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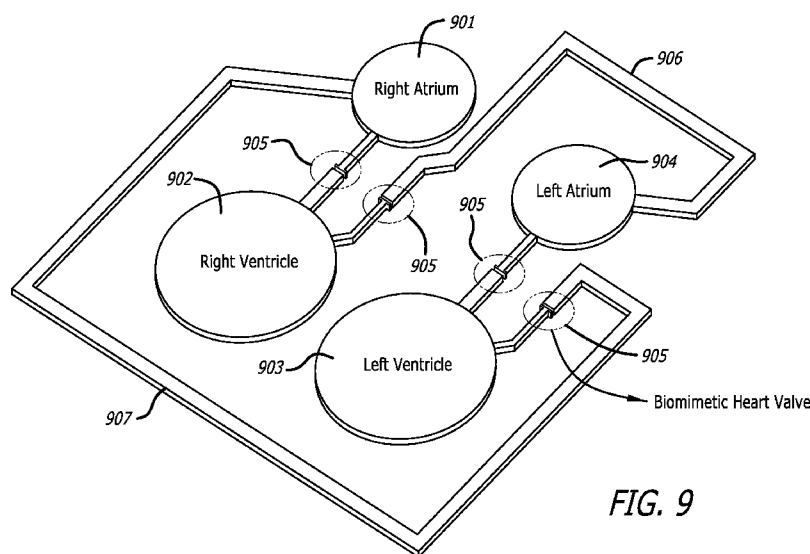


FIG. 9

(57) Abstract: Disclosed is a system for a biomimetic heart device simulating arterial flow and pulse properties thus allowing for a biomimetic microscale cardiac valve environment. The system's signaling and regulatory mechanisms linking mechano-sensing and cellular degenerative transformation provides details of force components and/or magnitudes leading heart valves to accelerated failure. The disclosed system supports a wide variety of scenarios for testing, diagnostics and drug delivery, and related products and services.



MICROFLUIDIC CARDIOVASCULAR SYSTEM AND METHOD

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5 CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to: provisional United States Patent Application Serial No. 62/020,811, filed on July 3, 2014, entitled “Biomimetic Cardiac Valve System” which provisional patent application is commonly assigned to the Assignee of the present invention and is hereby incorporated herein by reference in its entirety for all purposes.

TECHNICAL FIELD

10 [0003] The present invention relates in general to the cardiovascular field. In particular, the system of the present invention provides for a microfluidic-based, biomimetic organ-on-chip, particularly for the heart. The disclosed systems and methods support a wide variety of scenarios for cardiovascular research and related products and services.

STATEMENT OF FEDERALLY FUNDED RESEARCH

15 [0004] None.

BACKGROUND OF THE DISCLOSURE

[0005] Valvular heart disease accounts for 20,000 deaths and over 90,000 hospitalizations at a cost of over \$9 billion annually, according to the NIH. The most recent update of Heart Disease and Stroke Statistics publication indicates that the US prevalence of any valve
20 disease is 2.5%, with the mitral and aortic valves being the most affected.

[0006] The age-adjusted prevalence of valvular diseases is 2.5% and beyond 10% if only patients of age seventy-five or older are considered. The most commonly-affected valves are aortic and mitral. Regurgitation on left heart valves is the most frequently-observed disorder, with mitral regurgitation alone affecting 1.7% of the population. Technologies
25 exist for diagnosis and palliative treatment of secondary complications, but only surgical solutions exist for correcting the valvular structure. The lack of therapeutic strategies to

slow down valvular disease is directly correlated with the incomplete knowledge of the molecular mechanisms governing valvular structural changes.

5 [0007] Much has been accomplished to advance diagnosis and treatment of valvular diseases, including improved diagnostic imaging techniques, which have aided early detection of regurgitation and secondary myocardial changes, improvement of surgical techniques (e.g., catheter-based) and tissue-engineered replacement valves. However, to this day, no drug treatment has been identified capable of interrupting progression or reversing pathological changes on the valves.

10 [0008] Valvular regurgitation is the consequence of imperfect leaflet coaptation during closure, often associated with degenerative changes in the valve tissue. Different manifestations of valvular degeneration are observed in mitral and aortic valves. Mitral valves typically present myxomatous degeneration of the leaflets or annular calcification. Aortic valves usually present cusp calcification or sclerosis. Under these pathological conditions, valves are exposed to abnormal biomechanical environments, which aggravate
15 valvular degeneration. Descriptive studies of postmortem valves, *in vitro* and *in vivo* mouse models provide insights into the pathobiology of valve diseases. However, studies are still limited by tissue procurement, small sample sizes and long-term disease progress. It has recently been recognized that abnormal levels of mechanical stimulation can trigger degenerative processes in valvular tissues and cells.

