/p65 using the same conditions as demonstrating similar signal intensities and the flexibility to do several dilutions on the same sample.

Figure 3 shows detection of STAT3 phosphorylation in response to inflammatory stimuli. (A) Traditional immunoblots of RAW264.7 cell lysates showing phosphorylation of STAT3 in response to LPS stimulation. (B) Microfluidic immunoblot on same RAW264.7 cell lysates as (A). The microfluidic device allows for simultaneous monitoring of phospho-STAT3 and STAT3 in the same sample without the need for stripping or reprobing of the PVDF membrane.

Figure 4 shows protein immunoblots of the MAPK pathway with two different chemiluminescent detection modalities. (A) Traditional immunoblots of RAW264.7 cell lysates with or without LPS for 45 minutes. Membranes were probed for

phospho-JNK and phosphor-ERK using total ERK as the loading control. Primary antibodies were
detected using HRP chemiluminescent techniques. (B) Microfluidic immunoblots mirroring conditions in (A). (C) and (D) are identical to (A) and (B) except they were performed using alkaline phosphatase chemiluminescent secondary antibodies to detect primary antibodies.

DEFINITIONS

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity (Miller et al (2003) Jour. of Immunology 170:4854-4861; herein incorporated by reference in its entirety). Antibodies may be murine, human, humanized, chimeric, or derived from other species. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen. (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) Immuno Biology, 5th Ed., Garland Publishing, New York; herein incorporated by reference in its entirety). A target antigen generally has numerous binding sites, also called epitopes, recognized by CDRs on multiple antibodies. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen may have more than one corresponding antibody. An antibody includes a full-length immunoglobulin molecule or an immunologically active portion of a full-length immunoglobulin molecule, i.e., a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease. The immunoglobulin can be of any type (e.g., IgG, IgE, IgM, IgD, and IgA), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The immunoglobulins can be derived from any species, including human, murine, or rabbit origin.

As used herein, the term "antibody fragment" refers to a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab').sub.2, and Fv fragments; diabodies; linear antibodies; fragments produced by a Fab expression library, anti-idiotypic (anti-

Id) antibodies, CDR (complementary determining region), and epitope-binding fragments of any of the above which immunospecifically bind to antigens.

As used herein, the phrase "multiplex" or grammatical equivalents refers to the detection, analysis or amplification of more than one target sequence of interest. In one embodiment multiplex refers to >3, >5, >8, >10, >20> 50, >100, etc. "Multiplexability" refers to the quality of a particular device, reagent, system, platform, kit, etc. to be used in a multiplex fashion.

As used herein, the term "sample" is used in its broadest sense. In one sense, it is meant to include cells (e.g., human, bacterial, yeast, and fungi), an organism, a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and refers to a biological material or compositions found therein, including, but not limited to, bone marrow, blood, serum, platelet, plasma, interstitial fluid, urine, cerebrospinal fluid, nucleic acid, DNA, tissue, and purified or filtered forms thereof. Environmental samples include environmental material such as surface matter, soil, water, crystals and industrial samples. Such examples are not however to be construed as limiting the sample types applicable to the present invention.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The present invention provides microfluidic devices, systems, and methods for use in immunoblotting applications. In particular, devices and methods provided herein have the advantages of traditional Western blotting with increased throughput and multiplexability, and decreased time, sample, and reagent requirements.

Since its inception in 1979, protein immunoblotting has become the standard technique for profiling proteins in molecular biology and clinical diagnostics (Towbin et al. 1979; herein incorporated by reference in its entirety) Although, traditional protein immunoblotting remains a powerful technique, it has limitations including its slow throughput, the requirement for relatively large sample and antibody amounts, and the inability to probe for multiple proteins simultaneously (Wu et al. 2007; herein incorporated by reference in its entirety).

Several variations on protein immunoblotting have evolved to overcome some of the early limitations, including membrane stripping and reprobing that allows for sequential detection of distinct proteins, and the introduction of fluorescent secondary antibodies, that can be used to increase the different proteins detected from samples.

Despite the improvements, these variations have their own limitations, including loss of signal and signal variability. Additionally, none of these variations address the large sample and antibody amounts required by traditional immunoblotting.

Microfluidic technology has been applied to molecular biology and to clinical diagnostics. The small volumes and precise spatial control afforded by microfluidics make it an exciting complement to existing molecular biology technologies. The Herr group incorporated microfluidic technology with traditional protein immunoblotting (He and Herr 2009; Hughes and Herr 2012; herein incorporated by reference in their entireties). While an improvement, this approach could only detect single proteins and did not have the flexibility to interface with existing immunoblotting platforms. More recently, a microfluidic device that integrates the separation, transfer, and detection components of immunoblotting into a single device has been developed (Tia et al. 2011; herein incorporated by reference in its entirety). While the speed and multiplexing ability of this device is a significant enhancement, the ability to probe for specific proteins in complex biological matrices has yet to be demonstrated with such integrated microfluidic devices. Pan and colleagues made further advancements by introducing a fluorescence based microfluidic device that could detect multiple proteins and is compatible with existing protein immunoblotting methods (Pan et al. 2010; herein incorporated by reference in its entirety). None of these microfluidic devices allow for simultaneously probing for multiple proteins, and interface with existing immunoblotting equipment and chemiluminescent detection methods.

While traditional Western blotting can yield important information (e.g., about the interaction between a biological system (e.g., humans) and an outside stimulus (e.g., an anti-inflammatory drug)), they are expensive, both in terms of reagents and time. In certain embodiments, the present invention provides devices and methods that provide similar and/or greater information content as traditional Western blotting, but with decreased reagent and time consumption, and increased throughput and multiplexability. In some embodiments, the present invention provides a microfluidic device comprising sets of microfluidic channels configured to overlay the lanes present on a protein-containing membrane. In various embodiments, proteins on the membrane are probed by antibodies within the microfluidic channels. In some embodiments, a single protein lane on a membranes is overlayed by two or more microfluidic channels (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more), each containing a different antibody for probing the protein lane.

In some embodiments, the present invention provides numerous advantages over traditional immunoblotting. For example, instead of incubating the entire membrane with a single type of primary antibody, a much smaller quantity of antibody is provided in a single microfluidic channel per protein lane. In embodiments in which multiple microfluidic channels are provided per protein lane, a single protein lane may be probed by multiple different antibodies simultaneously. In some embodiments, the present invention allows for probing with more types of antibodies (e.g., 2… 5… 10… 20, or more per lane of a gel) using less reagent (e.g., microfluidic channel vs. incubating the entire membrane), less sample (e.g., a single lane on a gel vs. a different gel for each different antibody), and less time (e.g., a single gel and membrane transfer vs. a different gel and transfer) than traditional immunoblotting. In some embodiments, from a time perspective, devices and method of the present invention can complete 3, 5, 10, 20, or more immunoblots in the time it takes to complete one traditional Western blot. In some embodiments, from a cost perspective, devices and methods of the present invention can complete an immunoblot for less than 1% of the cost of a traditional Western Blot.

A. Device

In certain embodiments, microfluidic devices are provided that comprises one or more sets (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) of one or more microfluidic channels (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more). In some embodiments, the channels run along the flat surface of the device, such that fluid contained within the channels is exposed to the surface of the device. In some embodiments, each channel comprises a reservoir or other fluid-introduction means for introduction of antibody solution into the microfluidic channel.

