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(54) Title: COUPLED ORGAN-ON-CHIP SYSTEMS, DEVICES, AND METHODS OF USE FOR MIMICKING A DE-COUPLED ORGAN

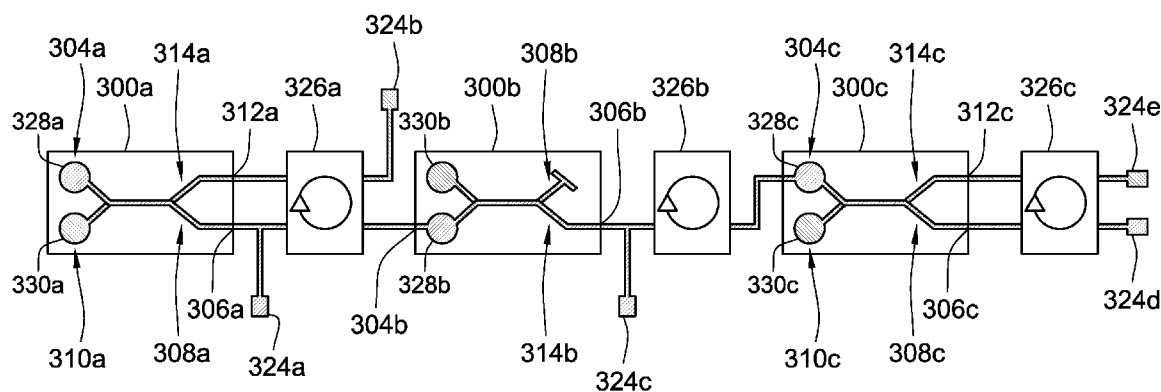


FIG. 3

(57) **Abstract:** A microfluidic system is disclosed that includes an influx microfluidic device, an efflux microfluidic device, and an intermediary microfluidic. The influx microfluidic includes first and second influx microchannels, and an influx membrane separating part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication. The efflux microfluidic includes first and second efflux microchannels, and an efflux membrane separating part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication. The intermediary microfluidic device includes an intermediary microchannel having an inlet and an outlet and being connected to the first influx microchannel outlet and the intermediary microchannel outlet being connected to the first efflux microchannel inlet; a reservoir; and an intermediary membrane separating the intermediary microchannel and the reservoir such that the intermediary microchannel and the reservoir are in communication.



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**COUPLED ORGAN-ON-CHIP SYSTEMS, DEVICES, AND METHODS OF USE
FOR MIMICKING A DE-COUPLED ORGAN**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 62/423,002, filed November 16, 2016, and entitled, “MICROFLUIDIC BRAIN NEUROVASCULAR SYSTEM, DEVICES, AND METHODS OF USE,” which is hereby incorporated by reference herein in its entirety.

GOVERNMENT FUNDING

[0002] This invention was made with government support under Contract No. W911NF-12-2-0036 awarded by DARPA. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates generally to microfluidic devices, and, more particularly, to discrete microfluidic devices coupled together to, as a whole, mimic an organ.

BACKGROUND OF THE INVENTION

[0004] Organ-on-chip devices (or microfluidic devices) allow for the investigation of physiological pathways and responses of organs *in vitro*. However, organ-on-chip devices do not allow for the investigation of pathways and responses that involve multiple discrete portions of organs and rely on the coupling of the discrete portions. The physiological pathways and responses related to brain homeostasis is an example of such a potential drawback for current organ-on-chip.

[0005] The neurovascular unit (NVU) is composed of endothelium-lined microvasculature, closely apposed pericytes within the perivascular niche, and surrounding astroglia and neurons that form the blood-brain barrier (BBB). The NVU regulates the transport of nutrients, metabolites, and drugs between the systemic blood circulation and the central nervous system (CNS), and the deregulation of its integrity results in rapid changes in brain function.

[0006] Brain homeostasis is dependent on neurovascular coupling that involves the dynamic influx of nutrients and efflux of metabolites across the NVU. Neuronal function, however, not only depends on regulated transport of molecules from the blood and to/from the brain parenchyma; it also is controlled through complex interplay between transport

events and metabolic activities that occur within all of the different cell types that comprise the NVU. To understand normal brain function and neuropathological states, it is helpful to analyze proteomic regulation as well as metabolic activity and fluxes throughout the CNS and across the BBB; however, it is extremely difficult to accomplish this using current experimental approaches.

[0007] Animal studies often fail to mimic human responses; typically due to species-specific differences in efflux transporter activities, tight junction functionality, and cell-cell signaling. Whole animals also do not offer possibilities to assess the function of discrete units, such as the metabolic contribution of the BBB compared to the brain parenchyma. In addition, normal human brain biopsies are rarely available and *in vitro* cultures of human brain microvascular endothelial cells (HBMVECs) and perivascular cells can establish only partial BBB function. Further, mature neuronal cells typically cannot be maintained in static cultures.

[0008] Therefore, there is a continuing need to solve the above and related problems.

SUMMARY OF THE INVENTION

[0009] According to one aspect of the present invention, a microfluidic system of discrete microfluidic devices metabolically coupled together to mimic an organ is disclosed. The discrete microfluidic devices of the system include an influx microfluidic device. The influx microfluidic device includes first and second influx microchannels each having an inlet and an outlet, and an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication through the influx membrane. The discrete microfluidic devices of the system also include an efflux microfluidic device. The efflux microfluidic device includes first and second efflux microchannels each having an inlet and an outlet, and an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication through the efflux membrane. The discrete microfluidic devices of the system include an intermediary microfluidic device. The intermediary microfluidic device includes an intermediary microchannel having an inlet and an outlet. The intermediary channel microchannel inlet is fluidically coupled to the first influx microchannel outlet. The intermediary microchannel outlet is fluidically coupled to the first efflux microchannel inlet. The intermediary microfluidic device also includes a chamber reservoir and an intermediary membrane

separating the intermediary microchannel and the chamber such that the intermediary microchannel and the chamber are in fluidic communication.

[0010] The system can include the following additional aspects, each one alone or in combination with any other aspect or combination of aspects. The chamber can contain human neural cells. The human neural cells can include glial cells and neurons. The neurons can be derived from primary hippocampal neural stem cells. The neurons can include one or more glutamatergic neurons, gabaergic neurons, dopaminergic neurons, serotonergic neurons, or a combination thereof. The glial cells can include astrocytes. The human neural cells can line the chamber including a chamber-side of the intermediary membrane. The first influx microchannel and the first efflux microchannel can contain pericytes and astrocytes. The pericytes and the astrocytes can form mono cell layers on the influx membrane facing the first influx microchannel and the efflux membrane facing the first efflux microchannel. As configured, the first influx microchannel and the second efflux microchannel can mimic perivascular channels within the blood-brain-barrier. The second influx microchannel and the second efflux microchannel can contain endothelial cells. The endothelial cells can be human brain microvascular endothelial cells. The endothelial cells can form monolayers on the influx membrane facing the second influx microchannel and the efflux membrane facing the second efflux microchannel. As configured, the second influx microchannel and the second efflux microchannel mimic vascular channels within a blood-brain-barrier. The system can further include one or more sample ports, with each sample port of the one or more sample ports being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device. The influx microfluidic device and the efflux microfluidic device can be blood-brain-barrier microfluidic devices, and the intermediary microfluidic device can be a brain microfluidic device. The system can include one or more pumps, with each pump of the one or more pumps being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device. The first influx microchannel can be about 0.2 millimeter high and the second influx microchannel can be about 1 millimeter high. The first efflux microchannel is about 0.2 millimeter high and the second efflux microchannel is about 1 millimeter high.

[0011] According to a further aspect of the present invention, a blood-brain barrier microfluidic device is disclosed. The device includes a body; a first microchannel formed within the body, the first microchannel having an inlet and an outlet relative to the body and containing pericytes and astrocytes; and a second microchannel formed within the body, the

second microchannel having an inlet and an outlet relative to the body and containing human brain microvascular endothelial cells. The device also includes a membrane configured as an interface between at least part of the first microchannel and the second microchannel to selectively pass material there between. The pericytes and the astrocytes form a monolayer on a first microchannel side of the membrane, and the human brain microvascular endothelial cells line the second microchannel, including a second microchannel side of the membrane.

[0012] The device can include the following additional aspects, each one alone or in combination with any other aspect or combination of aspects. The body can be formed of polydimethylsiloxane. The first microchannel can be about 2 centimeters long, about 1 millimeter wide, and about 1 millimeter tall. The second microchannel can be about 2 centimeters long, about 1 millimeter wide, and about 1 millimeter tall. The membrane can be formed of polyethylene terephthalate. The membrane can be about 10 to 20 micrometers thick and can be porous. The pores can be about 0.4 micrometer in diameter. The membrane can have a pore density of about 4,000,000 pores per square centimeter. The membrane can be coated with an extracellular matrix at least on a first side. The membrane can be coated with an extracellular matrix on both sides.

[0013] According to additional aspects of the present invention, a method of mimicking an organ *in vivo* with a microfluidic system of discrete microfluidic devices metabolically coupled together is disclosed. The includes providing an influx microfluidic device of the discrete microfluidic devices having first and second influx microchannels each having an inlet and an outlet; and an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication through the influx membrane. The method further includes providing an efflux microfluidic device of the discrete microfluidic devices having first and second efflux microchannels each having an inlet and an outlet; and an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication through the efflux membrane. The method further includes providing an intermediary microfluidic device of the discrete microfluidic devices having an intermediary microchannel having an inlet and an outlet and that is intermediary microchannel inlet being fluidically coupled to the first influx microchannel outlet and the intermediary microchannel outlet being fluidically coupled to the first efflux microchannel inlet; an chamber; and an intermediary membrane separating the intermediary microchannel and the chamber such that the intermediary microchannel and the chamber are in fluidic communication. The method further includes flowing a first fluid through the first

influx microchannel, the intermediary microchannel, and the first efflux microchannel, and flowing a second fluid through the second influx microchannel and the second efflux microchannel. The flowing of the first fluid and the second fluid metabolically couples the influx microfluidic device, the intermediary microfluidic device, and the efflux microfluidic device by passing components within the first fluid, the second fluid, the influx microfluidic device, the intermediary microfluidic device, and the efflux microfluidic device into, out of, and throughout the microfluidic system.

[0014] The method can include the following additional aspects, each one alone or in combination with any other aspect or combination of aspects. The first fluid is an artificial cerebral spinal fluid, and the second fluid is an artificial blood medium. The system can include one or more sample ports, with each sample port being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device. With the sample ports, the method can further include sampling the first fluid, the second fluid, or a combination thereof from at least one of the one or more sample ports for investigating metabolic pathways, metabolic responses, or a combination thereof of the components within the system. The influx microfluidic device, the efflux microfluidic device, the intermediary microfluidic device, or a combination thereof can further include one or more reservoirs containing the first fluid or the second fluid. With the reservoirs, the method can further include sampling the first fluid, the second fluid, or a combination thereof from at least one of the one or more reservoirs for investigating metabolic pathways, metabolic responses, or a combination thereof of the components within the system. The system can further include one or more pumps, with each pump being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device. With the pumps, the method can further include pumping the first fluid, the second fluid, or a combination thereof within the system and between the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device by the one or more pumps. The chamber can contain human neural cells. The human neural cells can include glial cells and neurons. The neurons can be derived from primary hippocampal neural stem cells. The neurons can include one or more glutamatergic neurons, gabaergic neurons, dopaminergic neurons, serotonergic neurons, or a combination thereof. The glial cells can include astrocytes. The human neural cells lining the chamber can include a chamber-side of the intermediary membrane. The first influx microchannel and the first efflux microchannel can contain pericytes and astrocytes. The pericytes and the astrocytes can form monolayers on the influx membrane facing the first influx microchannel and the

efflux membrane facing the first efflux microchannel. As configured, the first influx microchannel and the second efflux microchannel mimic perivascular channels of a blood-brain-barrier. The second influx microchannel and the second efflux microchannel can contain endothelial cells. The endothelial cells can be human brain microvascular endothelial cells. The endothelial cells can form monolayers on the influx membrane facing the second influx microchannel and the efflux membrane facing the second efflux microchannel. As configured, the second influx microchannel and the second efflux microchannel mimic vascular channels of a blood-brain-barrier.

[0015] According to yet additional aspects of the present invention, a method of mimicking endothelial-neural coupling in a neurovascular unit is disclosed. The neurovascular unit includes an influx microfluidic device, an efflux microfluidic device, and an intermediary microfluidic device. The influx microfluidic device includes: (i) first and second influx microchannels each having an inlet and an outlet, and (ii) an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication. The efflux microfluidic device includes: (i) first and second efflux microchannels each having an inlet and an outlet, and (ii) an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication. The intermediary microfluidic device includes: (i) an intermediary microchannel having an inlet and an outlet, the intermediary channel inlet being connected to the first influx channel outlet and the intermediary channel outlet being connected to the first efflux channel inlet, (ii) a reservoir, and (iii) an intermediary membrane separating the intermediary microchannel and the reservoir such that the intermediary microchannel and the reservoir are in fluidic communication. The method includes flowing a first fluid into the first influx channel, through the first influx channel to the intermediary channel, through the intermediary channel to the first efflux channel, and through the first efflux channel, the first fluid comprising a cerebral spinal fluid. The method further includes flowing a second fluid through the second influx channel, the second fluid comprising an artificial blood medium. The method further includes flowing a third fluid through the second efflux channel, the third fluid comprising an artificial blood medium. One or more components transfer between the first fluid and the second fluid within through the influx membrane in the influx microchannel device and between the first fluid and the third fluid through the efflux membrane within the efflux microchannel device mimicking the blood-brain barrier.