20 [0009] Recently, various microfluidic-based biomimetic organs-on-chips have been proposed to perform stem cell research, regenerative medicine, biomaterials and tissue engineering in three-dimensional (3D) *in vitro* models. Yet, despite advances in the art, there remains a need to improve biomimetic systems for purposes of these advanced models for research.

25 SUMMARY OF THE DISCLOSURE

[0010] It is therefore an object of the present invention to unveil the signaling and regulatory mechanisms linking mechano-sensing and cellular degenerative transformation, as well as provide an understanding of what force components and/or magnitudes can lead heart valves to accelerated failure.

30 [0011] Based on recent technology development for organs-on-chips, as an effort to uncover molecular disease mechanisms and improve clinical trials, development of the system of the

present invention addresses the field of degenerative valvular diseases, which: (1) contributes to rapid and low-cost research and development of new pharmaceuticals used in the treatment valvular diseases; and (2) serves as preliminary work for a complete heart-on-a-chip with the incorporation of complete myocardium and vessel structures.

5 [0012] The study of valvular diseases is usually limited by practical factors (long-term disease development, sample availability and size) and, so far, a complete understanding of degenerative mechanisms is lacking. The present invention provides an approach where biomimetic valvular constructs are built on the system's microfluidic system. This *in vitro* model will enable complex 3D cellular interactions and allow for multiple studies in shorter
10 time courses.

[0013] The system of the present invention supports the development of new tissue-engineered valves, where different combinations of extracellular matrix components and cells can be studied under pressures and flow conditions. In addition to all previously-described future applications, validation of the valve-on-a-chip model supports additional
15 investigation of valvular cell biomechanics and electrophysiology.

[0014] It is therefore an object of the present invention to provide a basis for the development of new tissue-engineered valves, where different combinations of extracellular matrix components and cells can be studied under pressures and flow conditions. In addition to all previously-described applications, the present invention addresses the limitations of the art
20 by providing validation of the valve-on-a-chip model which will open new avenues of investigation of valvular cell biomechanics and electrophysiology.

[0015] It is another object of the present invention to provide (1) demonstration of a novel valvular organ-on-a-chip system; (2) development of 3D biomimetic valvular systems based on primary porcine valve endothelial and interstitial cells; (3) discovery of mechanical
25 conditions eliciting degenerative cellular transformation; and lastly (4) confirmation of a link between mechanical regulation of serotonin signaling, differentiating myxomatous from calcific disease. The present invention addresses applications in drug screening for mechano-regulated pathways as well as addresses new drug delivery mechanisms. In addition, the present invention addresses key components of valvular tissue engineering, age-related
30 connective-tissue diseases and potentially other valvular disorders of bacterial origin.

[0016] It is yet another object of the present invention to provide a microfluidic device for cardiovascular flow profile generation comprising: a substrate; one or more microfluidic

channels; at least one microfluidic pump; and one or more valves comprising a vertical membrane actuatable as a lifting gate valve; wherein the actuated microfluidic pump mimics the pulsatile flow of fluid within a cardiovascular system.

5 [0017] It is a further object of the present invention to provide a method for evaluation of cardiovascular conditions, comprising: utilizing a microfluidic device for cardiovascular flow profile generation having one or more microfluidic channels; at least one microfluidic pump; and one or more valves comprising a vertical membrane actuatable as a lifting gate valve; actuating the microfluidic pump to mimic the pulsatile flow of fluid within a cardiovascular system; measuring the biomechanical conditions of the fluid flow within the microfluidic
10 device.

[0018] In considering the system and method of the present invention, various other aspects are included, including but not limited to: the microfluidic channel may be comprised of polydimethylsyloxane (PDMS); one or more vertical membrane valves may be passive; at least one of said channel and said membrane surfaces may be coated with at least one of
15 valvular interstitial and endothelial cells; multiple microfluidic channels can have different membrane thickness, which may range between 1 and 100 μm , between 4 and 51 μm ; the flow rate of the fluid may range between 0.01 and 50 $\mu\text{L}/\text{sec}$, or may further range between 0.09 and 11 $\mu\text{L}/\text{sec}$.; the pumping frequency of the microfluidic pump may range between 0.5 and 10 Hz., or may range between 1 and 5 Hz.