In some embodiments, a microfluidic device does not require valves, pumping, mixing chambers, and/or other more complex elements that are common to microfluidic devices, but increase the cost of a device and the complexity of its use. In some embodiments, a microfluidic device does not require integration with gel electrophoresis in order to carry out immunoblotting.

In some embodiments, a microfluidic device comprises one or more sets of channels, each containing one or more channels. Each set is configured to probe a single lane on of a protein gel. In some embodiments, each channel of a set is

configured to probe the protein lane with a different antibody. Each channel set may comprise the same or different number of channels.

In some embodiments, the device may be of any suitable size and dimensions. In some embodiments, a device is configured to match the dimensions of a particular gel or membrane. In some embodiments, a device is configured to be usable with gels and membranes of different sizes and/or dimensions. In some embodiments, devices comprise a footprint (e.g., membrane-contacting face) with dimensions between 1 cm and 50 cm or more (e.g., 1cm x 1 cm, 4 cm x 6 cm, 8 cm x 6 cm, 5 cm x 20 cm, 20 cm x 40 cm, etc.). In some embodiments, the length and/or width of the membrane-contacting face of a microfluidic device is 1 cm... 2 cm... 3 cm... 4 cm... 5 cm... 6 cm... 7 cm... 8 cm... 9 cm... 10 cm... 11 cm... 12 cm... 13 cm... 14 cm... 15 cm...16 cm... 17 cm... 18 cm... 19 cm... 20 cm... 21 cm... 22 cm... 23 cm... 24 cm... 25 cm... 26 cm... 27 cm... 28 cm... 29 cm... 30 cm... 31 cm... 32 cm... 33 cm... 34 cm... 35 cm... 36 cm... 37 cm... 38 cm... 39 cm... 40 cm... 41 cm... 42 cm... 43 cm... 44 cm... 45 cm... 46 cm... 47 cm... 48 cm... 49 cm... 50 cm.

In some embodiments, channels of a microfluidic device may be of any suitable size and dimensions. In some embodiments, channels are so dimensioned to allow the appropriate solutions to efficiently traverse and/or occupy the microfluidic channels. In some embodiments, the length of a channel is proportional to the length of the device. In some embodiments, the length of a channel is proportional to the length of the membrane and/or gel with which it is configured for use. In some embodiments, channels are between 1 cm and 50 cm in length (e.g., 1 cm... 2 cm... 3 cm... 4 cm... 5 cm... 6 cm... 7 cm... 8 cm... 9 cm... 10 cm... 11 cm... 12 cm... 13 cm... 14 cm... 15 cm...16 cm... 17 cm... 18 cm... 19 cm... 20 cm... 21 cm... 22 cm... 23 cm... 24 cm... 25 cm... 26 cm... 27 cm... 28 cm... 29 cm... 30 cm... 31 cm... 32 cm... 33 cm... 34 cm... 35 cm... 36 cm... 37 cm... 38 cm... 39 cm... 40 cm... 41 cm... 42 cm... 43 cm... 44 cm... 45 cm... 46 cm... 47 cm... 48 cm... 49 cm... 50 cm). In some embodiments, a channel is of appropriate width and/or depth to allow the necessary solutions (e.g., antibody solutions) to flow/traverse, occupy, and/or fill the channel. In some embodiments, the width of a channel is optimized to provide the desired number of channels in a set of a particular width. In some embodiments, as channels are narrowed to accommodate more lanes per set, channels are deepened to provide the necessary channel volumes. In some embodiments, a set of channels ranges in width from 1 mm to 50 cm (e.g., 1 mm... 2 mm... 3 mm... 4

mm… 5 mm… 6 mm… 7 mm… 8 m… 9 mm… 1 cm… 2 cm… 3 cm… 4 cm… 5 cm… 6 cm… 7 cm… 8 cm… 9 cm… 10 cm… 11 cm… 12 cm… 13 cm… 14 cm… 15 cm…16 cm… 17 cm… 18 cm… 19 cm… 20 cm… 21 cm… 22 cm… 23 cm… 24 cm… 25 cm… 26 cm… 27 cm… 28 cm… 29 cm… 30 cm… 31 cm… 32 cm… 33 cm… 34 cm… 35 cm… 36 cm… 37 cm… 38 cm… 39 cm… 40 cm… 41 cm… 42 cm… 43 cm… 44 cm… 45 cm… 46 cm… 47 cm… 48 cm… 49 cm… 50 cm). In some embodiments, set width is configured to match the lane width of a gel and/or membrane. In some embodiments, a set of microchannels comprises one or more channels (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10… 20… 30… 40… 50… 100… 500, etc.). In some embodiments, the number of channels per set is dependent upon the width of the set, the spacing between channels, and the width of the channels (e.g., which may be dependent upon the type of solution to be used within the channels). In some embodiments, channels widths range from 50 μm to 500 μm (e.g., 50 μm, 60 μm, 70 μm, 80 μm, 90 μm, 100 μm, 110 μm, 120 μm, 130 μm, 140 μm, 150 μm, 160 μm, 170 μm, 180 μm, 190 μm, 200 μm… 250 μm… 300 μm… 350 μm… 400 μm… 450 μm… 500 μm). In some embodiments, channel widths range from 100 μm to 200 μm (e.g., 125 μm to 175 μm, 140 μm to 160 μm, about 150 μm, 150 μm). In some embodiments, channel depths range from 25 μm to 200 μm (e.g., 25 μm, 30 μm, 40 μm, 50 μm, 60 μm, 70 μm, 80 μm, 90 μm, 100 μm, 110 μm, 120 μm, 130 μm, 140 μm, 150 μm, 160 μm, 170 μm, 180 μm, 190 μm, 200 μm). In some embodiments, channel widths range from 75 μm to 125 μm (e.g., 90 μm to 110 μm, about 100 μm, 100 μm). In some embodiments, channels accomdate between 0.1 μl and 5 μl of solution per channel (e.g., 0.1 μl, 0.2 μl, 0.3 μl, 0.4 μl, 0.5 μl, 0.6 μl, 0.7 μl, 0.8 μl, 0.9 μl, 1.0 μl, 1.1 μl, 1.2 μl, 1.3 μl, 1.4 μl, 1.5 μl, 1.6 μl, 1.7 μl, 1.8 μl, 1.9 μl, 2 μl… 3 μl… 4 μl… 5 μl).

In some embodiments, microfluidic devices are provided as part of a kit along with one or more of appropriately sized membranes, appropriately sized gel with lanes configured to match channels of the device, antibody solutions, buffer, activation solution, comb for pouring gels with lanes configured to match channels of the device, instructions, etc. In some embodiments, a device or kit is optimized for a particular assay. In some embodiments, a kit is provided with antibodies and lane configuration for performing a specific assay.

In some embodiments, the channel sets and/or microfluidic channels are not consistent across the device. Channels on a single device may vary across a single

device according to one or more of set width, number of microchannels per set, microchannel width, spacing, etc.