[0016] Additional aspects of the disclosure will be apparent to those of ordinary skill in the art in view of the detailed description of various embodiments, which is made with reference to the drawings, a brief description of which is provided below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] While the invention is susceptible to various modifications and alternative forms, specific embodiments have been shown by way of example in the drawings and will be described in detail herein. It should be understood, however, that the invention is not intended to be limited to the particular forms disclosed. Rather, the invention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

[0018] FIG. 1 illustrates a perspective view of a microfluidic device, in accord with aspects of the present disclosure.

[0019] FIG. 2 illustrates a cross-sectional view along the line 2-2 of the microfluidic device in FIG. 1, in accord with aspects of the present disclosure.

[0020] FIG. 3 represents a schematic view of a microfluidic system formed of multiple microfluidic devices to mimic the NVU, in accord with aspects of the present disclosure.

[0021] FIG. 4 illustrates a cross-sectional view of the NVU system of FIG. 3, in accord with aspects of the present disclosure.

[0022] FIG. 5A illustrates a principle component analysis (PCA) that was used to assess the expression variations and abundance of all the metabolites, in accord with aspects of the present disclosure.

[0023] FIG. 5B illustrates changes in biochemical pathways associated with significant metabolic changes identified by mass spectrometric analysis, in accord with aspects of the present disclosure.

[0024] FIG. 5C also illustrates changes in biochemical pathways associated with significant metabolic changes identified by mass spectrometric analysis, in accord with aspects of the present disclosure.

[0025] FIG. 6A illustrates graphic depictions of the distribution of the C₁₃-labeled metabolites, pyruvate, lactate, glutamine, and GABA measured by mass spectrometric analysis when C₁₃-labeled glucose was provided as the only glucose source in the linked NVU system of FIG. 3, in accord with aspects of the present disclosure.

[0026] FIG. 6B illustrates isotype distribution of pyruvate, lactate and Gln, and GABA in unlinked brain chips supplied with C₁₃-labeled glucose (Glc), lactate (lac), and pyruvate (pyr) respectively, in accord with aspects of the present disclosure.

DETAILED DESCRIPTION

[0027] While this systems, devices, and methods disclosed herein are susceptible of embodiment in many different forms, there is shown in the drawings and will herein be described in detail preferred embodiments with the understanding that the present disclosure is to be considered as an exemplification of the principles of the invention and is not intended to limit the broad aspect of the invention to the embodiments illustrated. For purposes of the present detailed description the singular includes the plural and vice versa (unless specifically disclaimed); the words “and” and “or” shall be both conjunctive and disjunctive; the word “all” means “any and all”; the word “any” means “any and all”; and the word “including” means “including without limitation.” Where a range of values is disclosed, the respective embodiments include each value between the upper and lower limits of the range.

[0028] The present disclosure provides systems, devices, and methods that compartmentalize portions of an organ into discrete organ-on-chip devices that are metabolically coupled to mimic a human (or any animal) organ *in vivo* but in a de-coupled state. Thus, an organ, that may have various different portions that all work together to achieve the organ functionality, can be de-coupled, such that each portion of the organ can be implemented by a separate organ-on-chip device. To achieve the same functionality as the organ, each separate organ-on-chip device is coupled together into a system but responsible for performing different and discrete functionality that, overall and in totality of all devices, achieves the functionality of the organ.

[0029] Although the NVU is discussed throughout as an example organ or system that can be divided into the discrete organ-on-chip devices to better represent the NVU as a whole, the concepts disclosed herein can apply to any organ or system, such as the liver, kidney, lungs, heart, bladder, skin, etc. For example, the lung can be de-coupled into various airway passages and vascular passages that can each be represented by a different microfluidic device. Each microfluidic device can then be coupled together to form a system that mimics the lung as a whole. Similarly, the liver can be de-coupled into, for example, the arterial part of the liver and the venal part of the liver. The two parts, represented by two separate and discrete microfluidic devices, can then be coupled together by coupling the discrete devices to achieve the overall effect of the liver. As applied to the NVU, as an

example, the compartmentalized but metabolically coupled microfluidic devices permit analysis of influx- and efflux-mediated downstream changes in cell metabolism within microvascular, perivascular, and neuronal sub-compartments of the NVU, and provide the ability to gain greater insight into how metabolic activity within brain microvascular endothelial cells might influence neuronal cell viability and functionality that a single organ-on-chip (or microfluidic device) cannot achieve. The same effect can be achieved for other organs that are de-coupled and represented by separate and discrete microfluidic devices that are metabolically coupled back together within a system.

[0030] FIGS. 1 and 2 illustrate one type of an organ-on-chip (“OOC”), which is also referred to herein as a microfluidic device 100, in accord to aspects of the present concepts. Referring to FIG. 1, the device 100 includes a body 102 that is typically comprised of an upper body segment 102a and a lower body segment 102b. Although the body 102 can be comprised of the upper and lower body segments 102a 102b, alternatively the body 102 can be formed as a single portion. The upper body segment 102a and the lower body segment 102b are preferably made of a polymeric material, such as polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), polycarbonate, cyclic olefin copolymer (COP), cyclic olefin polymer (COC), polyurethane, styrene-butadiene-styrene (SBS) and/or poly(styrene-ethylene/butylene-styrene) (SEBS) block copolymers, etc. The upper body segment 102a includes a fluid inlet 104 and a fluid outlet 106. A first fluid path for a first fluid includes the fluid inlet 104, an upper microchannel 108, and the fluid outlet 106. The lower body segment 102b includes a fluid inlet 110 and a fluid outlet 112. A second fluid path for a second fluid includes the fluid inlet 110, a lower microchannel 114, and the fluid outlet 112.

[0031] Although referred to herein as a fluid inlet 104 and a fluid outlet 106, the fluid inlet 104 and the fluid outlet 106 are both an inlet and an outlet, such as in the case of bi-directional flow of fluid through the microchannel 108. By way of example, fluid can flow into the fluid inlet 104 and then flow out of the fluid outlet 106. Subsequently, the fluid can flow back into the fluid outlet 106 and then flow out of the fluid inlet 104. Thus, the terms inlet and outlet are used for purposes of convenience and should not be interpreted as limiting. Similarly, the fluid inlet 110 and the fluid outlet 112 also can be both fluid inlets and fluid outlets, such as in the case of the bi-directional flow of fluid through the microchannel 114. Depending on the intended configuration of the device 100, one of the microchannel 108 or 114 can be sealed or the device 100 can exclude the fluid inlet and fluid outlet of the microchannel 108 or 114. For example, the device 100 may not include the fluid inlet 110 and the fluid outlet 112. In which case, the microchannel 114 is instead a

microchamber. As discussed below, fluids and matter can still be transported into and out of such a microchamber through a membrane (membrane 216; FIG. 2) that separates the microchamber from the microchannel 108. Thus, as used throughout, a microchamber is a microchannel without the fluid inlet and outlet. As used herein, compartment refers to both microchannels and microchambers.

[0032] FIG. 2 illustrates a cross-sectional view of the device 100 of FIG. 1 along the line 2-2, in accord with of the present disclosure. The device 100 includes a membrane 216 extends between and separates the upper body segment 102a and the lower body segment 102b. The membrane 216 is preferably an inert, polymeric membrane having uniformly or randomly distributed pores. The pores can be various sizes depending on the intended use of the device 100. By way of example, the pores can have a diameter of about 0.2 μm to about 5 μm , and the membrane 216 can have pores at a density of about 4×10^6 pores/ cm^2 . The membrane may, for example, be micro-molded, track-etched, laser-machined, fiber-based, or otherwise produced. The thickness of the membrane 216 is generally in the range of about 0.5 μm to about 100 μm . The membrane 216 can include a bilayer of cells, as illustrated. In particular, a cell layer 220 can be adhered to one side of the membrane 216, and a cell layer 222 can be adhered to an opposing side of the membrane 216. The cell layer 220 can include the same type of cells as the cell layer 222, or a different type of cells than the cell layer 222. Although a single layer of cells is shown for the cell layer 220 and the cell layer 222, the cell layer 220 and the cell layer 222 may include multiple cell layers. Further, while the illustrated embodiment includes a bilayer of cells on the membrane 216, the membrane 216 may include only a single cell layer of cells disposed on one of its sides. Further, while the illustrated embodiment shows cells only on the membrane 216, the cells also can coat the sides of the microchannels 108 and 114.

[0033] The device 100 is configured to simulate a biological function that typically includes cellular communication between the cell layer 220 and the cell layer 222, as would be experienced *in vivo* within organs. Depending on the application, the membrane 216 is designed to have a porosity to permit the migration of components, such as cells, particulates, media, proteins, and/or chemicals, between the upper microchannel 108 and the lower microchannel 114 (or microchamber 114). The working fluids within the microchannels 108 and 114 may be the same fluid or different fluids. As one example, a device 100 simulating a lung may have air as the fluid in one channel and a fluid simulating blood in the other channel. As another example, a device 100 simulating the NVU may have a fluid simulating blood as the fluid in one microchannel and a fluid simulating cerebral spinal fluid in the other

microchannel. When developing the cell layers 220 and 222 on the membrane 216, the working fluids may be one or more tissue-culturing fluids.

[0034] As discussed further below, where multiple discrete devices are coupled together to mimic the NVU, the cell layers 220 and 222 can include endothelial cells, such as HBMVECs, as the cell layer 222, and pericytes, such as brain microvascular pericytes, intermingled with astrocytes as the cell layer 220 for a device mimicking the BBB. The cell layers 220 and 222 also can include a mixed population of human neural cells (~60% glial cells and ~40% neurons, including glutamatergic, gabaergic, dopaminergic, and/or serotonergic neurons) as the cell layer 222 for a device mimicking the brain.

[0035] As discussed above, although the present disclosure primarily discusses the NVU, the disclosure is applicable to any organ in a coupled arrangement of multiple microfluidic devices. By way of example, the cell layers 220 and 222 can include human airway epithelial cells (*e.g.*, bronchiolar, bronchial, or tracheal cells) for mimicking a lung. Specifically, the cell layer 220 within the microchannel 108 can include differentiated (pseudostratified ciliated) epithelial cells. The cell layer 222 within the microchannel 36 can include other lung cell types, such as endothelium, macrophages, fibroblasts, and/or other immune cells. Accordingly, the cells can be cells from one or more parts of the airways or respiratory system, including from the lungs (and the various scales of the airway tubes within the lungs, including the alveoli), the windpipe, and the nasal canal. With the cell layer 220 within the microchannel 108, the microchannel 108 resembles an airway lumen of the human respiratory tract.

[0036] In one embodiment, the active region 218 defined by the microchannels 108 and 114 has a length of less than about 10 cm, a height of less than 1.5 mm, and a width of less than 2000 μm . The device 100 can include an optical window that permits viewing of the fluids, media, particulates, etc. as they move across the cell layer 220 and/or the cell layer 222. Various image-gathering techniques, such as spectroscopy and microscopy, can be used to quantify and evaluate the effects of the fluid flow in the microchannels 108 and 114, as well as cellular behavior and cellular communication through the membrane 216. More details on the device 100 can be found in, for example, U.S. Patent No. 8,647,861, which is incorporated by reference in its entirety. Consistent with the disclosure in U.S. Patent No. 8,647,861, the membrane 216 can be capable of stretching and expanding in one or more directions to simulate the physiological effects of expansion and contraction forces that are commonly experienced by cells.

[0037] Although FIGS. 1 and 2 describe a specific type of device 100, the aspects of the present concepts can be applied to various other types of devices without departing from the spirit and scope of the present disclosure. By way of example, the present concepts disclosed herein can apply to any microfluidic device (or a plurality of microfluidic devices), and particularly to any type of microfluidic cell-culture device that does not necessarily include a membrane (*e.g.*, membrane 216) that separates one or more microchannels. Further, although two microchannels are described, the devices contemplated herein can have one or more than two microchannels. Further, one or more of the microchannels can instead be microchambers, as described above. Thus, the aspects of the present concepts related to microfluidic devices are not restricted to only membrane-based devices or the specific device 100 illustrated in FIGS. 1 and 2.

[0038] FIG. 3 illustrates a microfluidic system 300 formed of multiple microfluidic devices 100 that mimic the NVU, in accord with aspects of the present disclosure. Specifically, to model the NVU while being able to separately study and challenge its constitutive parts, three different microfluidic devices (*e.g.*, device 100) were constructed—two BBB devices and one brain neuronal cell device (or brain device), as described below. The constructed devices were then connected in series to be metabolically coupled to form the system 300. In particular, the system 300 includes a microfluidic device 300a configured as an influx microfluidic device or BBB chip (as discussed further below), a microfluidic device 300b configured as an intermediary microfluidic device or a brain chip (as discussed further below), and a microfluidic device 300c configured as an efflux microfluidic device or efflux BBB chip.