20 [0019] In one aspect, the microfluidic device may further comprise a flow sensor, instrumentation for measurement of membrane displacement, and may further be actuated using phase-shifted pumping.

[0020] It is another object of the present invention to allow for measurement of biomechanical conditions occur using instrumentation selected from the group consisting of:
25 flow sensors, cameras, high-speed cameras, and force feedback sensors. Further, the system allows for evaluation of one or more microfluidic devices having at least one of normal conditions and pathological conditions. The fluid can utilize various forms of culture medium, including but not limited to using DMEM:F12 supplemented with between 5-15% bovine growth serum and between 0.1 and 5% total of at least one of antibiotics and
30 antimycotics. The system and method of the present invention may further be utilized to assess and cardiovascular conditions such as valvular degeneration, shear stress, and other valvular conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The foregoing and other objects, features, and advantages of the disclosure are apparent from the following description of embodiments as illustrated in the accompanying drawings, in which reference characters refer to the same parts throughout the various views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating principles of the disclosure:

[0022] FIG. 1A depicts the system of the present invention integrated with a microfluidic pulsatile pump for valvular study having multiple microchannel sizes.

[0023] FIG. 1B depicts a heartbeat profile generation.

[0024] FIG. 1C depicts valvular endothelial cell-lined biomimetic cardiac valve in the closed position.

[0025] FIG. 1D depicts the valvular endothelial cell-lined biomimetic cardiac valve in the open position.

[0026] FIG. 2A depicts the demonstration of the valving capability in the system of the present invention by providing fluorescent dye solution pumped through a passive valve from left to the right.

[0027] FIG. 2B depicts a valving capability image where a vacuum was applied to the right-side of the channel resulting in immediate valve closure.

[0028] FIG. 2C depicts a pumping profile generated by a standard pumping sequence in a micro-controller.

[0029] FIG. 3 depicts confluent cell cultures on modified PDMS membrane.

[0030] FIG. 4 depicts an exemplary microfluidic flow profile generator of the present invention.

[0031] FIG. 5 depicts a graph having simulated cardiac like E and A waves.

[0032] FIG. 6 depicts a bar graph showing wall shear stresses on VIC for both E and A waves obtained from 250, 300 and 500 μm of channel width.

[0033] FIG. 7 depicts VIC's that are elongated along the flow direction (0.02 $\mu\text{l/sec.}$ of continuous flow versus a static culture.

[0034] FIG. 8A depicts a lifting gate structure of the pneumatic pump of the present invention.

[0035] FIG. 8B depicts a membrane structure of a PDMS microvalve within a microfluidic channel.

5 [0036] FIG. 8C depicts a graph showing the mimicked systolic and diastolic features of the pneumatic pump structure.

[0037] FIG. 8D depicts a graph defining exemplary strain measurement of a mitral valve anterior leaflet.

[0038] FIG. 9 depicts a schematic of a full cardiovascular system of the present invention.

10 [0039] FIG. 10A depicts a graph showing shear stress of multiple pump sizes through multiple channel sizes.

[0040] FIG. 10B depicts a graphical representation of various microchannels and related fluid flow.

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DETAILED DESCRIPTION OF THE DISCLOSURE

[0041] While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts, goods, or services. The specific embodiments discussed herein are merely illustrative of
20 specific ways to make and use the disclosure and do not delimit the scope of the disclosure.

[0042] All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this disclosure pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually
25 indicated to be incorporated by reference.

[0043] The present invention will now be described more fully hereinafter with reference to the accompanying drawings, which form a part hereof, and which show, by way of illustration, specific example embodiments. Subject matter may, however, be embodied in a variety of different forms and, therefore, covered or claimed subject matter is intended to be
30 construed as not being limited to any example embodiments set forth herein; example

embodiments are provided merely to be illustrative. Likewise, a reasonably broad scope for claimed or covered subject matter is intended. Among other things, for example, subject matter may be embodied as methods, devices, components, or systems. The following detailed description is, therefore, not intended to be taken in a limiting sense.

5 [0044] Throughout the specification and claims, terms may have nuanced meanings suggested or implied in context beyond an explicitly stated meaning. Likewise, the phrase “in one embodiment” as used herein does not necessarily refer to the same embodiment and the phrase “in another embodiment” as used herein does not necessarily refer to a different embodiment. It is intended, for example, that claimed subject matter include combinations of
10 example embodiments in whole or in part.