B. Method

In some embodiments, devices of the present invention are utilized to perform immunoblot experiments, using any suitable techniques and reagents. \

An exemplary procedure for immunoblotting with a device of the present invention is as follows. A protein-containing sample is loaded into the wells of a gel (e.g., polyacrylamide gel) and proteins present in the sample are separated by gel electrophoresis (e.g., native gel electrophoresis, SDS-PAGE, etc.). A membrane (e.g., PVDF membrane) is placed atop the gel to allow transfer of the proteins onto the membrane. The membrane is then placed on a glass slide. A microfluidic device is placed on top of membrane, and the microfluidic channels are aligned with the protein lanes. Blocking buffer (e.g., Tween-20 and BSA) is optionally injected in each of the channels. In embodiments with a blocking step, the inlets/outlets of the channels are covered (e.g., with tape) to minimize evaporation during incubation. Antibodies are injected into the appropriate channels (e.g., different antibodies in each microchannel of a set). The inlets/outlets are covered (e.g., with tape) to minimize evaporation during incubation. Following incubation (e.g., 1 hours), the microfluidic device is removed and the membrane is transferred to a solution of an appropriate secondary antibody (e.g., the membrane is left in this solution for 1 hour, or until the membrane is completely wet). The membrane is removed from the secondary antibody solution and washed with fresh blocking buffer (e.g., 3 minutes, 5 times each). The membrane is transferred to a developing solution (e.g., 30 minutes), and the membrane is removed and rinsed with DI water. The membrane is then allowed to dry. In some embodiments, any or all of the above steps may be altered for an alternative procedure or to accommodate a specific use. For example, in some embodiments, the device is placed onto the membrane with antibodies pre-loaded into the channels. In other embodiments, the blocking, secondary antibody solution, washing, and developing solutions are applied through the microfluidic channels. In some embodiments, a sample is placed on a membrane directly or from a surface other than a gel (e.g., microtiter plate, array, etc.).

In some embodiments, the same sample under the same conditions is run on multiple wells of a gel. In such cases, the microfluidic channels of multiple sets of

channels may all be loaded with different antibodies to interrogate the same sample. In other embodiments, different samples (or the same sample under different conditions) are run on two or more lanes of a gel. In such cases, the various channel sets may be loaded identically, to probe each sample with the same set of antibodies.

In some embodiments, the device and/or membrane comprise hydrophobic surfaces (e.g., PDMS and PVDF). Experiments conducted during development of embodiments of the present invention revealed that it is challenging to achieve a suitable seal between the device and membrane. In some embodiments, to overcome this challenge, an activation step (e.g., of the device and/or of the membrane) is performed to enhance the seal between the device and membrane. In some embodiments, the seal is activated through the microfluidic channels. In some embodiments, an activating solution is applied to the membrane-contacting surface of the device. In some embodiments, an activating solution is applied to the membrane. In some embodiments, the activation takes advantage of the hydrophobic/hydrophilic differences between the PDMS/PVDF surfaces and the aqueous solutions used.

In some embodiments, solutions are added to microfluidic channels through any suitable structure or method. In some embodiments, solutions are injected into channels. In some embodiments, solutions are injected into reservoirs at one end of the channels. In some embodiments, pumps and/or valves are not required for solution addition. In some embodiments, a needle (e.g., 10-40 gauge (e.g., 12 gauge… 16 gauge… 20 gauge… 24 gauge… 27.5 gauge… 30 gauge… 36 gauge… 40 gauge) is used to introduce solutions (e.g., antibody solutions) into the channel. In some embodiments, introduction of solutions from an appropriately sized needle (e.g., 27.5 gauge) into an appropriately sized reservoir produces a “tamponade” effect around the needle that greatly enhances the seal and facilitates filling of the microfluidic channel.

C. Fabrication

In some embodiments, devices of the present invention are fabricated using microfluidics fabrication techniques that are known in the field. A variety of exemplary fabrication methods are described in Fiorini and Chiu, 2005, "Disposable microfluidic devices: fabrication, function, and application" Biotechniques 38:429-46; Beebe et al., 2000, "Microfluidic tectonics: a comprehensive construction platform for microfluidic systems." Proc. Natl. Acad. Sci. USA 97:13488-13493; Rossier et al.,

2002, "Plasma etched polymer microelectrochemical systems" Lab Chip 2:145-150; Becker et al., 2002, "Polymer microfluidic devices" Talanta 56:267-287; Becker et al., 2000, "Polymer microfabrication methods for microfluidic analytical applications" Electrophoresis 21:12-26; U.S. Pat. No. 6,767,706 B2, e.g., Section 6.8 "Microfabrication of a Silicon Device"; Terry et al., 1979, A Gas Chromatography Air Analyzer Fabricated on a Silicon Wafer, IEEE Trans. on Electron Devices, v. ED-26, pp. 1880-1886; Berg et al., 1994, Micro Total Analysis Systems, New York, Kluwer; Webster et al., 1996, Monolithic Capillary Gel Electrophoresis Stage with On-Chip Detector in International Conference On Micro Electromechanical Systems, MEMS 96, pp. 491496; and Mastrangelo et al., 1989, Vacuum-Sealed Silicon Micromachined Incandescent Light Source, in Intl. Electron Devices Meeting, IDEM 89, pp. 503-506. Each of these references is incorporated herein by reference in their entireties for all purposes. In some embodiments, the methods used in fabrication of a microfluidic device may vary with the materials used, and include soft lithography methods, microassembly, bulk micromachining methods, surface micro-machining methods, standard lithographic methods, wet etching, reactive ion etching, plasma etching, stereolithography and laser chemical three-dimensional writing methods, modular assembly methods, replica molding methods, injection molding methods, hot molding methods, laser ablation methods, combinations of methods, and other methods known in the art or developed in the future.

An exemplary method of device fabrication is as follows. A computer aided design (CAD) document is generated that provides the design specifications for the device (e.g., device dimensions, channel dimensions, channel spacing, number of channels per set, number of sets of channels, channel shape, presence of reservoirs, reservoir shape/size, etc.). From the CAD specification, a mask (e.g., glass, polymer, etc.) is produced that provides a tangible design of the device. From the mask, a mold (e.g., silicon wafer mold) is produced by pouring uncured polymer (e.g., polydimethylsiloxane (PDMS)) into the mold, followed by heating of the mask and polymer to cure the polymer (e.g., polymerize, crosslink, etc.), yielding a solid microfluidic device. In some embodiments, a single mask is reused to produce multiple (e.g., 10, 100, 1000, 10,000, or more) molds. In some embodiments, a single mold is reused to produce multiple (e.g., 10, 100, 1000, 10,000, or more) devices.

In some embodiments, microfluidic devices are fabricated from polymethylsixoxane (PDMS) using soft-lithography techniques; although, and suitable polymer(s) and techniques are within the scope of the present invention.

D. Antibodies/Reagents

In some embodiments, solutions for use with the devices described herein are be configured to fill microfluidic channels is an efficient and reproducible manner. To this end, soultions should have appropriate viscosity, hydrophilicity, etc. Experiments were conduted during development of embodiments of the present invention to develop solutions with appropriate characteristic for deployment in the microfluicid channels of the devices described herein. In some embodiments, solutions comprise one or more surfactants, detergents, emulsifiers, solubilizers, etc. to provide acceptable/optimal filling the microfluidic channels in a fast and reproducible manner. In some embodiments, solutions comprise one or more of: ammonium lauryl sulfate, sodium lauryl sulfate (SDS, sodium dodecyl sulfate), sodium laureth sulfate, sodium myreth sulfate, dioctyl sodium sulfosuccinate, perfluorooctanesulfonate (PFOS), perfluorobutanesulfonate, linear alkylbenzene sulfonates (LABs), sodium stearate, sodium lauroyl sarcosinate, perfluorononanoate, perfluorooctanoate, alkyltrimethylammonium salts (e.g., cetyl trimethylammonium bromide), cetylpyridinium chloride (CPC), benzalkonium chloride (BAC), benzethonium chloride (BZT), 5-Bromo-5-nitro-1,3-dioxane, dimethyldioctadecylammonium chloride, cetrimonium bromide, dioctadecyldimethylammonium bromide (DODAB), CHAPS, cocamidopropyl hydroxysultaine, lecithin, polyoxyethylene glycol alkyl ethers, polyoxypropylene glycol alkyl ethers, glucoside alkyl ethers, polyoxyethylene glycol octylphenol ethers (e.g., Triton X-100), Polyoxyethylene glycol alkylphenol ethers, glycerol alkyl esters, polyoxyethylene glycol sorbitan alkyl esters (e.g., Polysorbate (e.g., Polysorbate 20, Polysorbate 40, Polysorbate 60, Polysorbate 80, etc.)), sorbitan alkyl esters, cocamide MEA, cocamide DEA, dodecyldimethylamine oxide, block copolymers of polyethylene glycol and polypropylene glycol, polyethoxylated tallow amine (POEA), etc.