[0039] The devices 300a-300c are connected in series so that one or more outputs of one device (*e.g.*, device 300a) are one or more inputs of another device (*e.g.*, device 300b). By way of example, the fluid outlet 306a of the upper microchannel 308a (or influx microchannel relative to the system 300) of the device 300a can be connected via tubing to a sample port 324a and a pump 326a (*e.g.*, a peristaltic pump, although various other pumps can be used) external to the device 300a before connecting via tubing to the fluid inlet 304b of the upper microchannel 308b or (intermediary microchannel relative to the system 300) of the device 300b. The fluid outlet 312a of the lower microchannel 314a (or second influx microchannel relative to the system 300) of the device 300a can be connected via tubing to the pump 326a followed by a sample port 324b. The upper microchannel 308b of the brain device 300b can be connected through a membrane (*e.g.*, membrane 216) to a bottom chamber 314b (in place of the lower microchannel) within the brain device 300b. Further,

the fluid outlet 306b of the upper microchannel 308b of the brain device 300b can be connected via tubing to a sample port 324c and a pump 326b (e.g., a peristaltic pump) external to the device 300b before connecting to the fluid inlet 304c of the upper microchannel 308c (or efflux microchannel relative to the system 300) of the BBB device 300c. The fluid outlet 312a of the lower microchannel 314a of the BBB device 300a can be connected via tubing to the pump 326a before connecting to a sample port 324b. The fluid outlet 312a of the lower microchannel 314c (or second efflux microchannel relative to the system 300) of the BBB device 300c can be connected via tubing to the pump 326c before connecting to a sample port 324e. In one or more embodiments, the system 300 can include more or less numbers of sample ports 324 and pumps 326. However, the sample ports allow for the removal of fluids and components contained therein. The removal of fluids throughout the system 300, such as after each microfluidic device 300a-300c can for each specific microchannel allows for the investigation that each specific compartment, and the cells and components there, has on the system. Thus, the separate and discrete nature of the microfluidic device 300a-300c within the system 300, along with the separate sampling ports 324, allows for greater control over sampling to provide better understanding of the pathways and responses within the organ.

[0040] In some embodiments, the fluid inlet 304a of the BBB device 300a optionally can include an upper reservoir 328a, such as to aid in reducing shearing forces in the device 300a and/or provide a reservoir for sampling fluid. The fluid inlet 310a of the BBB device 300a also optionally can include a lower reservoir 330a for similar reasons. Similarly, the fluid inlet 304c of the BBB device 300c optionally can include an upper reservoir 328c, and the fluid inlet 310c of the BBB device 300c optionally can include a lower reservoir 330c for similar reasons. Similarly, the fluid inlet 304b of the upper microchannel 308b of the brain device 300b optionally can include an upper reservoir 328b. The bottom chamber 314b of the brain device 300b connected to the upper microchannel 308b through a membrane also optionally can include a reservoir 330b.

[0041] To mimic the NVU within the system 300, the BBB device 300a can be configured as described above to be a PDMS device containing two parallel microchannels. The microchannels can have the dimensions discussed above. In one embodiments, one microchannel (e.g., upper microchannel 308a, 308c) can be 0.2 mm tall, and the other microchannel (e.g., lower microchannel 314a, 314c) can be 1 mm tall. Such a configuration helps to control the flow of fluids through the microfluidic device 300a and 300c and reduce shear stress. In one embodiments, a cell layer (e.g., cell layer 222) of endothelial cells, such

as HBMVECs, can be cultured as a continuous cell monolayer on the lower side of the membrane as well as all three remaining sides of the bottom microchannel 314a. In one embodiment, brain microvascular pericytes intermingled with astrocytes can be cultured on the opposite surface of the same porous membrane (e.g., cell layer 220) to mimic a portion of a hollow brain microvessel. In one embodiment, the membrane (e.g., membrane 216) can be configured to have a porosity of about 0.4 μm diameter pores at a density of about 4×10^6 pores/ cm^2 . In one embodiment, the membrane also can be coated with an extracellular matrix (ECM).

[0042] In one embodiment, the BBB devices 300a and 300c can be formed using molds by soft lithography. For example, the BBB devices 300a and 300c can be made using a degassed 10:1 base:crosslinking mix of PDMS poured onto a mold that is allowed to crosslink at 80 °C for 18 hours. In one embodiment, the fluid inlets 304a, 304c and 310a, 310c and the fluid outlets 306a, 306c and 312a, 312c can be formed to be a diameter of about 1.5 mm via a punch in the molded PDMS and the devices 300a and 300c can be bonded to a 100 μm layer of spincoated PDMS by pre-treating with oxygen plasma at 50 Watts for 20 seconds and then pressing the surfaces together. In one embodiment, a porous polyester membrane (e.g., membrane 216) of polyethylene terephthalate can be sandwiched between the two microchannels 308a, 308c and 314a, 314c during bonding to form the membrane.

[0043] In one embodiment, the devices 300a and 300c can be coated with fibronectin and collagen IV at 200 $\mu\text{g/ml}$ respectively in cell culture grade water for about 4 hours and seeded at a density of 1×10^6 cells/ml in the upper microchannels. After 1 hour of attachment time, HCBMVCs were seeded at a density of 4.8×10^6 cells/ cm^2 in the lower microchannels 314a and 314c, incubated for 30 minutes, and followed by upside down incubation for 1 hour to allow attachment on both the porous membrane and the bottom of the devices microchannels 314a and 314c. The devices 300a and 300c were fed daily by a gravity driven flow of ~ 50 $\mu\text{L/min}$ for 4 minutes until a complete endothelial monolayer was formed. Media was exchanged to BBB media with 250 μM cyclic adenosine monophosphate (cAMP) and 17.4 μM RO20-1724, and the devices 300a and 300c were connected to pressurized media inlets (~ 10 cm H_2O) and a peristaltic pump running at 1 $\mu\text{L/min}$. The design of the BBB devices 300a and 300c resulted in a 25-fold lower shear stress (0.02 dyne/ cm^2 , 7×10^{-4} dyne/ cm^2 in each microchannel) on the astrocytes and pericytes compared to the endothelium while maintaining a symmetric volumetric flow rate (0.06 ml/hr).

[0044] In one embodiment, the brain device 300b can be formed of two parts of polycarbonate (e.g., body segments 102a and 102b) and a porous membrane (e.g., 216). In one embodiment, the membrane can be formed to have a porosity of about 5 μm diameter pores at a density of about 4×10^6 pores/cm². The membrane also can be coated with an extracellular matrix (ECM). In one embodiment, the two polycarbonate parts can be produced by micromachining. The polycarbonate parts can then be sonicated twice for 15 minutes in soap water and once in water to remove residual oils from the machining process. Thereafter, the polycarbonate parts can be dried with condensed air and incubated overnight at 650°C for drying. In one embodiment, the polycarbonate parts can be briefly exposed to dichloromethane (DCM) vapors and dried in a dust free environment at room temperature for 24 hours for polishing. The porous polycarbonate membrane can be sandwiched in between the two polycarbonate parts. The brain device 300b can be cultured to contain human neural cells (~60% glial cells and 40% neurons, including glutamatergic, gabaergic, dopaminergic and serotonergic neurons) differentiated from hippocampus-derived neuronal stem cells in the bottom chamber 314b below the upper microchannel 308b. By restricting active flow entirely to the upper microchannel 308b of the brain device 300b, the flow velocity over the neuronal cells in the bottom chamber 314b approaches zero, causing diffusion-mediated molecular transport to dominate.

[0045] In one embodiment, the brain device 300b can be coupled to a base with manifolds for the device 300b. In one embodiment, the reservoirs 328b and 330b can be fixed on the manifold and can consist of, for example, 5 mL syringes from which the top was cut. The plungers can be cut and a punch used to create a minimal opening to the atmosphere. Connectors were fixed to the manifold. The connector linked to the bottom microchannel 314b can be blocked and the connector linked to the upper microchannel 308a can be connected to the peristaltic pumps 324a and/or 324b. This configuration resulted in the bottom chamber 314b and prevented any shear stress on the neuronal cells while enabling diffusion through the polycarbonate porous membrane.

[0046] With the devices 300a-300c metabolically coupled as described above, the NVU system 300 was further configured by flowing a vascular culture medium through the lower microchannels 314a and 314c, and by flowing a neuronal medium through the perivascular upper microchannels 308a and 308c. As described above, the fluid efflux from the upper microchannel 308a of the BBB device 300a was directly transferred to the upper microchannel 308b of the brain device 300b, allowing fluids and materials (e.g., cells,

metabolites, etc.) to transfer to and from the bottom chamber 314b by diffusion through the membrane in the brain device 300b. The efflux from the upper microchannel 308b of the brain device 300b was then transferred to the upper microchannel 308c of the BBB device 300c. The efflux of both microchannels 308c and 314c of the BBB device 300c were collected for subsequent analysis. Thus, in this model of the NVU, the BBB device 300a modeled influx from the blood to the parenchyma of the brain device 300b, and the BBB device 300c modeled the efflux from the parenchyma to the blood. The larger dimensions of the upper microchannels in the BBB devices 300a and 300c resulted in a 5-fold lower flow velocity and 25-fold lower shear stress on the astrocytes and pericytes in the perivascular upper microchannels 308a and 308c compared to endothelium in the vascular lower microchannels 314a and 314c (0.06 ml/min and 0.02 dyne/cm² versus 7×10^{-4} dyne/cm², respectively) while maintaining a symmetric volumetric flow rate. By restricting active flow entirely to the upper microchannel 308a of the brain device 300b, the flow velocity over the neuronal cells in the bottom chamber 314b approached zero, and chemical transport was dominated by diffusion. Thus, these linked microfluidic devices 300a-300c minimize shear forces on neurons and astrocytes and more closely mimic the microenvironment of the brain parenchyma *in vivo* than conventional static cultures where each medium exchange exposes the cells to non-physiological flow-induced forces.

[0047] FIG. 4 illustrates a cross-sectional view of the NVU system 300 of FIG. 3, in accord with aspects of the present disclosure. Specifically, the left portion represents and illustrates the influx device 300a, the center portion represents and illustrates the brain device 300b, and the right portion represents and illustrates the efflux 300c. As described above, endothelial cells 432 are on all four walls of the lower microchannels 314a and 314c of the devices 300a and 300c, respectively, and a mixture of brain astrocytes 434 and pericytes 436 are in the upper microchannels 308a and 308c of the devices 300a and 300c, respectively. Human brain neuronal cells 438 and astrocytes 434 are in the bottom chamber 314b of the device 300b. The astrocytes 434 extend processes that contact both the basement membrane surrounding endothelium and neuronal cells.

[0048] In one embodiment, an artificial cerebral spinal fluid (aCSF) cell culture medium 438 is flowed into the upper microchannel 308a of the BBB device 300a, and a cell culture medium 440 mimicking blood is flowed separately through the lower microchannel 314a. Fluid and matter (e.g., cells, metabolites, etc.) that pass through the BBB into the perivascular fluid in the upper microchannel 308a in the BBB device 300a are then transferred to the upper microchannel 308b of the brain device 300b where they pass by diffusion into and out

of the bottom chamber 314b, and from there into the upper microchannel 308c of the BBB device 300c, where some components pass back out into the lower microchannel 314c.

[0049] The devices 300a-300c and the system 300 as a whole can be used to investigate various pathways and responses within the NVU. According to one investigation of the biological effects of human NVU coupling in the NVU system 300, the proteomes of each of coupled and uncoupled cell-lined compartments (e.g., microchannels and chambers) were compared. The identification of a set of expressed proteins characteristic for each compartment in a single chip configuration verified their baseline *in vitro* phenotype. When the different NVU compartments were fluidically coupled using the devices 300a-300c, there were significant changes in protein expression profiles within both the BBB devices 300a and 300c and the brain device 300b. In the coupled system 300, the neuronal cells were stimulated by factors secreted by perivascular cells, as well as by factors secreted by the vascular endothelium or transported by these cells. The efflux of the efflux BBB device 300c contained compounds produced or transported by cells lining the entire upstream system of linked BBB and brain devices 300a-300c. Analysis of the proteome maps revealed the dominant biological functions of each compartment of the NVU system 300, and again, linking produced specific changes characteristic for each compartment. For example, vesicular transport proteins were downregulated in the vascular compartments, which could be attributed to the lower degree of transcytotic vesicular transport found in functional BBB endothelium compared to other endothelia. In the upper microchannels, fluidic coupling upregulated biosynthesis of proteins, such as GFAP and A2M, which indicated an increased astrocytic phenotype. The bottom chamber 312b of the brain device 300b was dominated by proteins classified by cytoskeletal function compared to the other NVU compartments, which is consistent with past studies of the human brain proteome and validates the system 300 as a way of mimicking the NVU. Moreover, network analysis of the differential protein expression in the brain device 300b revealed four clusters; two were associated with increases in expression of proteins involved in translation and transcription, one with increases in signal transmission hub molecules, and a fourth cluster contained ECM proteins that were downregulated under these conditions, which could translate to a lower degree of cellular stress and astrocyte reactivity. Importantly, these experiments also revealed that fluidic coupling between these different compartments of the NVU system 300 resulted in upregulation of carbon metabolism-associated proteins, such as ENO1. Taken together, these results demonstrate that fluidic coupling between the different compartments of the NVU system 300 changes the phenotype of each of the individual constituent cell compartments.