[0045] In general, terminology may be understood at least in part from usage in context. For example, terms, such as “and”, “or”, or “and/or,” as used herein may include a variety of meanings that may depend at least in part upon the context in which such terms are used. Typically, “or” if used to associate a list, such as A, B or C, is intended to mean A, B, and C,
15 here used in the inclusive sense, as well as A, B or C, here used in the exclusive sense. In addition, the term “one or more” as used herein, depending at least in part upon context, may be used to describe any feature, structure, or characteristic in a singular sense or may be used to describe combinations of features, structures or characteristics in a plural sense. Similarly, terms, such as “a,” “an,” or “the,” again, may be understood to convey a singular usage or to
20 convey a plural usage, depending at least in part upon context. In addition, the term “based on” may be understood as not necessarily intended to convey an exclusive set of factors and may, instead, allow for existence of additional factors not necessarily expressly described, again, depending at least in part on context.

[0046] The present invention incorporates an organ-on-a-chip concept, specifically, valve-on-a-chip. The microfluidic device of the present invention is designed to mimic blood flow
25 patterns through a biomimetic heart valve. The device comprises polydimethylsyloxane (PDMS) channels and membrane surfaces coated with valvular interstitial and endothelial cells. The valve itself comprises a vertical PDMS membrane capable of opening and closing according to a set heart rate, and where all dimensions were scaled down based on a
30 mouse heart. The three-dimensional cell structure used in this device will resemble the native structure of the valve leaflet and provide a physiologic context to test mechano-chemical signaling pathways.

[0047] The main pathway targeted is serotonin signaling based on a previous demonstration of its activation in valves stretched *in vitro*. The system of the present invention provides high-throughput mechano-chemical screening capability enabling parametric studies, as well as real-time observations of cellular-level changes. The system further enables *in situ* immunoassay development for the discovery of pathways active in heart valve diseases in a much shorter timescale and reduce the need for *in vivo* testing.

[0048] Recently, various microfluidic-based biomimetic organs-on-chips have been proposed to perform stem cell research, regenerative medicine, biomaterials and tissue engineering in three-dimensional (3D) *in vitro* models. However, a biomimetic heart system simulating arterial flow, pulse properties and architectural environment is not available in cardiovascular research. In one embodiment, the present invention provides for a biomimetic cardiovascular system (BioCAV), containing a biomimetic microscale cardiac valve environment, which provides both physiological relevance and mechanical similarity for cardiac mechanistic studies and drug development. The BioCAV comprises four one-way valves and four fluidic pumps (two fluidic upper chambers and two lower fluidic chambers) to mimic atria and ventricles, respectively. During the simulation of blood circulation, fluid (blood) passes through the valves to fill each chamber of the chip, thus mimicking a beating heart. After blood passes through the open valves, backward flow is prevented with closure of the one-way valves, which precisely mimics the valvular mechanical environment.

[0049] In another embodiment, heart valve functions such as pumping frequency, loading volume and distribution of mechano-chemical signals can be mimicked *in vitro* by utilizing the system of the present invention to understand the progression of valvular disease and the parameters that culminate in heart failure. Furthermore, the system can serve as a tool for multiple applications in pharmaceutical compound screening, disease mechanism studies, and personalized healthcare. Additionally, the present invention allows for small molecule screening with numerous applications in pharmaceutical compound testing, heart valve disease modeling such as regurgitation, stenosis, and atresia.

[0050] Both disease mechanism studies and drug tests generally require whole animals or freshly dissected body parts. Such experiments are costly, and can raise ethical issues. *In vitro* biomimetic systems could reduce these issues and provide alternative models. The systems are particularly well-suited for multi-variable bioassays, offering systematic control of all experimental variables. In addition, the biomimetic system can be replicated to create a

high-throughput experimental platform for hundreds of experiments simultaneously. With these advantages, the biomimetic system reduces discovery time and the consumption of expensive reagents and cells, and allows the integration of detection assays directly in the microfluidic material (e.g., transparent PDMS). In a further embodiment the present invention allows for the limitations of existing cell culture and animal studies to be resolved by imitating complex structures and functions of living organs.