In some embodiments, solutions (e.g., antibody solutions, activating solutions, blocking solutions, washing solutions, etc.) comprise tailored concentrations of Tween (e.g., Tween-20) and bovine serum albumin (BSA). In some embodiments,

useful concentrations of Tween (e.g., Tween-20) and BSA are utilized to provide for efficient flowing of solutions for the length of the microfluidic channels. In some embodiments, useful concentrations of Tween (e.g., Tween-20) and BSA are utilized to provide activation/wetting of the membranes through the microfluidic channels. In some embodiments, solutions comprise between 0.01% and 5% BSA (e.g., 0.01%... 0.02%... 0.05%... 0.1%... 0.2%... 0.5%... 1%... 2%... 5%). In some embodiments, solutions comprise between 0.01% and 5% Tween (e.g., Tween20) (e.g., 0.01%... 0.02%... 0.05%... 0.1%... 0.2%... 0.5%... 1%... 2%... 5%). In some embodiments, solutions comprise about 0.1% BSA and about 0.1% Tween20. In some embodiments, solutions comprise 0.1% BSA and 0.1% Tween20.

In some embodiments, solutions (e.g., blocking, activating, washing, antibody, etc.) comprise appropriate salts, buffers, metals, etc. as would be understood by one of skill in the art.

In some embodiments, antibody binding to target proteins are visualized and/or detected through the use of a detectable/observable moieties and/or labels. Suitable labels and/or moieties are detected spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. In some embodiments, primary or secondary antibodies are linked to a detection moiety. In some embodiments, detection is performed enzymatically using, for example alkaline phosphatase or horseradish peroxidase. Some embodiments, utilize fluorescent detection. Fluorophores contemplated to be useful in the present disclosure include Alexa dyes (e.g., Alexa 350, Alexa 430, etc.), AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy2, Cy3, Cy5, 6-FAM, Fluorescein, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, ROX, TAMRA, TET, Tetramethylrhodamine, Texas Red, etc. One of skill in the art will recognize that these and other detection moieties not mentioned herein can be used with success in various embodiments.

Any antibodies, antibody fragments, or other molecules capable of specifically and stably binding a target, analyte, or antigen find use as the primary and secondary antibodies described herein (e.g., dendrimer, nucleic acid, affinity tag, etc.). Although the embodiments described herein have typically utilized antibodies (e.g., primary antibodies, secondary antibodies) for interrogating the protein contents of a sample,

the present invention is not limited to the use of antibodies. Embodiments should be read broadly as also encompassing other binding molecules.

E. Applications

Embodiments of the present invention find use in any application in which traditional Western blotting has been traditionally used. Moreover, due to removal of the cost, time, reagent, throughput, and multiplexability limitations of Western blotting, devices and method described herein find use in additional research, clinical, diagnostic, and epidemiological application to which traditiona Western blotting is insufficient. For example, rapid, parallel profiling of signaling pathways has been a long sought after goal in biological sciences and clinical medicine. To fully understand the dynamics of these signaling pathways, their protein components must be profiled. These components are typically profiled with protein immunoblotting. Although traditional protein immunoblotting is a powerful technique, it has several limitations including the large sample requirements, high antibody amounts, and limitations in assay throughput. The microfluidic immunoblotting devices of the present invention provide rapid, parallel
profiling of the protein components of signaling pathways. The utility of the present devices and methods have been demonstrated by profiling common inflammatory signaling pathways (e.g., NFκB, JAK-STAT, and MAPK) in cell models and clinical samples. Experiments conducted during development of embodiments of the present invention demonstrate that microfluidic immunoblotting devices can profile endogenous proteins with accuracy similar to that of traditional protein immunoblotting but with 2800-fold less antibody and the ability to multiplex. Additionally, the present microfluidic device interfaces with commonly available immunoblotting equipment and is compatible with existing protein detection methodologies.

EXPERIMENTAL

Over the past decade, inflammation has been recognized as a driver of chronic diseases including cancer and heart disease (Saleh and Trinchieri 2011; Hansson 2005; herein incorporated by reference in their entireties). Although many regulatory steps are involved, protein modifications are defining features of inflammatory responses (Anderson 2010; Anderson 2008; Akira and Takeda 2004; herein

incorporated by reference in their entireties). As appreciation of the importance of protein activation in inflammatory responses has grown, there is an increasing need for more robust techniques to monitor these dynamic responses.

Example 1

Materials and Methods

Microfluidic protein immunoblotting

After gel electrophoresis, the gels were blotted onto a PVDF membrane (Invitrogen) for 1 hour at 30V. The electroblotted PVDF membrane was dried overnight before proceeding to microfluidic immunoblotting. The microfluidic assembly was created by sandwiching the electroblotted PVDF membrane between a glass support and the PDMS microfluidic device. The PDMS microfluidic device was aligned to the ladder to ensure accurate channel placement over the sample lanes. To complete the assembly, two glass slides were placed on top of the PDMS device for further support. The spacing between the two glass slides was approximately 0.5mm and was used as the injection site. The activating and antibody solutions were injected using a 1ml syringe with 27G ½ needles. The PVDF membrane was activated through the microfluidic channels using a 0.1% BSA and 0.1% Tween20 TBS solution. Primary antibodies were injected through the microfluidic channels and incubated for 1 hour. Both the primary and secondary antibody dilutions were prepared in 0.1% BSA and 0.1% Tween20 TBS solutions.

Following the primary antibody incubation period, the microfluidic device was removed and the membrane was washed, blocked for 1 hour in 0.1% BSA and 0.1% Tween20 TBS, and then stained with the secondary antibody for 1 hour prior to chemiluminescent detection with either alkaline phosphatase or horseradish peroxidase.