The system 300, therefore, extends the proteomic characterization of human neural and neurovascular cells in an *in vivo*-like configuration, providing the ability to study human tissue proteomics and human tissue transcriptomics, as well as human astrocytes and endothelial cells.

[0050] More specifically, the canonical pathways that show significant change in both of their metabolic and proteomic expression due to fluidic coupling of the NVU compartments are as follow: for the lower microchannels 314a, 314c tRNA charging, l-carnitine biosynthesis, glutathione biosynthesis, γ -glutamyl cycle, purine ribonucleosides degradation to ribose-1-phosphate, cysteine biosynthesis iii (mammalia), histamine degradation, dopamine receptor signaling, and l-cysteine degradation; for the upper microchannels 308a, 308 tRNA charging, l-carnitine biosynthesis, glutathione biosynthesis, γ -glutamyl cycle, purine ribonucleosides degradation to ribose-1-phosphate, cysteine biosynthesis iii (mammalia), histamine degradation, dopamine receptor signaling, glycine degradation (creatine biosynthesis), and adenosine nucleotides degradation ii; and tRNA charging, l-carnitine biosynthesis, glutathione biosynthesis, γ -glutamyl cycle, purine ribonucleosides degradation to ribose-1-phosphate, cysteine biosynthesis iii (mammalia), histamine degradation, dopamine receptor signaling, glycine degradation (creatine biosynthesis), and tyrosine biosynthesis iv.

[0051] the system 300 also can be used to gain insight into the metabolic interplay between the BBB and the brain by using an untargeted mass spectroscopy analysis of the differences in small ($66.7 < (m/z) < 1000$) secreted molecules between each compartment. brain metabolites were analyzed in human cerebral spinal fluid samples and a limited set of biopsies, whereas animal studies have provided increased resolution of metabolomics of cells in different brain regions. Such metabolites can include, for example, adenine, d-(-)-fructose, l-(+)-lactic acid, l-aspartic acid, l-threonic acid, indole-3-acetic acid, 4-toluic acid, hippuric acid, pyruvic acid, uric acid, n-acetylneuraminic acid, 4-oxoprolinemethionine, glycine, linoleic acid, citric acid, maltol, n-acetylaspatic acid, 4-acetamidobutanoic acid, vanillin, and hypoxanthine, to name a few examples. However, the compartmentalized *in vitro* NVU system 300 according to the present disclosure enables metabolic analysis of each organ unit individually as well as the interactive effects of the secretome, a capability which is not possible in whole animal or human clinical studies. Principle component analysis produced clustering of the identified compounds, where each specific vascular and perivascular compartment demonstrated higher resemblance to each other in the influx and efflux devices 300a and 300b compared to the brain device 300b. This untargeted analysis of

metabolism enabled identification of compounds and matching against the Kyoto Encyclopedia of Genes and Genomes database to reveal chemicals that exhibit significant alterations (>2 fold change, $p < 0.05$) in each of the devices 300a, 300b, and 300c. Greater numbers of changes in secreted molecules were observed in the devices 300a and 300c compared to the brain device 300b, with the dominant alteration being changes in metabolic pathways. In fact, methamphetamine is known to change metabolic processes in chronic abusers, and acutely in rats, with the citric acid pathway being a major target. Similar alterations were observed in the citric acid pathway. Moreover, the perivascularity was identified as the most susceptible to metabolic change as a result of methamphetamine. This can be observed both by the amount of pathways that change and the verity of the pathways. In addition to changes in the citric acid pathway (TCA), both glycolysis and glutamate drug metabolisms show changes due to methamphetamine.

[0052] Metabolic interplay between and within the vasculature, perivascularity and brain parenchyma is critical for regulation of neuronal function and signaling. In one embodiment, the compartmentalized nature of the NVU system 300 can be used to decipher these processes. Past studies have revealed that the synthesis and signaling of the key neurotransmitters—glutamate and GABA—are coupled to levels of pyruvate and lactate via the TCA cycle and glutamine-glutamate shuttling. However, the localization where each component is produced, as well as where basal energy and amino acid metabolic conversions take place within the NVU, remain poorly understood. What is known is that brain endothelial cells and astrocytes are primarily dependent on glycolysis, whereas neurons typically rely on lactate and pyruvate as primary energy sources.

[0053] The NVU 300 can be used to gain better insight into these processes. By way of example, glucose- C_{13} was used as an energy source to analyze the contribution of each cellular compartment to the flux through the major metabolic pathways associated with glycolysis, the TCA cycle, and the glutamine-glutamate cycle. By supplying glucose- C_{13} only into the lower microchannel 314a of the influx BBB device 300a, while fluidically coupling the device 300a to the brain device 300b and efflux BBB device 300c, as described above, glucose- C_{13} and C_{13} -labelled metabolites generated by the endothelial cells in the influx BBB device 300a can be allowed to penetrate through the system 300. The glucose- C_{13} also was able to cross the endothelium of the influx BBB device 300a, resulting in penetration of this metabolic component throughout the whole system NVU 300.

[0054] In the efflux BBB device 300c, unlabeled glucose was flowed through the lower microchannel 314c to avoid endothelial cell hypoglycemia. Importantly, the bidirectional

glucose fluxes observed in the brain *in vivo* were recapitulated, with a net glucose flux from blood to brain using this system 300 as a result of the flux of glucose- C_{13} between the perivascular channels of the BBB devices 300a and 300c and the brain device 300b and the lower microchannel 314c of the efflux BBB device 300c. Metabolic changes within cells in the compartments of the three linked devices 300a-300c resulted in changes in the amounts and ratios of the carbon isotopes of the various targeted metabolites, including pyruvate, lactate, glutamine, and glutamate. Analysis of the metabolic paths in the NVU system 300 revealed that glycolysis occurred in each organ-mimicking compartment, with metabolites feeding into the TCA cycle resulting in production of both glutamine and glutamate, but also one specific neuronal product—glucose- C_{13} -labeled neurotransmitter gamma-aminobutyric acid (GABA)—which was exclusively secreted by the brain device 300b. The endothelial cell contribution to secretion of the primary glycolytic metabolites, pyruvate and lactate, was also demonstrated by an increase in the respective C_{13} -labelled isotopes in the lower chamber 314b as well as the perivascular upper microchannel 308a. Likewise, the cells in the upper microchannel 308b carried out glycolysis. The evidence of neural glycolysis, seen as an increase in C_{13} -labeled pyruvate and lactate downstream of the brain device 300b, is more likely to be carried out by the astrocyte population in the brain device 300b, given the preferential utilization of pyruvate as a neuronal energy source. Moreover, the consistency in the relative changes in C_{13} -labeled pyruvate and lactate throughout the system is consistent with rapid enzymatic conversion between these two metabolites. This data is consistent with previous reported *in vitro* and *in vivo* observations; however, the fluidically linked system 300 allows one to analyze the contributions of the human cell compartments of the NVU of the brain device 300, individually and collectively, thereby providing information that is not available using conventional *in vitro* or *in vivo* analytical approaches. The increase in concentration of C_{13} -labeled lactate and pyruvate in the vascular channel compared to the perivascular in the NVU system 300 also revealed a previously unknown contribution of endothelial metabolism to the nutrient supply of the neural compartment.

[0055] To understand more deeply the most important source of substrates used for the synthesis of neuronal GABA and glutamate, as well as related glutamine shuttling, results of studies were compared with the coupled NVU system 300 to results from experiments using uncoupled brain devices supplied with C_{13} -labeled form of glucose, lactate, and pyruvate at the same concentrations that they were detected in the sampling point in the perivascular outlet of the influx BBB device 300a in the linked setup. Neuronal synthesis of glutamate and GABA is highly dependent on an external supply of glutamine, typically provided by

astrocytes. Consistent with this observation, glutamine synthesis was observed in all the compartments that contained astrocytes (i.e., perivascularity of the BBB devices 300a and 300c and the brain device 300b), with higher C₁₃-glutamine levels (and isotope ratio) being observed in the brain device 300b where astrocytic glutamine secretion could be stimulated by communication with the neuronal population. Furthermore, in an uncoupled device, C₁₃-glutamine secretion was significantly lower compared to the coupled brain device 300b, demonstrating that metabolites from the vasculature and perivascularity directly influence glutamine production in the neural compartment.

[0056] In addition to being a key neurotransmitter, glutamate is also known to be synthesized by endothelial cells, and glutamate synthesis was observed within endothelial cells in the vascular microchannels 314a and 314c in the studies. Separate cultures of astrocytes or pericytes alone demonstrated net uptake and secretion of glutamate, respectively, whereas in the coupled BBB devices 300a and 300c, a net increase of C₁₃-glutamate over the perivascular microchannel 308a of the BBB device 300a was observed. While the fluidically linked brain device 300b demonstrated a change in distribution of C₁₃-labeled glutamate, but not in the absolute concentration, an uncoupled brain device did not show distribution change. Again, the metabolites released into the perivascular microchannels 308a and 308c of the BBB devices 300a and 300c were found to influence the metabolic processes of the neural compartment in the brain device 300b. Glutamate transferred to the brain device 300b is likely to be taken up by astrocytes and shuttled to the neurons via conversion to glutamine, which in turn will synthesize their own glutamate intracellularly. Synthesis of the neurotransmitter GABA only occurred in the brain device 300b, which is the only compartment in which GABAergic neurons are present in the NVU system 300. GABA synthesis is highly dependent upon availability of primary energy sources (glucose, lactate, and pyruvate), and on glutamate and glutamine supply.

[0057] In one embodiment, the NVU system 300 was configured for drug modeling studies, in particular the psychoactive drug, methamphetamine, which is known to transiently change barrier function by inducing reversible degradation of the BBB after acute administration *in vivo* and *in vitro*, as well as in chronic abusers. Mass spectroscopic analysis of effluents of the devices 300a-300c revealed that when 1.5 mM methamphetamine was administered into the lower microchannel 314b of the influx BBB device 300a, approximately 10% of the methamphetamine penetrated into the upper microchannel 308a and passed into the brain device 300b, resulting in drug levels (~100 μ M) similar to those

observed *in vivo* in the brains of chronic drug abusers. Methamphetamine in this concentration range did not have an effect on the viability of the neural cells in the brain device 300b.

[0058] Small or large fluorescent molecules (CB and BSA-555, respectively), or an antibody that targets a neuronal-specific extracellular domain of the Glutamate receptor 2 (GluR2), were then introduced into the lower microchannel 314a of the influx BBB device 300a to assess changes in BBB function. In the absence of methamphetamine, the GluR2 antibody was fully excluded from the brain device 300b, verifying that the influx device 300a has a tight permeability barrier similar to that exhibited by the BBB *in vivo*.

[0059] Previous animal studies demonstrated that exposure to methamphetamine for 1 day resulted in significant increases in BBB permeability. Similarly, when treated with methamphetamine, increased penetration of CB, BSA, and the GluR2 antibody through the BBB and into upper microchannel 308b of the brain device 300b was observed, which reversed within 1 day after withdrawal of the drug. The concentration of methamphetamine in the efflux of the upper microchannel 308c of the efflux BBB device 300c was well below the IC₅₀ for barrier alteration (1.5 mM), and consequently, there was no significant change in permeability in the efflux BBB device 300c. The increased passage of GluR2 antibody across the barrier induced by methamphetamine was evidenced by greatly enhanced binding and labeling of surface GluR2 receptor epitopes on the surface of live neurons within the brain device 300b. Thus, acute methamphetamine administration resulted in reversible alteration of the human BBB in this *in vitro* NVU system 300 to such an extent that large biological molecules, including whole antibodies, were able to cross to the BBB and retain their physiological binding activities.

[0060] FIGS. 5A-5C show untargeted metabolic analysis of the cellular compartments within the fluidically coupled NVU system 300, in accord with aspects of the present disclosure. Specifically, FIG. 5A shows a principle component analysis (PCA) that was used to assess the expression variations and abundance of all the metabolites (~3000) detected in the different compartments of the coupled NVU system 300 by mass spectrometry. The components are organized within clusters that correspond to the different NVU microfluidic compartments: fluid inlet (Vessel 1 In) and fluid outlet (Vessel 1 Out) of the lower microchannel of the influx BBB device 300a, fluid inlet (Perivasc 1 In) and fluid outlet (Perivasc 1 Out) of the upper microchannel of the same BBB device 300a, fluid inlet (Vessel 2 In) and fluid outlet (Vessel 2 Out) of the lower microchannel of the efflux BBB device 300c, and the fluid inlet (Perivasc 2 In) and fluid outlet (Perivasc 2 Out) of the upper

microchannel of the efflux BBB device 300c (note that Perivasc 1 and Perivasc 1 Out are also the inflow and outflow of the brain device 300b, respectively).

[0061] FIGS. 5B and 5C show changes in biochemical pathways associated with significant metabolic changes identified by mass spectrometric analysis within cells in the lower microchannels 314a, 314c of the influx or efflux BBB device 300a, 300c (Vessel 1 or 2, respectively), upper microchannel of the influx or efflux BBB device 300a, 300c (Perivasc 1 or 2, respectively), or the lower compartment of the brain device 300b within the coupled NVU system 300 under control conditions (FIG. 5B) or exposed to methamphetamine for 24 hrs (FIG. 5C).