[0051] In another embodiment, the present invention provides a system which mimics the cardiovascular system, generating physiological and pathological heart environments, e.g., cardiac arrhythmias or coronary artery disease (CAD) without using animal models. In addition, the present invention estimates multiple cardiovascular tissue responses to drugs rapidly. The system of the present invention may be interconnected with other well-developed organ-on-a-chip systems such as gut, kidney and lung to expand their capabilities. For instance, by connecting lung-on-a-chip with the system of the present invention, the capabilities mimicking a coordinated thoracic system may further serve as a model for pulmonary hypertension, among other diseases which incorporate blood flow and gas exchange. Novel in vitro disease models can thus lead the discoveries of new therapeutic methods and appropriate drugs.

[0052] In one embodiment, the system provides for heart valve mechanobiology, along with microfabrication techniques, which will provide solutions for tissue engineering and fabrication of semi-synthetic valves. Exemplary embodiments of the present invention include the design of a microfluidic cardiovascular system (**FIG. 9**) which may further be represented as a biomimetic cardiac valve system (**FIG. 1A**) or a biomimetic microfluidic flow profile generator (**FIG. 10A**). Turning to **FIG. 9**, an exemplary embodiment of the present invention provides a microfluidic cardiovascular system having one or more pneumatic actuated pumps which represent a right atrium **901**, a right ventricle **902**, a left atrium **904** and a left ventricle **903**. Biomimetic cardiac valve systems **905** are represented in between each designated pump **901**, **902**, **903**, **904**, and appropriate determinations of valvular behavior may be measured. First, dimensions observed in mice mitral valves were used to scale down the model system. Second, the design was created and mold developed for chip fabrication (**FIG. 2A**). Lastly, for exemplary purposes, confluent valve interstitial cell cultures were established on PDMS membranes modified with a cross-linker and arginylglycylaspartate (RGD) adhesive peptide (**FIG. 3**), although other synthetic fibers or

composite materials may be utilized for substrates or membranes, particularly with regard to anisotropic formations.

[0053] In a further embodiment the system, the current preliminary data demonstrates the ability to: (1) design a microfluidic platform to mimic blood flow through heart valves; and
5 (2) modify the hydrophobic surface into an environment that promotes cell growth to confluence. Additionally, the present invention provides for the establishment of tissue cultures on the valve structure and demonstration of the three-dimensional architecture of the constructs. Additional tools include the application of pulsatile flow to the tissue constructs, use of inhibitors (pharmacologic or RNAi) to manipulate mechanically regulated pathways,
10 and microscopic imaging to verify signaling pathways and cell phenotype change.

[0054] In one embodiment, the valve and channel structure of the present invention is comprised of a homogenous and isotropic material such as PDMS. The platform may be developed using soft lithography, which means a family of techniques for fabricating or replicating structures using elastomeric stamps, molds, and conformable photomasks. Soft
15 lithography is generally used to construct features measured on the micrometer to nanometer scale. The term “soft” is used because the process uses elastomeric materials, most notably PDMS. PDMS stamps are pieces of PDMS that have been patterned usually against a master to form a relief pattern used in soft lithography. This PDMS stamp can be used in either its current form as a relief surface for techniques such as microcontact printing or can also be
20 attached to an external source by tubing so that liquid may be passed through channels on its surface. In this second case it will often be laminated to a surface, referred to as a substrate, so that chemistry can be performed on that surface producing a pattern of the PDMS stamp on to the surface. Alternatively a PDMS stamp can be laminated to a second piece of PDMS to form a contained device. It is possible to pattern PDMS with nanometer resolution. Many
25 techniques have been developed to modify the basic setups to perform a range of tasks such as assays on small volumes, all of which are considered in light of the present invention. These kinds of devices are often referred to as microfluidic devices. Because of the small dimensions of these devices flow is laminar not turbulent which can lead to many useful properties.

[0055] In another embodiment, the valve structure of the present invention is comprised of
30 artificial fibers or composite materials which are anisotropic or have nonlinear anisotropic mechanical properties of native and engineered tissues in order to ensure integrity in stress

distribution throughout the valve structure. In yet another embodiment, various gas concentration environments are included by attaching a microfluidic perfusion system for realizing adequate gas concentration environments.

[0056] The examples below provide illustrative embodiments of the present invention. While various embodiments have been described for purposes of this disclosure, such embodiments should not be deemed to limit the teaching of this disclosure to those embodiments. Various changes and modifications may be made to the elements and operations described above to obtain a result that remains within the scope of the systems and processes described in this disclosure.