Gel electrophoresis and protein immunoblotting

For peripheral blood mononuclear cells (PBMCs), healthy individuals who agreed to participate in this institutional review board study were provided with written informed consent. PBMCs were isolated using BD Vacutainer CPT Cell Preparation Tubes with Sodium Citrate. Following isolation, PBMC cell lysates were collected using RIPA buffer as described below. The murine macrophage cell line (RAW264.7) was purchased from American Type Culture Collection (ATCC;

Manassas, VA) and cultured in RPMI medium (Invitrogen; Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (Invitrogen) at a density of 3.0 X 106. After the cells were allowed to adhere for 4 to 6 hours, the media was changed to RPMI media supplemented with 0.5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and incubated overnight (14-18 h). Endotoxin stimulation was initiated by incubating cells in 0.5% FBS medium containing lipopolysaccharide (LPS) from Escherichia coli O55:B5 (Sigma-Aldridge; St Louis, MO) at a concentration of 100 ng/ml. Cells were then harvested and lysed using RIPA buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM Na3VO4, and 10 mM NaF (phosphatase inhibitors) and 0.1% SDS with 10 μl of Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL) added for each 1 ml of buffer. Samples were separated using SDS-PAGE electrophoresis and transferred to a PVDF membrane. PVDF membranes were then probed with rabbit antibodies against active-JNK (phospho-JNK1/2; Promega; Madison, WI), active-MAPK (phospho-ERK; Promega), and ERK-1 (Santa Cruz Biotechnology; Santa Cruz, CA), NF-κB p65 (Cell Signaling Technology; Beverly, MA), STAT3 (sc-483, Santa Cruz Biotechnology), and phospho-STAT3 (Cell Signaling Technology). Horseradish peroxidase or alkaline phosphatase goat anti-rabbit secondary antibodies (Thermo Scientific) were used at a dilution of 1:20000.

Example 2

Results

Design and interface of microfluidic device

Poly(dimethysiloxane) (PDMS) microfluidic devices were designed to interface with existing immunoblotting equipment. The devices, which were approximately 8 cm long and 6 cm wide, consisted of 5 lanes spaced at intervals corresponding to the space between the protein lanes in a traditional Western blot. Within each lane resided 3 microfluidic channels (6 cm long, 150 μM wide, 100 μM deep.).

The devices were fabricated using standard soft-lithography techniques.(Wu et al. 2003) Briefly, a transparency mask was printed using a CAD file that contained the design of the microfluidic device (CAD/Art Services, Inc., Bandon, OR). Soft-lithography was used to fabricate a silicon master mold from the transparency mask. The microfluidic devices were formed by mixing the pre-polymer and curing agent at

a mass ratio of 12:1 (Sylgard 184 Silicone Elastomer Kit, Dow Corning) and then pouring the mixture on to the silicon master. The uncured device was degassed to remove any air bubbles formed during mixing before curing at 60° C for 1 hour.

The approach consists of three stages including gel electrophoresis, transfer to a PVDF membrane, and immunoblotting for target proteins using the microfluidic device (**Figure 1**). The first two stages are similar to traditional immunoblotting that allows our microfluidic device to easily interface with existing equipment.

Following the separation and transfer of samples, the PVDF membrane was dried. The PVDF membrane was then sandwiched between a glass support and the PDMS microfluidic device. The PVDF membrane was activated by injecting blocking solution through the microfluidic and allowing it to sit for 20 minutes. After activating the PVDF membrane, the primary antibody was injected through the microfluidic channels and incubated for 1 hour. After incubation with the primary antibody, the microfluidic device was removed and the PVDF membrane was washed and blocked prior to proceeding to the detection phase. To detect the primary antibody, the entire PVDF membrane was incubated with the appropriate secondary antibody and detected with either alkaline phosphatase or horseradish peroxidase chemiluminescent methodologies.

Comparison of traditional and microfluidic immunoblotting

To directly assess the accuracy of our microfluidic immunoblotting device compared to traditional immunoblotting, peripheral blood monocytes (PBMC) were collected from healthy volunteers and isolated cell lysates as previously described. 5 and 0.5 micrograms of protein were separated using gel electrophoresis followed by transfer to the PVDF membrane. Using a primary antibody to RelA (p65), a member of the NF-κB transcriptional family, the signals between microfluidic and traditional immunoblotting were compared at two different primary antibody dilutions and protein loading amounts (**Figure 2**). For the two loading amounts and antibody dilutions, both approaches demonstrated similar signals for p65. Importantly, with the microfluidic device obtained similar signal intensities using the same antibody dilutions as used with the traditional immunoblotting. For the PVDF membrane size used in these experiments, a traditional immunoblotting assay for 1 protein requires approximately 10 mL of primary antibody solution. Based on the dimensions of a microfluidic channel (0.015, 0.010, and 6.000 cm), each channel requires

approximately 0.0009 mL of primary antibody solution. Using the microfluidic device, to assay a PVDF membrane for 1 protein requires 0.0036 mL of primary antibody solution (4 lanes *0.0009 mL of antibody/lane = 0.0036 mL antibody per protein). Even with conservative assumptions and ignoring its multiplexing capacity, microfluidic immunoblotting translates into approximately 2800-fold reduction in antibody amounts compared with traditional protein immunoblotting.

Monitoring inflammatory pathway activation and protein modifications

Inflammatory pathways are often regulated by posttranslational modifications of signaling proteins (Medzhitov 2008, 2010; herein incorporated by reference in its entirety). Monitoring these protein modifications is essential to understand the dynamics of inflammatory responses. Similar to the NF- κB transcriptional family, the signal transducer and activator of transcription (STAT) family is a key mediator of inflammatory responses (Ho et al. 2009; herein incorporated by reference in its entirety) In response to cytokine and growth factors, STAT family members are phosphorylated by receptor-associated kinases (Hu et al. 2007; O'Sullivan et al. 2007; herein incorporated by reference in their entireties). Protein phosphorylation is typically monitored with protein immunoblotting but also requires parallel monitoring of total protein levels. In the context of STAT3 activation, protein immunoblotting must be used to monitor both phosphorylated and total STAT3 levels from the same sample. These types of experiments are well suited for microfluidic immunoblotting because the microfluidic device enables the simultaneous profiling of phosphorylated and total protein from the same sample lane. To evaluate our microfluidic device in this capacity, RAW264.7 cells, a macrophage cell line, were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for 24 hours. After 24 hours, the cell lysates were collected and probed for phospho-STAT3 and STAT3 using the microfluidic device and traditional immunoblotting. Similar to traditional immunoblotting, microfluidic immunoblotting detected phospho-STAT3 in LPS stimulated macrophages consistent with inflammatory activation of STAT signaling (**Figure 3**).

These results suggest that the microfluidic device can be used to monitor posttranslational modifications associated with inflammatory responses. The ability to monitor multiple proteins from the same sample without the need for membrane stripping greatly improves the assay throughput and strengthens conclusions that can be drawn from these types of immunoblotting experiments.

Monitoring multicomponent, inflammatory pathways

The mitogen activated protein kinase (MAPK) pathway is another pathway that coordinates inflammatory responses.(Chang and Karin 2001; herein incorporated by reference in its entirety) More specifically, MAPKs are serine/threonine-specific protein kinases that transduce extracellular signals and regulate diverse cellular responses including cell proliferation, differentiation, and apoptosis. One of the difficulties in monitoring this pathway is that it has multiple protein components with different patterns of activation. Because of these complexities, monitoring the MAPK pathway with traditional immunoblotting is time and resource intensive.