[0062] The NVU system 300 demonstrated that the following pathways are expressed in the lower microchannel 314a of the influx microfluidic device 300a before the introduction of methamphetamine: leukotriene metabolism; glycerophospholipid metabolism; xenobiotics metabolism; pyruvate metabolism; β -alanine metabolism; lysine metabolism; prostaglandin formation from arachidonate, valine, leucine and isoleucine degradation; arginine and proline metabolism; glycine, serine, alanine and threonine metabolism; and vitamin h (biotin) metabolism, in addition to purine metabolism; drug metabolism other enzymes; aspartate and asparagine metabolism; ascorbate (vitamin c) and aldarate metabolism; putative anti-inflammatory; metabolites formation from epa; urea cycle/amino group metabolism; and d4 & e4 neuroprostanes formation, with the addition of methamphetamine.

[0063] The NVU system 300 demonstrated that the following pathways are expressed in the upper microchannel 308a of the influx microfluidic device 300a before the introduction of methamphetamine: Arginine and Proline Metabolism; Urea cycle/amino group metabolism; Lysine metabolism; Glycerophospholipid metabolism; Vitamin B3 (nicotinate and nicotinamide) metabolism; Drug metabolism other enzymes; Aspartate and asparagine metabolism; Glycine, serine, alanine and threonine metabolism; Alanine and Aspartate Metabolism; Nitrogen metabolism; Fructose and mannose metabolism; β -Alanine metabolism; C21steroid hormone biosynthesis and metabolism; and Glycosphingolipid metabolism; with the addition of Glutamate metabolism; Sialic acid metabolism; Hexose phosphorylation; TCA cycle; Putative anti-inflammatory metabolites formation from EPA; Glycolysis and Gluconeogenesis; Glutathione Metabolism; Histidine metabolism; Glycolysis; and Gluconeogenesis, in addition to purine metabolism; drug metabolism other enzymes; aspartate and asparagine metabolism; ascorbate (vitamin c) and aldarate metabolism; putative anti-inflammatory; metabolites formation from epa; urea cycle/amino group metabolism; and d4 & e4 neuroprostanes formation, with the addition of methamphetamine.

[0064] The NVU system 300 demonstrated that the following pathways are expressed in the microfluidic device 300b prior to the introduction of methamphetamine: lysine metabolism; glycerophospholipid metabolism; pyruvate metabolism; xenobiotics metabolism; leukotriene metabolism; valine, leucine and isoleucine degradation; glycine, serine, and alanine and threonine metabolism, followed by lysine metabolism; bile acid biosynthesis; squalene and cholesterol biosynthesis; arginine and proline metabolism; drug metabolism cytochrome p450; and urea cycle/amino group metabolism with the addition of methamphetamine.

[0065] The NVU system 300 demonstrated that the following pathways are expressed in the upper microchannel 308c of the efflux microfluidic device 300c before the introduction of methamphetamine: histidine metabolism; methionine and cysteine metabolism; glycine, serine, alanine and threonine metabolism; purine metabolism; pyruvate metabolism; nglycan biosynthesis; urea cycle/amino group metabolism; lysine metabolism; caffeine metabolism; phosphatidylinositol phosphate metabolism; glycosphingolipid biosynthesis globoseries; pyrimidine metabolism; glycerophospholipid metabolism; propanoate metabolism; selenoamino acid metabolism, in addition to purine metabolism; drug metabolism other enzymes; aspartate and asparagine metabolism; ascorbate (vitamin c) and aldarate metabolism; putative anti-inflammatory; metabolites formation from epa; urea cycle/amino group metabolism; and d4 & e4 neuroprostanes formation, and including Arginine and Proline Metabolism with the addition of methamphetamine.

[0066] The NVU system 300 demonstrated that the following pathways are expressed in the lower microchannel 314c of the efflux microfluidic device 300c before the introduction of methamphetamine: drug metabolism other enzymes; d4&e4neuroprostanes formation; butanoate metabolism; propanoate metabolism; vitamin b5 coa biosynthesis from pantothenate; coa catabolism; vitamin h (biotin) metabolism; leukotriene metabolism; pyruvate metabolism; vitamin b2 (riboflavin) metabolism; bile acid biosynthesis; lysine metabolism; and alkaloid biosynthesis ii, and drug metabolism other enzymes; d4&e4neuroprostanes formation; butanoate metabolism; propanoate metabolism; vitamin b5 coa biosynthesis from pantothenate; coa catabolism; vitamin h (biotin) metabolism; leukotriene metabolism; pyruvate metabolism; vitamin b2 (riboflavin) metabolism; ubiquinone biosynthesis; drug metabolism cytochrome p450; and urea cycle/amino group metabolism after the introduction of methamphetamine.

[0067] FIGS. 6A and 6B show a linked cell metabolism within the fluidically coupled NVU system 300, in accord with aspects of the present disclosure. In particular, FIG. 6A

shows graphic depictions of the distribution of the C₁₃-labeled metabolites, pyruvate, lactate, Glutamine, and GABA measured by mass spectrometric analysis when C₁₃-labeled glucose was provided as the only glucose source in the linked NVU system 300. The number of carbons in respective compounds is illustrated in the ball-and-stick molecular illustration, where the balls show number of carbons incorporated in each glycolysis-TCA cycle turnover. The pie charts, showing percentage of C₁₃-labelled carbons for each analyte, are positioned above or below the sampling points along the coupled NVU system 300 diagrammed at the center. FIG. 6B shows isotype distribution of pyruvate, lactate and Gln, and GABA in unlinked brain chips supplied with C₁₃-labeled glucose (Glc), lactate (lac), and pyruvate (pyr) respectively.

[0068] To provide a deeper insight into the functional effects of fluidic coupling within the NVU system 300, vesicular transport processes were down-regulated in the vascular endothelium, which could be attributed to higher physiological resemblance to human brain microvessels since a lower degree of transcytotic vesicular transport is found in functional BBB endothelium compared to other endothelia. In the upper microchannels 314a, 314c of the BBB devices 300a and 300c, fluidic coupling up-regulated the processes of amino acid synthesis as well as biosynthesis of proteins. In comparison, the brain device 300b was more dominated by cytoskeletal function proteins in both coupled and un-coupled configuration, which is consistent with neuronal expression patterns in the human brain proteome. The fluidic coupling in the NVU system 300 also resulted in up-regulation of carbon metabolism-associated proteins, which was shown to decrease DNA strand breakage, oxidative stress, and apoptosis.

[0069] This discrete, coupled organ-on-chip approach described here permits dissection of how living cells within distinct tissue compartments of a human organ interact with each other, communicate metabolically, and respond to drugs, which is not possible with existing culture systems, animal models, or even human studies. The use of modular compartmentalized organ-on-chip models provides a way to measure drug penetrance and localized drug target and off-target effects in more direct way than conventional methods. The NVU system 300 also enables the ability to discover that the efficiency of neuronal synthesis and secretion of the neurotransmitters, glutamate and GABA, increased as a result of coupled metabolic reactions involving direct neuronal utilization of vascular endothelial metabolites. These results were previously not possible with conventional systems.

[0070] Each of the embodiments disclosed above and obvious variations thereof are contemplated as falling within the spirit and scope of the claimed invention, which is set forth

in the following claims. Moreover, the present concepts expressly include any and all combinations and sub-combinations of the preceding elements and aspects.

CLAIMS

What is claimed is:

1. A microfluidic system of discrete microfluidic devices metabolically coupled together to mimic an organ, the system comprising:
 - an influx microfluidic device of the discrete microfluidic devices comprising:
 - first and second influx microchannels each having an inlet and an outlet; and
 - an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication through the influx membrane;
 - an efflux microfluidic device of the discrete microfluidic devices comprising:
 - first and second efflux microchannels each having an inlet and an outlet; and
 - an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication through the efflux membrane; and
 - an intermediary microfluidic device of the discrete microfluidic devices comprising:
 - an intermediary microchannel having an inlet and an outlet, the intermediary microchannel inlet being fluidically coupled to the first influx microchannel outlet and the intermediary microchannel outlet being fluidically coupled to the first efflux microchannel inlet;
 - an chamber; and
 - an intermediary membrane separating the intermediary microchannel and the chamber such that the intermediary microchannel and the chamber are in fluidic communication.
2. The system of claim 1, wherein the chamber contains human neural cells.
3. The system of claim 2, wherein the human neural cells include glial cells and neurons.
4. The system of claim 3, wherein the neurons are derived from primary hippocampal neural stem cells.

5. The system of claim 3, wherein the neurons include one or more glutamatergic neurons, gabaergic neurons, dopaminergic neurons, serotonergic neurons, or a combination thereof.

6. The system of claim 3, wherein the glial cells include astrocytes.

7. The system of claim 2, wherein the human neural cells line the chamber including a chamber-side of the intermediary membrane.

8. The system of claim 1, wherein the first influx microchannel and the first efflux microchannel contain pericytes and astrocytes.

9. The system of claim 8, wherein the pericytes and the astrocytes form monolayers on the influx membrane facing the first influx microchannel and the efflux membrane facing the first efflux microchannel, and the first influx microchannel and the second efflux microchannel mimic perivascular channels within a blood-brain-barrier.

10. The system of claim 1, wherein the second influx microchannel and the second efflux microchannel contain endothelial cells.

11. The system of claim 10, wherein the endothelial cells are human brain microvascular endothelial cells.

12. The system of claim 10, wherein the endothelial cells form monolayers on the influx membrane facing the second influx microchannel and the efflux membrane facing the second efflux microchannel, and the second influx microchannel and the second efflux microchannel mimic vascular channels within a blood-brain-barrier.

13. The system of claim 1, further comprising one or more sample ports, each sample port of the one or more sample ports being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device.

14. The system of claim 1, further comprising one or more pumps, each pump of the one or more pumps being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device.

15. The system of claim 1, wherein the first influx microchannel is about 0.2 millimeter high and the second influx microchannel is about 1 millimeter high, and the first efflux microchannel is about 0.2 millimeter high and the second efflux microchannel is about 1 millimeter high.

16. A blood-brain-barrier microfluidic device comprising:

a body;

a first microchannel formed within the body, the first microchannel having an inlet and an outlet relative to the body and containing pericytes and astrocytes;

a second microchannel formed within the body, the second microchannel having an inlet and an outlet relative to the body and containing human brain microvascular endothelial cells; and

a membrane configured as an interface between at least part of the first microchannel and the second microchannel to selectively pass material there between,

wherein the pericytes and the astrocytes form a monolayer on a first microchannel side of the membrane, and the human brain microvascular endothelial cells line the second microchannel, including a second microchannel side of the membrane.

17. The device of claim 16, wherein the body is formed of polydimethylsiloxane.

18. The device of claim 16, wherein the first microchannel is about 2 centimeters long, about 1 millimeter wide, and about 1.2 millimeters tall.

19. The device of claim 16, wherein the second microchannel is about 2 centimeters long, about 1 millimeter wide, and about 1.2 millimeters tall.

20. The device of claim 16, wherein the membrane is formed of polyethylene terephthalate.

21. The device of claim 16, wherein the membrane is about 0.4 to 20 micrometers thick.

22. The device of claim 16, wherein the membrane is porous.

23. The device of claim 22, wherein the pores are about 0.4 micrometer in diameter.

24. The device of claim 23, wherein the membrane has a pore density of about 4,000,000 pores per square centimeter.

25. The device of claim 16, wherein the membrane is coated with an extracellular matrix at least on a first side.

26. The device of claim 16, wherein the membrane is coated with an extracellular matrix on both sides.

27. A method of mimicking an organ *in vivo* with a microfluidic system of discrete microfluidic devices metabolically coupled together, the method comprising:

providing an influx microfluidic device of the discrete microfluidic devices having:

first and second influx microchannels each having an inlet and an outlet; and

an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication through the influx membrane;

providing an efflux microfluidic device of the discrete microfluidic devices having:

first and second efflux microchannels each having an inlet and an outlet; and

an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication through the efflux membrane;

providing an intermediary microfluidic device of the discrete microfluidic devices having:

an intermediary microchannel having an inlet and an outlet, the intermediary microchannel inlet being fluidically coupled to the first influx microchannel outlet and the intermediary microchannel outlet being fluidically coupled to the first efflux microchannel inlet;

an chamber; and

an intermediary membrane separating the intermediary microchannel and the chamber such that the intermediary microchannel and the chamber are in fluidic communication;

flowing a first fluid through the first influx microchannel, the intermediary microchannel, and the first efflux microchannel; and

flowing a second fluid through the second influx microchannel and the second efflux microchannel,

wherein the flowing of the first fluid and the second fluid metabolically couples the influx microfluidic device, the intermediary microfluidic device, and the efflux microfluidic device by passing components within the first fluid, the second fluid, the influx microfluidic device, the intermediary microfluidic device, and the efflux microfluidic device into, out of, and throughout the microfluidic system.

28. The method of claim 27, wherein the first fluid is an artificial cerebral spinal fluid.

29. The method of claim 27, wherein the second fluid is an artificial blood medium.

30. The method of claim 27, wherein the system includes one or more sample ports, each sample port of the one or more sample ports being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device, the method further comprising:

sampling the first fluid, the second fluid, or a combination thereof from at least one of the one or more sample ports for investigating metabolic pathways, metabolic responses, or a combination thereof of the components within the system.