One of the unique aspects of the present microfluidic immunoblotting approach is the interface between the PDMS microfluidic device and the PVDF membrane. To achieve an efficient interface, the composition and the duration of the activation solution are critical parameters. In order to maintain an efficient seal between the PDMS device and the PVDF membrane, the initial blocking step that is used with traditional immunoblotting was eliminated. However, because of the cross-reactivity of some antibodies and the elimination of the blocking step, there was the potential for more non-specific signals with microfluidic immunoblotting compared with traditional immunoblotting that would be compounded by the complexity of the MAPK signaling pathway. To evaluate the ability of our microfluidic device to monitor the multicomponent, MAPK pathway, RAW264.7 cells were incubated with LPS (100 ng/ml) for 45 minutes and collected cell lysates as described above. The samples were probed for phospho-JNK, phospho-ERK, and total ERK with traditional and microfluidic protein immunoblotting (**Figure 4**). In contrast to traditional immunoblotting that required three different PVDF membranes, the microfluidic approach allowed simultaneous monitoring of these protein components with only two sample lanes from half of a PVDF membrane. By simultaneously probing for total ERK from the same sample, time, resources, and variability were minimized compared with traditional immunoblotting. Importantly, there was equivalent background with both approaches even with the elimination of the blocking step in the microfluidic immunoblotting approach.

The flexibility of the microfluidic immunoblotting technique was evaluated with other commonly used chemiluminescent detection methods. Both horseradish peroxidase and alkaline phosphatase have excellent sensitivity. However, this higher

sensitivity also makes them more susceptible to background noise. A microfluidic immunoblot was run using the same cell lysates and MAPK antibodies as above. Then used a horseradish peroxidase secondary antibody was used to detect the primary antibodies. The efficacy of HRP detection was equivalent to alkaline phosphatase (AP) detection using the microfluidic immunoblotting device (**Figure 4**) further expanding the utility of this approach.

All publications and patents mentioned in the present application and/or listed below are herein incorporated by reference. Various modification, recombination, and variation of the described features and embodiments will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although specific embodiments have been described, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes and embodiments that are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.

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CLAIMS

We claim:

1. A microfluidic immunoblotting device comprising a flat membrane-contacting surface and a plurality of non-connected parallel microfluidic channels, wherein the entire length of said microfluidic channels are open to the membrane-contacting surface.

2. The microfluidic immunoblotting device of claim 1, wherein said microfluidic channels are spatially grouped into two or more channel sets.

3. The microfluidic immunoblotting device of claim 2, wherein each of the two or more channel sets comprises 2-20 microfluidic channels.

4. The microfluidic immunoblotting device of claim 3, wherein the distance separating microfluidic channels with a channel set is less than the distance between channel sets.

5. The microfluidic immunoblotting device of claim 1, wherein each of the microfluidic channels comprises a reservoir at one end of the microfluidic channel.

6. The microfluidic immunoblotting device of claim 1, wherein each of the microfluidic channels is 100-200 μm wide.

7. The microfluidic immunoblotting device of claim 6, wherein each of the microfluidic channels is 140-160 μm wide.

7. The microfluidic immunoblotting device of claim 1, wherein each of the microfluidic channels is 50-150 μm deep.

8. The microfluidic immunoblotting device of claim 1, wherein each of the microfluidic channels is 90-110 μm deep.

9. The microfluidic immunoblotting device of claim 1, wherein said device comprises polydimethylsiloxane (PDMS).

10. The microfluidic immunoblotting device of claim 1, wherein said device consists essentially of polydimethylsiloxane (PDMS).

11. A method of immunoblotting comprising applying antibody solutions to a membrane using a device of one of claims 1-10.

12. A system comprising a device of one of claims 1-10 and a membrane.

13. The system of claim 12, further comprising a activating solution between said device and said membrane.

14. The system of claim 13, wherein said activating solution comprises Tween-20 and BSA.

15. The system of claim 14, wherein said activating solution comprises 0.05-0.15% Tween-20 and 0.05-0.15% BSA.

16. The system of claim 15, wherein said activating solution comprises 0.1% Tween-20 and 0.1% BSA.

17. The system of claim 12, wherein said membrane comprises membrane nitrocellulose or polyvinylidene fluoride (PVDF).

18. The system of claim 12, wherein said membrane comprises peptides, polypeptides, or proteins on or within it.

19. The system of claim 12, further comprising one or more antibody-containing solutions within the microfluidic channels.

20. The system of claim 19, wherein each microfluidic channel of a channel set comprises a different antibody-containing solution.

21. The system of claim 20, wherein each channel set comprises the same combination of antibody-containing solutions.

22. The system of claim 19, wherein each microfluidic channel comprises a different antibody-containing solution.

23. A method of immunoblotting comprising applying antibody solutions to a membrane using system of one of claims 12-22.

24. A method of immunoblotting comprising:

(a) transferring proteins from a sample onto a membrane;

(b) placing a membrane-contacting face of a microfluidic immunoblotting device onto the membrane;

(c) injecting an activating buffer into microfluidic channels of said device;

(d) injecting primary antibody solutions into the microfluidic channels of said device;

(e) detecting binding of antibodies with said antibody solutions to said proteins.

FIG. 1

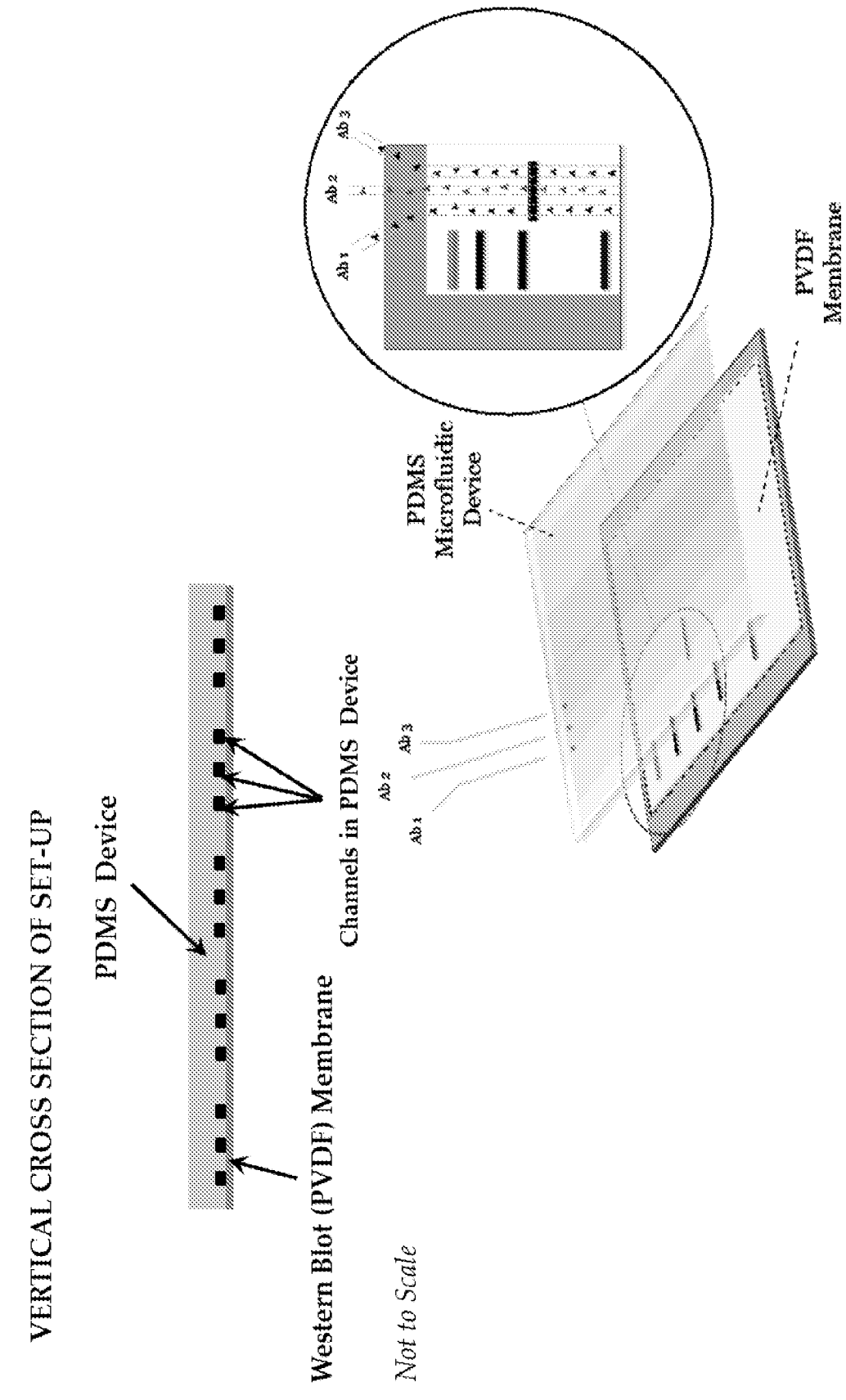

VERTICAL CROSS SECTION OF SET-UP
PDMS Device
Channels in PDMS Device
Western Blot (PVDF) Membrane
Not to Scale
Ab 1
Ab 2
Ab 3
PDMS Microfluidic Device
PVDF Membrane

FIG. 2

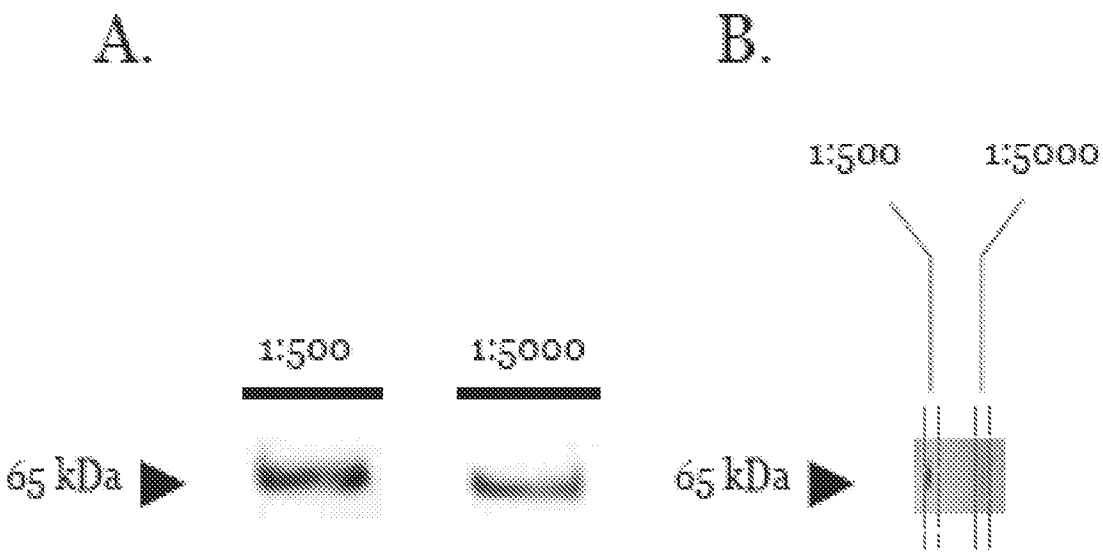


FIG. 3

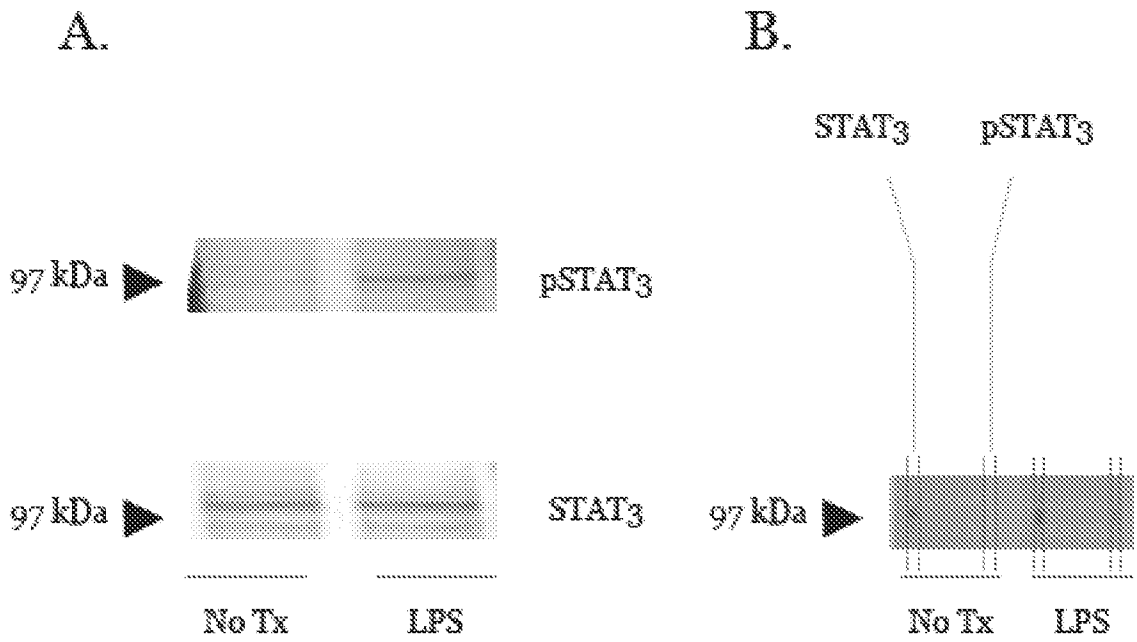
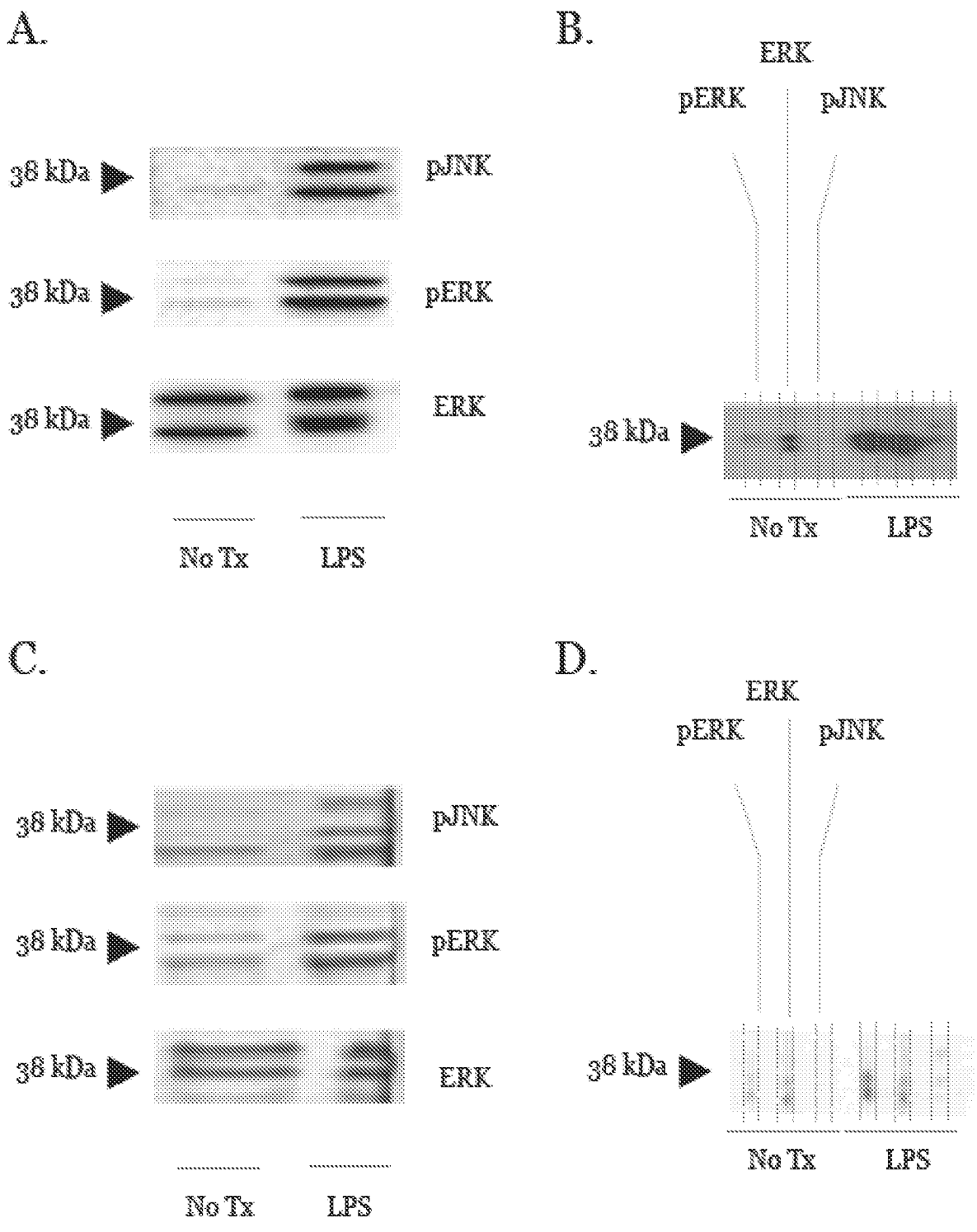