31. The method of claim 27, wherein the influx microfluidic device, the efflux microfluidic device, the intermediary microfluidic device, or a combination thereof include one or more reservoirs containing the first fluid or the second fluid, the method further comprising:

sampling the first fluid, the second fluid, or a combination thereof from at least one of the one or more reservoirs for investigating metabolic pathways, metabolic responses, or a combination thereof of the components within the system.

32. The method of claim 27, wherein the system includes one or more pumps, each pump of the one or more pumps being fluidically coupled between two of the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device, the method further comprising:

pumping the first fluid, the second fluid, or a combination thereof within the system and between the influx microfluidic device, the efflux microfluidic device, or the intermediary microfluidic device by the one or more pumps.

33. The method of claim 27, wherein the chamber contains human neural cells.

34. The method of claim 33, wherein the human neural cells include glial cells and neurons.

35. The method of claim 34, wherein the neurons are derived from primary hippocampal neural stem cells.

36. The method of claim 34, wherein the neurons include one or more glutamatergic neurons, gabaergic neurons, dopaminergic neurons, serotonergic neurons, or a combination thereof.

37. The method of claim 34, wherein the glial cells include astrocytes.

38. The method of claim 33, wherein the human neural cells lining the chamber include a chamber-side of the intermediary membrane.

39. The method of claim 27, wherein the first influx microchannel and the first efflux microchannel contain pericytes and astrocytes.

40. The method of claim 39, wherein the pericytes and the astrocytes form monolayers on the influx membrane facing the first influx microchannel and the efflux membrane facing the first efflux microchannel, and the first influx microchannel and the second efflux microchannel mimic perivascular channels of a blood-brain-barrier.

41. The method of claim 27, wherein the second influx microchannel and the second efflux microchannel contain endothelial cells.

42. The method of claim 41, wherein the endothelial cells are human brain microvascular endothelial cells.

43. The method of claim 41, wherein the endothelial cells form monolayers on the influx membrane facing the second influx microchannel and the efflux membrane facing the second efflux microchannel, and the second influx microchannel and the second efflux microchannel mimic vascular channels of a blood-brain-barrier.

44. A method of mimicking endothelial-neural coupling in a neurovascular unit, the neurovascular unit comprising: an influx microfluidic device, an efflux microfluidic device, and an intermediary microfluidic device, the influx microfluidic device comprising (i) first and second influx microchannels each having an inlet and an outlet, and (ii) an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication, the efflux microfluidic device comprising (i) first and second efflux microchannels each having an inlet and an outlet, and (ii) an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication, the intermediary microfluidic device comprising (i) an intermediary microchannel having an inlet and an outlet, the intermediary microchannel inlet being connected to the first influx microchannel outlet and the intermediary microchannel outlet being connected to the first efflux microchannel inlet, (ii) a chamber, and (iii) an intermediary membrane separating the intermediary microchannel and the chamber such that the intermediary microchannel and the chamber are in fluidic communication, the method comprising:

flowing a first fluid into the first influx microchannel, through the first influx microchannel to the intermediary microchannel, through the intermediary microchannel to the first efflux microchannel, and through the first efflux microchannel, the first fluid comprising a cerebral spinal fluid;

flowing a second fluid through the second influx microchannel, the second fluid comprising an artificial blood medium; and

flowing a third fluid through the second efflux microchannel, the third fluid comprising an artificial blood medium,

wherein one or more components transfer between the first fluid and the second fluid within through the influx membrane in the influx microchannel device and between the first fluid and the third fluid through the efflux membrane within the efflux microchannel device mimicking a blood-brain barrier.

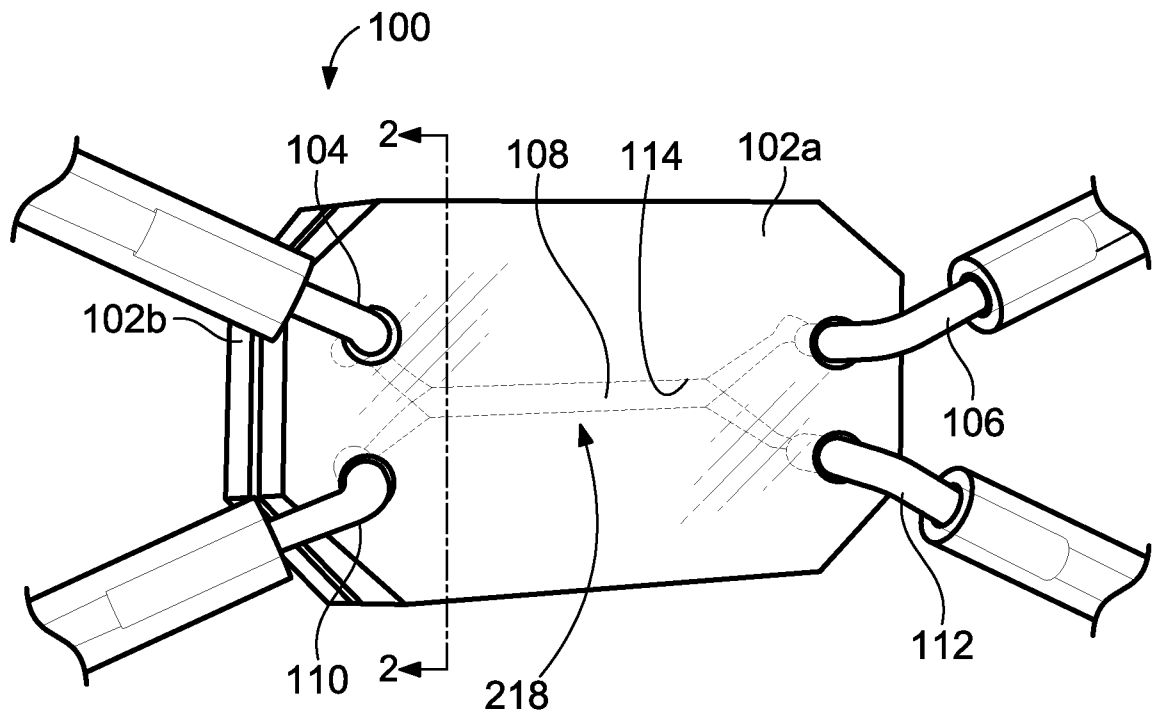


FIG. 1

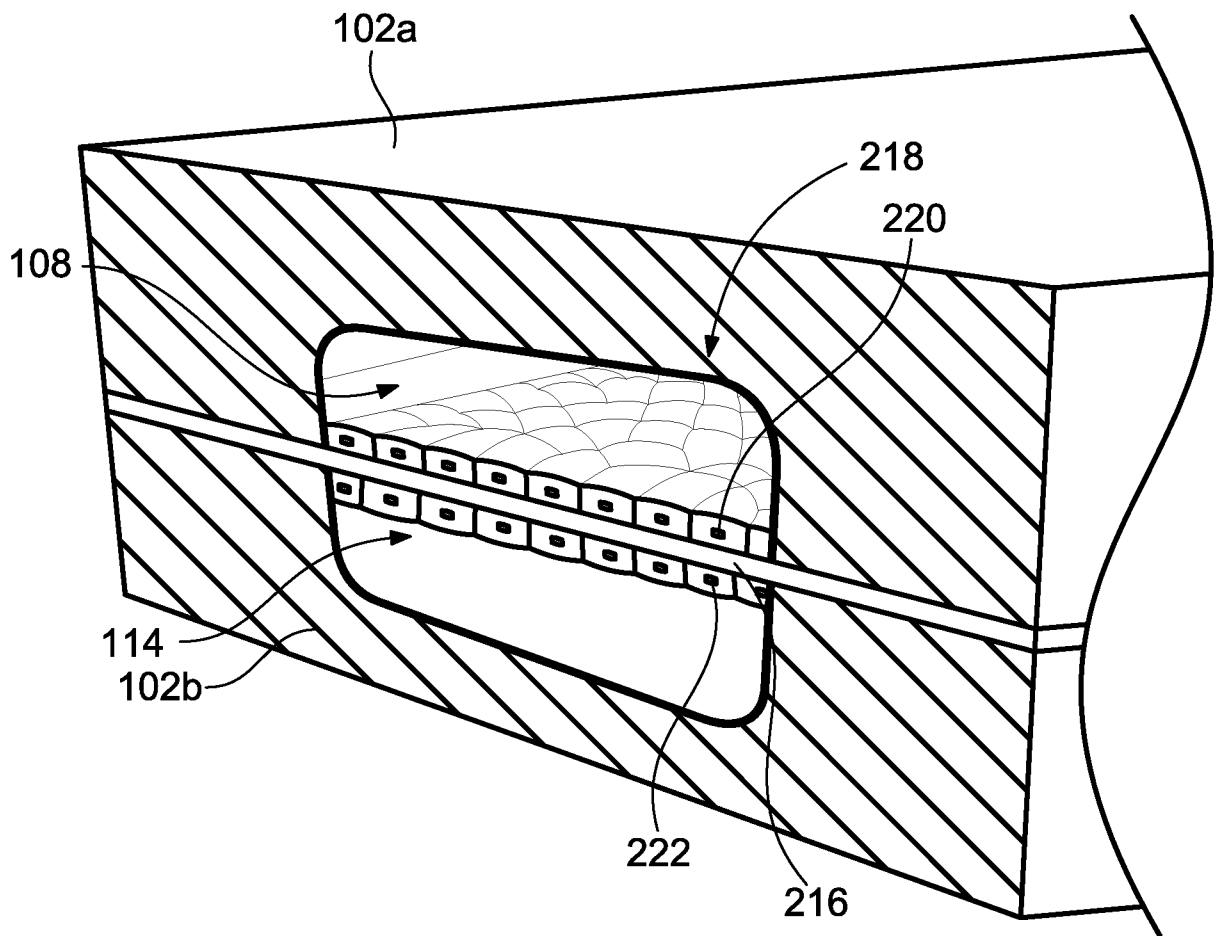


FIG. 2

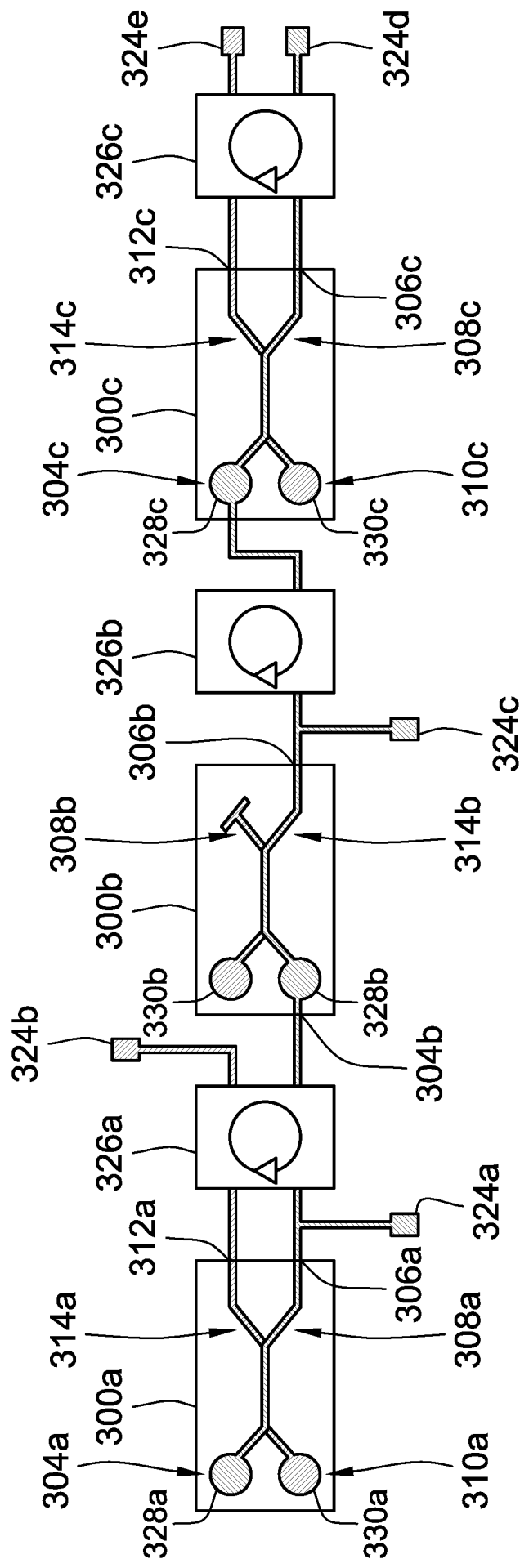


FIG. 3

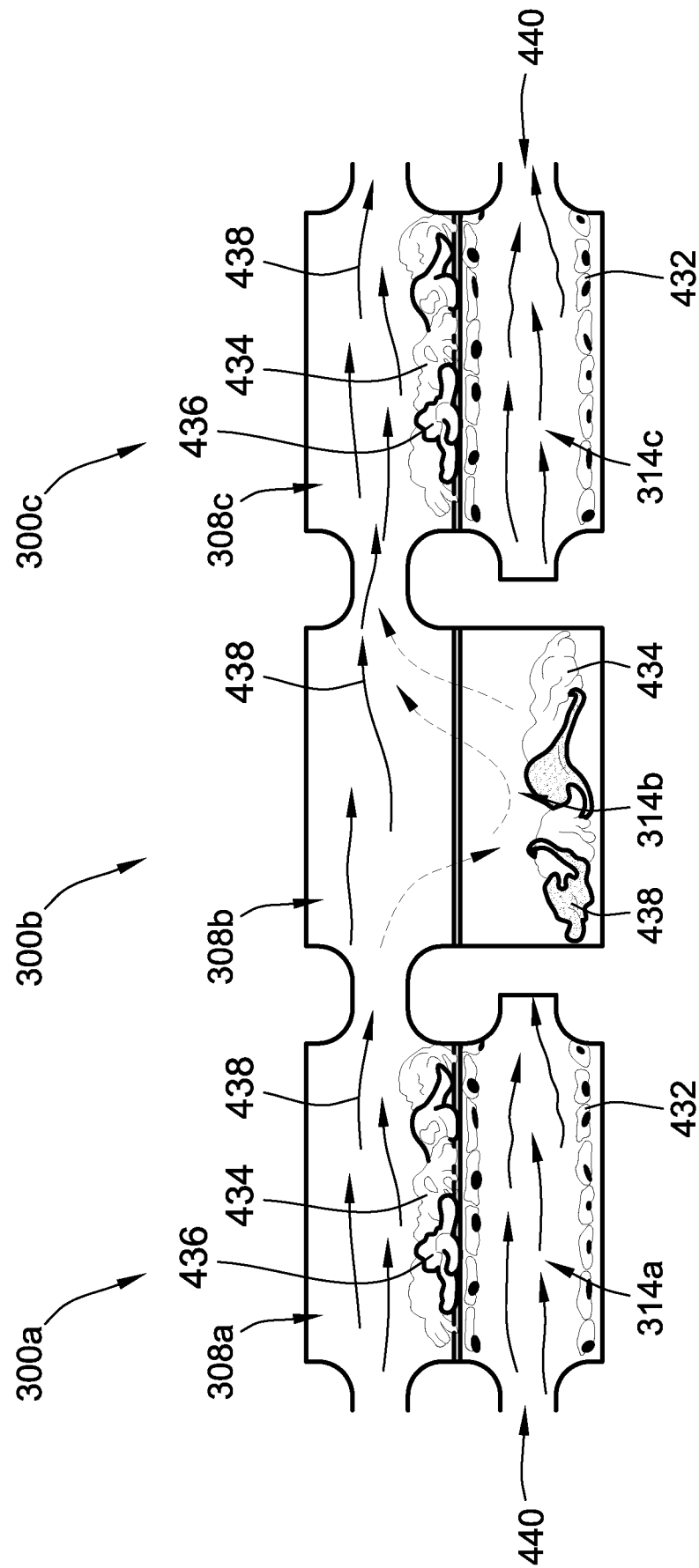


FIG. 4

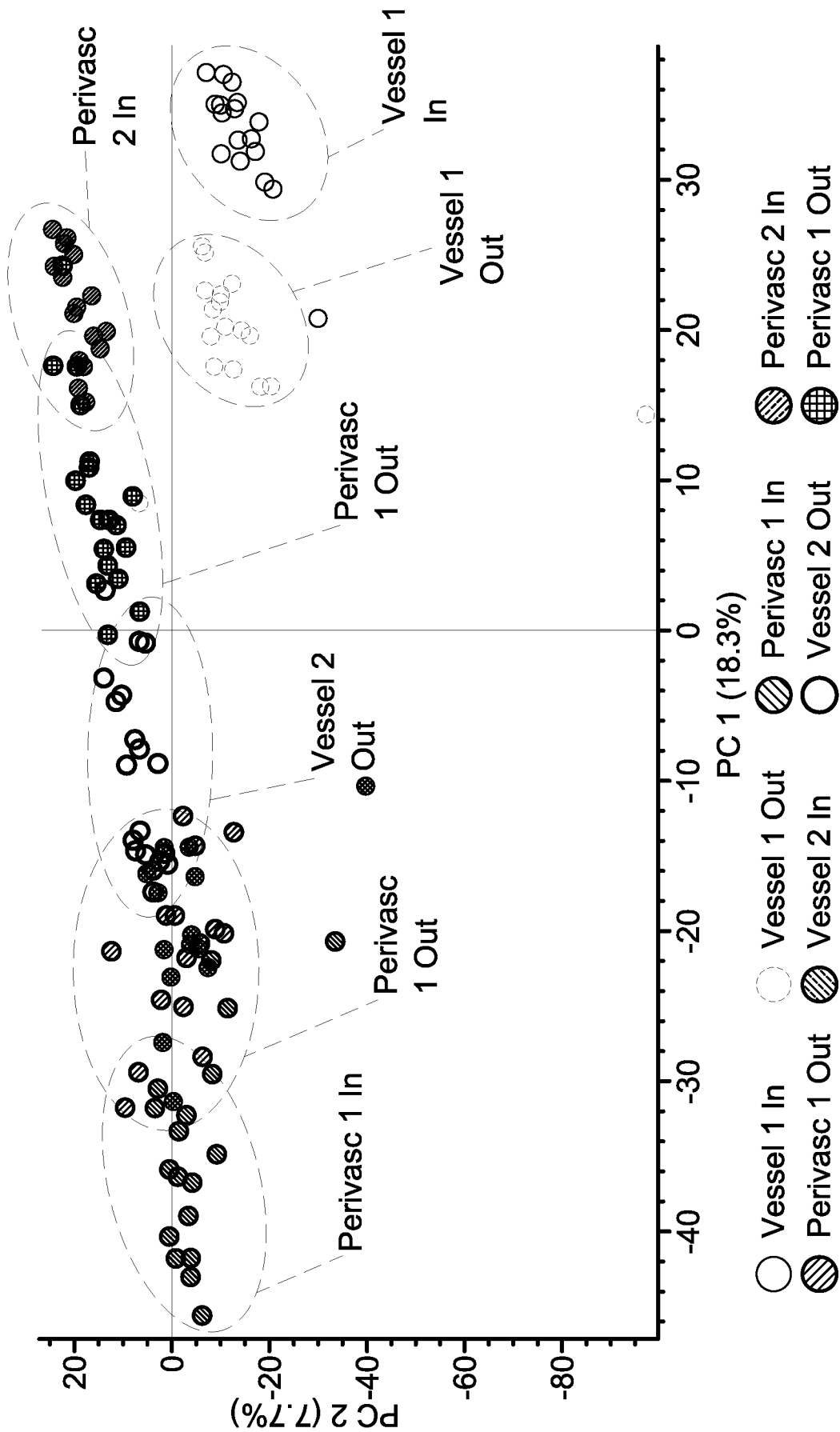
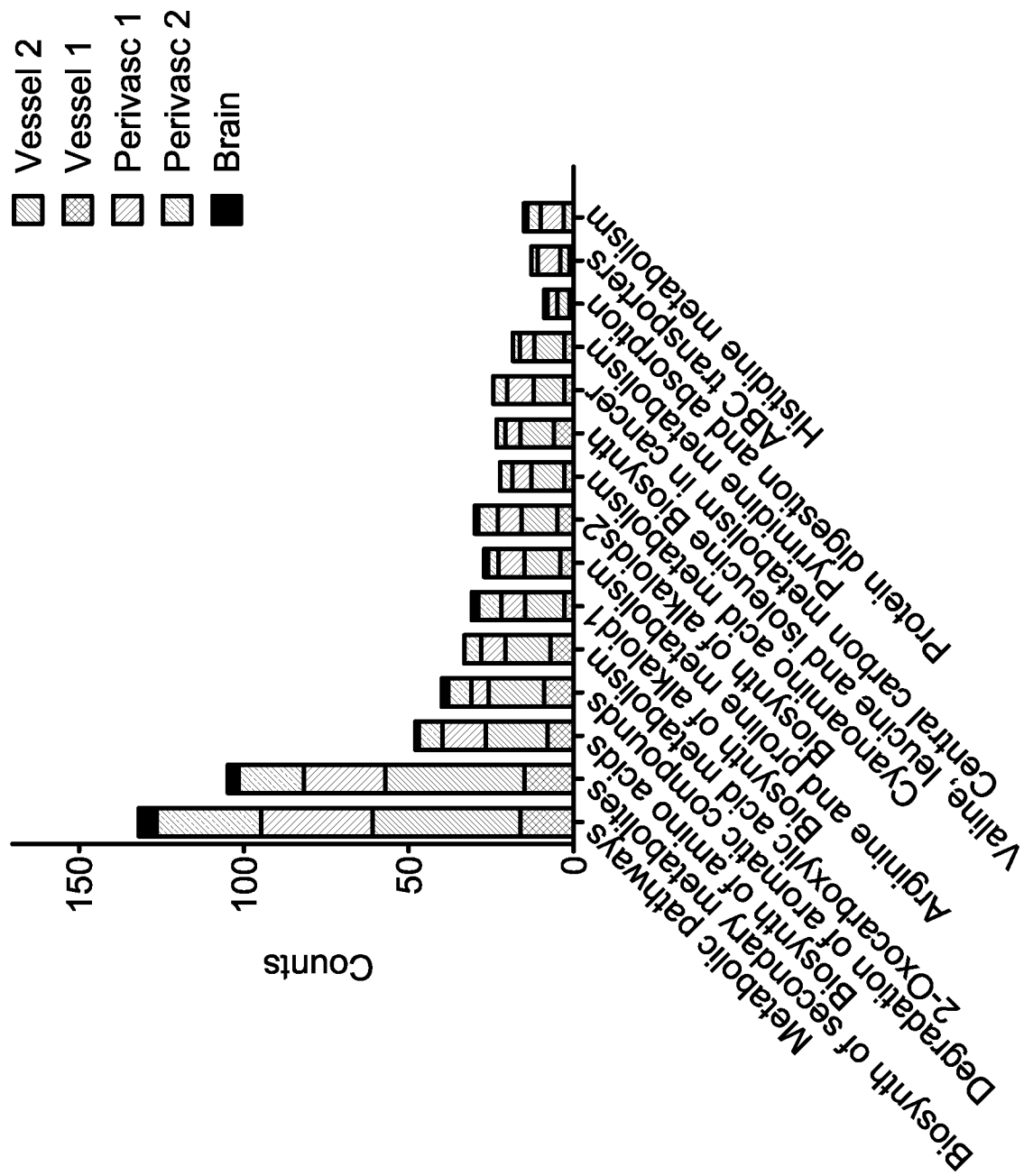