A.
97 kDa
pSTAT3
97 kDa
STAT3
No Tx
LPS
B.
STAT3
pSTAT3
97 kDa
No Tx
LPS

FIG. 4


A.
38 kDa
pJNK
38 kDa
pERK
38 kDa
ERK
No Tx
LPS
B.
ERK
pERK
pJNK
38 kDa
No Tx
LPS
C.
38 kDa
pJNK
38 kDa
pERK
38 kDa
ERK
No Tx
LPS
D.
ERK
pERK
pJNK
38 kDa
No Tx
LPS

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/24684

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/561, 33/49, 33/53 (2014.01)
USPC - 204/453, 455, 604
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)
IPC(8): G01N 33/561, 33/49, 33/53, 33/68, 35/08, 27/447 (2014.01)
USPC: 204/453, 455, 604, 605; 435/6.19, 287.2, 288.5; 436/94, 89, 503, 504

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)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; ProQuest; Microfluidic, immunoblot, western blot, surface, membrane, exterior, outward, top, flat, even, plane, contact, connect, non-connected, parallel, lateral, channels, medium, device, apparatus.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/106693 A2 (KENNEDY, R et al.) September 1, 2011; paragraphs [007], [0024], [0029], [0032], [0034], [0044], [0047], [0057], [0087], [0013], [0041], [0071], [0078]	1-3, 5-6, 7B, 11/1-3, 11/5-6, 11/7B, 12/1-3, 12/5-6, 12/7B, 13/12/1-3, 13/12/5-6, 13/12/7B, 17/12/1-3, 17/12/5-6, 17/12/7B, 18/12/1-3, 18/12/5-6, 18/12/7B, 19/12/1-3, 19/12/5-6, 19/12/7B, 20/19/12/1-3, 20/19/12/5-6, 20/19/12/7B, 21/20/19/12/1-3, 21/20/19/12/5-6, 21/20/19/12/7B, 22/19/12/1-3, 22/19/12/5-6, 22/19/12/7B, 24
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Y		4, 7A, 8-10, 11/4, 11/7A, 11/8-10, 12/4, 12/7A, 12/8-10, 13/12/4, 13/12/7A, 13/12/8-10, 14/13/12/1-6, 14/13/12/7A, ...continued below...

☒ Further documents are listed in the continuation of Box C. ☐

* 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
13 June 2014 (13.06.2014)	11 JUL 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/24684

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
		...continued from above... 14/13/12/7B, 14/13/12/8-10, 15/14/13/12/1-6, 15/14/13/12/7A, 15/14/13/12/7B, 15/14/13/12/8-10, 16/15/14/13/12/1-6, 16/15/14/13/12/7A, 16/15/14/13/12/7B, 16/15/14/13/12/8-10, 17/12/4, 17/12/7A, 17/12/8-10, 18/12/4, 18/12/7A, 18/12/8-10, 19/12/4, 19/12/7A, 19/12/8-10, 20/19/12/4, 20/19/12/7A, 20/19/12/8-10, 21/20/19/12/4, 21/20/19/12/7A, 21/20/19/12/8-10, 22/19/12/4, 22/19/12/7A, 22/19/12/8-10
Y	US 2011/0312804 A1 (SILVERBROOK, K et al.) December 22, 2011; figure 100	4, 11/4, 12/4, 13/12/4, 14/13/12/4, 15/14/13/12/4, 16/15/14/13/12/4, 17/12/4, 18/12/4, 19/12/4, 20/19/12/4, 21/20/19/12/4, 22/19/12/4
Y	US 2011/0177618 A1 (HERR, AE et al.) July 21, 2011; paragraph [0062]	7A, 8, 11/7A, 11/8, 12/7A, 12/8, 13/12/7A, 13/12/8, 14/13/12/7A, 14/13/12/8, 15/14/13/12/7A, 15/14/13/12/8, 16/15/14/13/12/7A, 16/15/14/13/12/8, 17/12/7A, 17/12/8, 18/12/7A, 18/12/8, 19/12/7A, 19/12/8, 20/19/12/7A, 20/19/12/8, 21/20/19/12/7A, 21/20/19/12/8, 22/19/12/7A, 22/19/12/8
Y	US 2012/0211373 A1 (EL-SAYED, M et al.) August 23, 2012; paragraphs [0067], [0069]	9-10, 11/9-10, 12/9-10, 13/12/9-10, 14/13/12/9-10, 15/14/13/12/9-10, 16/15/14/13/12/9-10, 17/12/9-10, 18/12/9-10, 19/12/9-10, 20/19/12/9-10, 21/20/19/12/9-10, 22/19/12/9-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/24684

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0202219 A1 (AGNEW, HD et al.) August 9, 2012; paragraph [0098]	14/13/12/1-6, 14/13/12/7A, 14/13/12/7B, 14/13/12/8-10, 15/14/13/12/1-6, 15/14/13/12/7A, 15/14/13/12/7B, 15/14/13/12/8-10, 16/15/14/13/12/1-6, 16/15/14/13/12/7A, 16/15/14/13/12/7B, 16/15/14/13/12/8-10
A	WO 2007/060580 A1 (WIMBERGER-FRIEDL, R et al.) May 31, 2007; entire document	1-6, 7A, 7B, 8-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/24684

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 23
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.