FIG. 5A



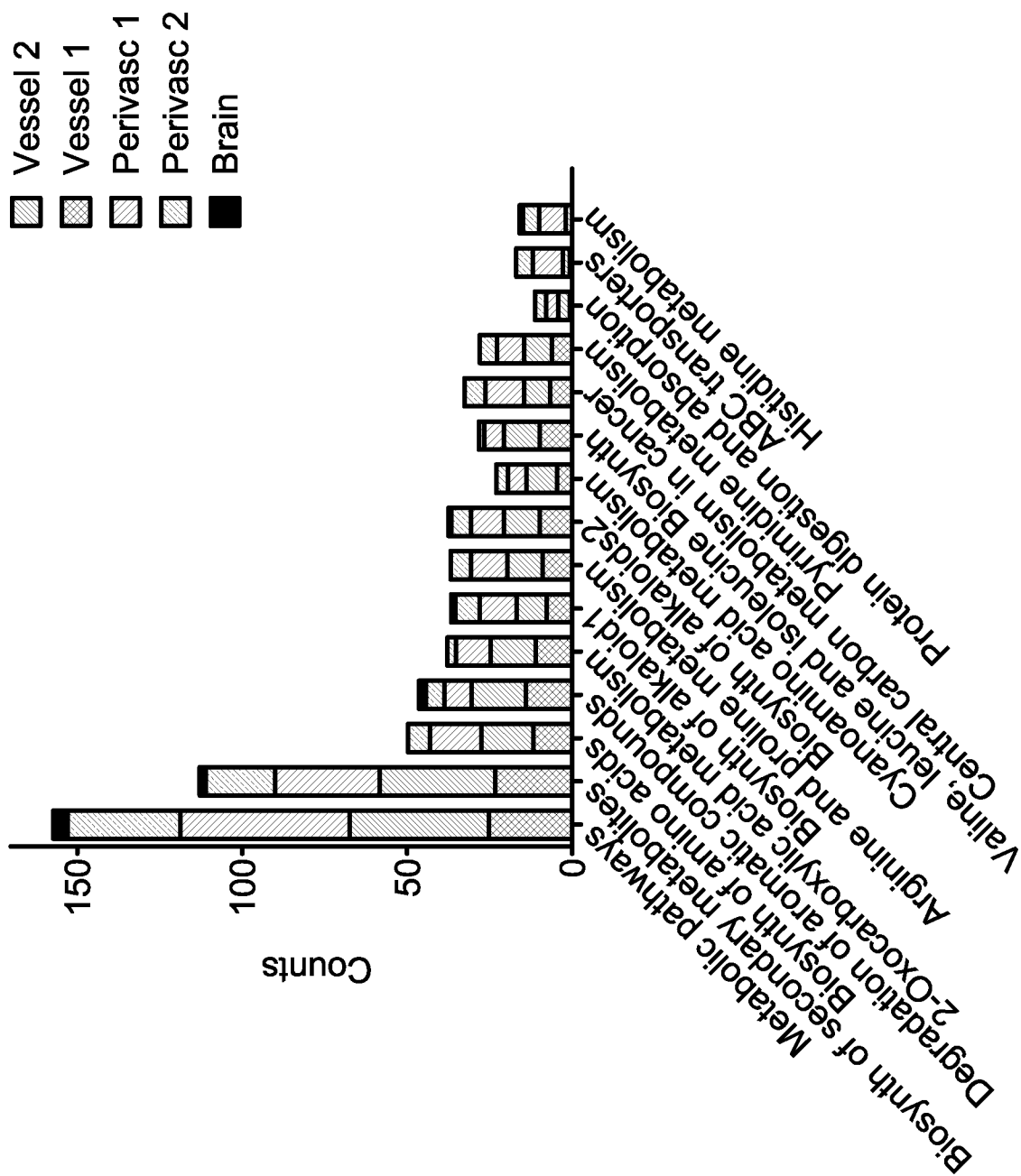


FIG. 5C

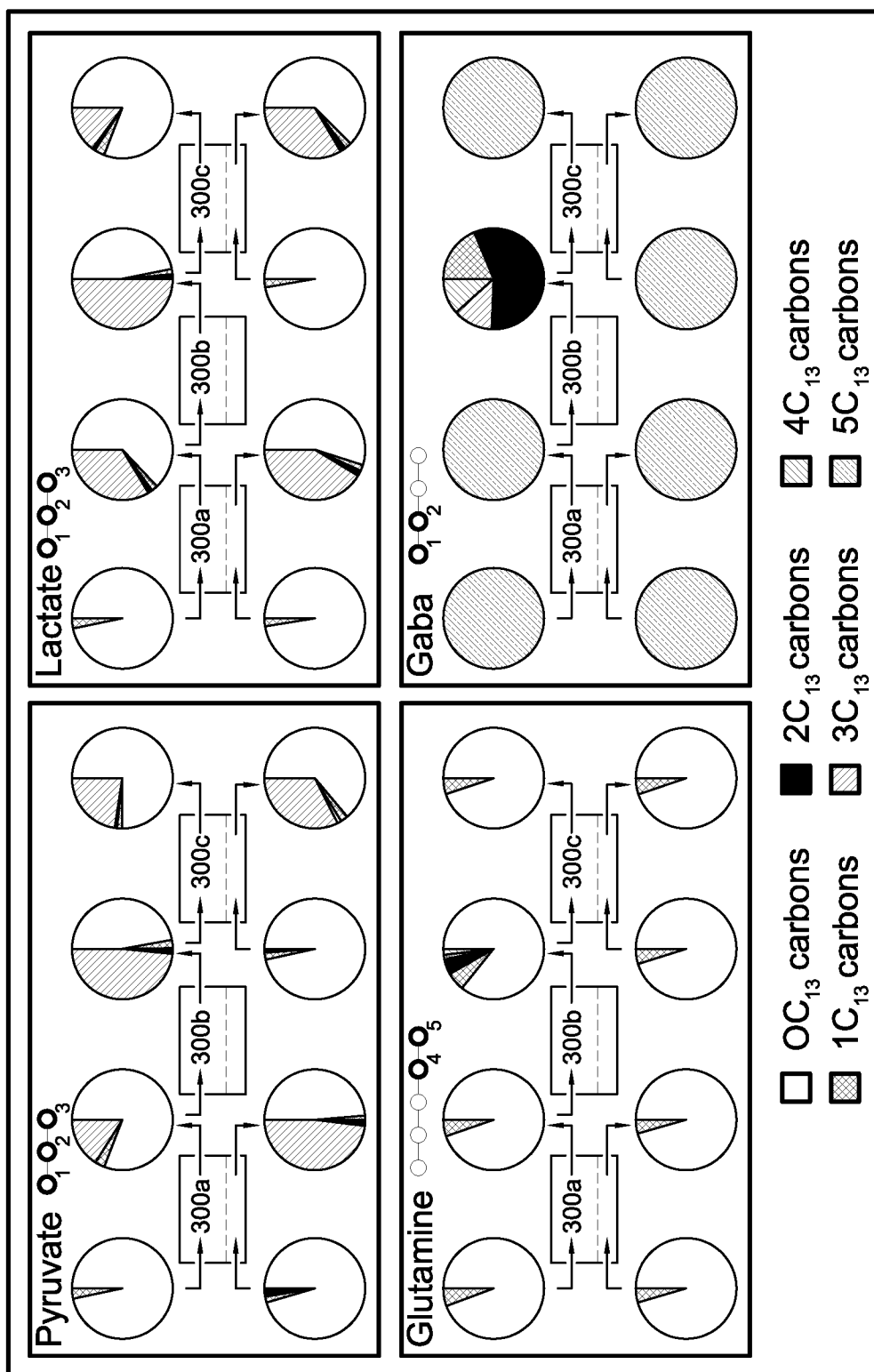


FIG. 6A

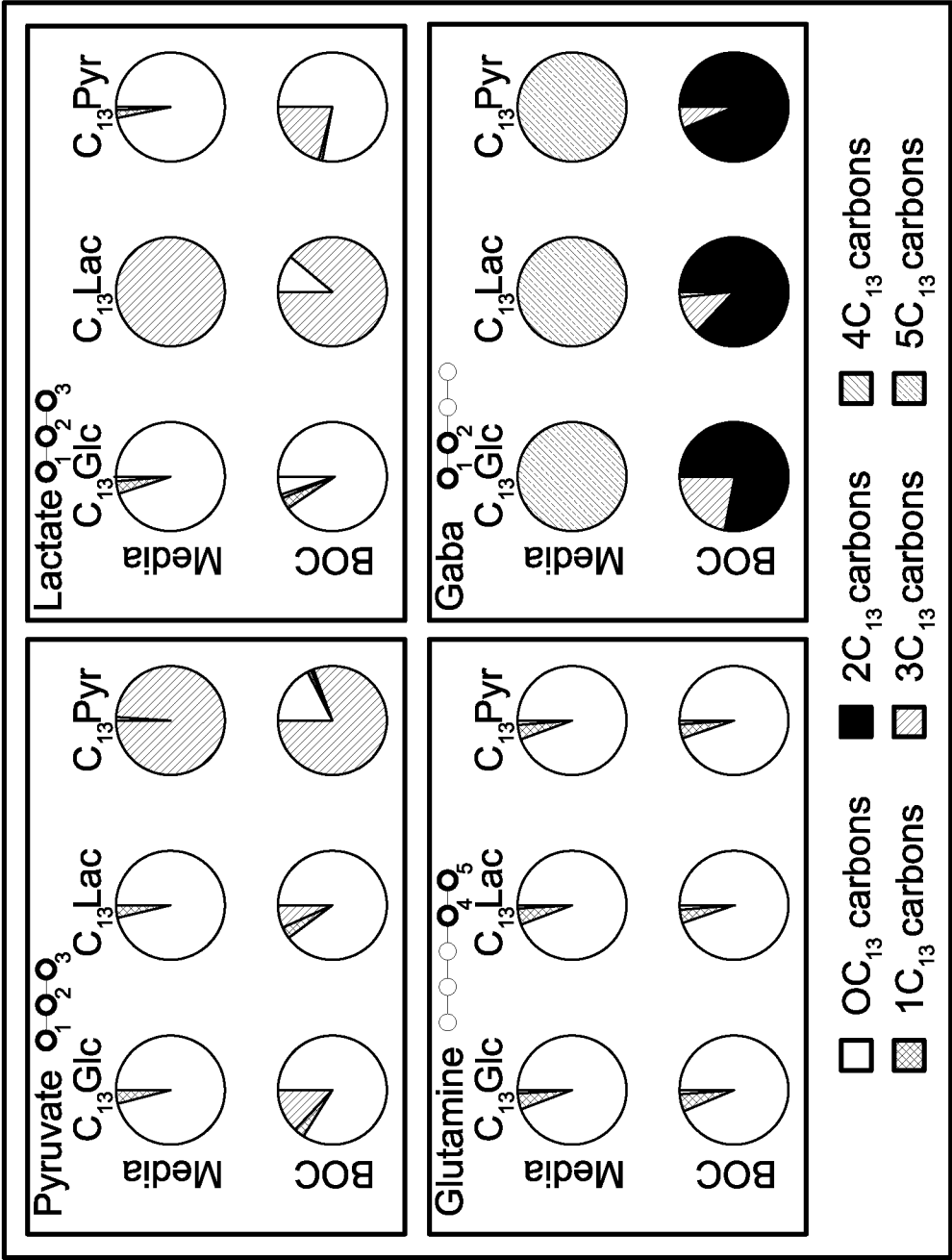


FIG. 6B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/61952

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12M 3/00 (2018.01)

CPC - C12M 3/00, C12N 5/0697, C12M 25/02, G01N 33/5058, B01L 3/5027, C12M 23/44, B01L 2200/028, C12M 23/34

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2014/0342445 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 20 November 2014 (20.11.2014) the entire document, and more specifically: para [0006], [0036], [0039], [0045], [0064], [0069], [0232]	1-15 and 27-44
A	WO 2016/100695 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 23 June 2016 (23.06.2016) pg 4, ln 4-15; pg 19, ln 4-6; abstract	1-15 and 27-44
A	"Over a century of neuron culture: From the Hanging drop to Microfluidic devices" (MILLET et al.); Yale Journal of Biology and Medicine; Volume 85, Issue 4 (December 2012); pg 501-521 (515, col 1, para 3; pg 515, col 2, para 1-2; pg 516, col 1, para 1)	1-15 and 27-44
A	US 2012/0211373 A1 (EL-SAYED et al.) 23 August 2012 (23.08.2012); the entire document	1-15 and 27-44
A	US 2016/0046897 A1 (CHILDRENS MEDICAL CENTER CORPORATION) 18 February 2016 (18.02.2016); the entire document	1-15 and 27-44
A	US 2014/0308688 A1 (RESEARCH TRIANGLE INSTITUTE) 16 October 2014 (16.10.2014); the entire document	1-15 and 27-44

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

☐ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

05 March 2018

Date of mailing of the international search report

20 MAR 2018

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. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/61952

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.:
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-15 and 27-44, directed to a microfluidic system with an influx microfluidic device, an efflux microfluidic device and an intermediary microfluidic device.

Group II: Claims 16-26, directed to a blood-brain-barrier microfluidic device.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

****please see the continuation at the end of this form****

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.:
1-15 and 27-44

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.

continuation of Box III (Lack of Unity)

Special Technical Features:

Group I requires an influx microfluidic device of the discrete microfluidic devices comprising: first and second influx microchannels each having an inlet and an outlet; and an influx membrane separating at least part of the first and second influx microchannels such that the first and second influx microchannels are in fluidic communication through the influx membrane; an efflux microfluidic device of the discrete microfluidic devices comprising: first and second efflux microchannels each having an inlet and an outlet; and an efflux membrane separating at least part of the first and second efflux microchannels such that the first and second efflux microchannels are in fluidic communication through the efflux membrane; and an intermediary microfluidic device of the discrete microfluidic devices comprising: an intermediary microchannel having an inlet and an outlet, the intermediary microchannel inlet being fluidically coupled to the first influx microchannel outlet and the intermediary microchannel outlet being fluidically coupled to the first efflux microchannel inlet; and an intermediary membrane separating the intermediary microchannel and the chamber such that the intermediary microchannel and the chamber are in fluidic communication, not required by group II.

Group II requires the first microchannel containing pericytes and astrocytes; the second microchannel containing human brain microvascular endothelial cells; the membrane configured as an interface between at least part of the first microchannel and the second microchannel to selectively pass material there between, wherein the pericytes and the astrocytes form a monolayer on a first microchannel side of the membrane, and the human brain microvascular endothelial cells line the second microchannel, including a second microchannel side of the membrane, not required by group I.

Common Technical Features:

Groups I and II share the technical feature of blood-brain-barrier; microfluidic device; a first microchannel having an inlet and an outlet; a second microchannel having an inlet and an outlet; a membrane between at least part of the first microchannel and the second microchannel; and endothelial. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being obvious over US 2015/0004077 A1 to President And Fellows Of Harvard College et al. (hereinafter Harvard). Harvard teaches blood-brain-barrier; microfluidic device (abstract; para [0005]; Integrated Organ-on-Chip microphysiological systems. Organ Chips are microfluidic devices that comprise living human cells cultured within the microfluidic devices, and mimic Organ- level functions of living Organs, such as blood-brain barrier.); a first microchannel having an inlet; a second microchannel having an inlet; a membrane between at least part of the first microchannel and the second microchannel (para [0063]; The membrane is configured to separate the central microchannel to form a first central microchannel and a second central microchannel, wherein a first fluid is applied through the first central microchannel (first inlet) and a second fluid is applied through the second central microchannel (second inlet).); and endothelial (para [0057]; vascular endothelial cells). Harvard does not explicitly teach the first microchannel having an outlet, and the second microchannel having an outlet. Harvard though teaches the use of outlets (para [0161]). Therefore, it would have been obvious to one ordinary skilled in the art to have outlets for the microchannels, such as for example: the first microchannel having an outlet, and the second microchannel having an outlet; in order to direct a fluid flow to a downstream Organ Cartridge, a downstream Organ Chip, a fluid-sampling port and/or a waste port (para [0161]).

As the shared technical features were known in the art at the time of the invention, they cannot be considered common technical features that would otherwise unify the groups. Therefore, Groups I-II lack unity under PCT Rule 13.