10 EXAMPLE 1: Development of biomimetic cardiac valve (BioCAV) system for in vitro valvular degeneration study.

[0057] Complex mechanical loading conditions govern heart valve function, in both healthy and diseased states. Under degenerative conditions, leaflets are thickened, with eventual calcification, often associated with increased biomechanical stresses along with valve prolapse and/or regurgitation. Thus, a systemic understanding of the motion of cardiac valve and cellular response is a critical factor in order to develop proper therapies for valvular degeneration. The objective of Example 1 is to develop a biomimetic cardiac valve (BioCAV) system consisting of a simple passive microvalve and a pulsatile microfluidic pump. By tuning the geometry of the passive valve and the pumping profile in the BioCAV, we will generate the specific biomechanical conditions mimicking various valvular disease models (FIG. 1).

[0058] Turning to FIG. 1, an exemplary form factor of the present invention is provided wherein the system 100 is integrated with a microfluidic pulsatile pump 101, having one or more microchannels 103 having one or more cardiac valves 102 represented within the microchannels 103. To create the BioCAV platform of the present example, soft-lithography using a SU-8 mold was used to prepare micropumps 102 and microchannels 103, including vertical membranes in polydimethylsiloxane (PDMS) layers. First, two SU-8 molds were fabricated to obtain a microchannel structure and a free-standing membrane. After silanizing the SU-8 molds using chemical vapor deposition, PDMS (Dow Corning) was poured on the molds and cured on a 65°C hot- plate for 1 hour. Holes were punched in the PDMS microchannel replica for inlets and outlets 104 and the microchannel structure was irreversibly bonded to the substrate containing the free standing membrane after plasma

activation. Using a similar method, we fabricated the micropumps to generate desired pumping profiles. The valves **102** are then capable of providing various transmissions of fluid representing blood flow through the channels **103** through to each outlet **104**. **FIG. 1B** presents the heartbeat profile generation achieved by the microfluidic pulsatile pump comprising a first position **105**, a second position **106**, and a third position **107**. **FIG 1C**, showing one embodiment of the present invention having a valve construct **111** capable of opening and closing as a passive valve responding to a directional flow of fluid **110**, causing the valve **111** to be in an open position, wherein the application of back-pressure **109** causes the valve to return to the closed position **108**. Additionally, cell growth may be achieved on the valve **111** as exemplified further in the present invention. As a preliminary experiment, a FITC- fluorescence solution was flowed through the System of the present invention to confirm valving capability. **FIGS. 2A** and **2B** present the fluorescence images during the experimental demonstration of valving capability of the valves with no back-flow. A fluorescent dye solution **202** was pumped through the passive valve **203** from left **202** to right **201**. From the images the flow pattern controlled by the passive valve can be verified. A vacuum was then applied to the right side of the channel **206** resulting in immediate valve closure **205**, where fluid remains present on the left side **204**. In addition, pulsatile flow is generated with a pneumatically-actuated microfluidic pump. **FIG. 2C** shows a standard pumping profile measured by a flow sensor. Almost any flow profile can be created by changing the pump size and pulsatile regimen to provide a cardiac-like pumping cycle.

[0059] By integrating the microfluidic pulsatile pumps with the passive valves **102** (**FIG. 1**), studies are performed to investigate biomechanical behaviors under various membrane thicknesses, flow rates and pumping frequencies. Multiple channels of different membrane thicknesses, within the range of 5-50 μm , are fabricated and different combinations of flow rates (0.1~10 $\mu\text{L}/\text{sec}$) and pumping frequencies (1~5 Hz) are used in experiments for the determination of mechanical behavior. In another embodiment, the membrane thickness ranges between 1 and 100 μm . In another embodiment, the membrane thickness ranges between 4 and 51 μm . In another embodiment the flow rate of the fluid ranges between 0.01 and 50 $\mu\text{L}/\text{sec}$. In another embodiment the flow rate of the fluid ranges between 0.09 and 11 $\mu\text{L}/\text{sec}$. In another embodiment, the pumping frequency of the microfluidic pump ranges between 0.5 and 10 Hz.

[0060] FIG 8A presents additional detail with regard to the microfluidic pulsatile pumps capable of achieving the pump positions as set forth in FIG. 1B, such as having an inlet 801, and, sequentially, the first position 802 open, a second position 803 open, and finally a third position 804 open. The resulting impact on the fluid and related passive valves are represented in FIG. 8B, wherein, similar to FIG. 1C (closed) and FIG. 1D (open), FIG. 8B provides a fluid flow 806 which passes fluid through 809 an open valve 807 and continuing through 810 the valve. When the valve closes 808 due to the pulsatile effect of the pneumatic microfluidic pumps, the backflow is prevented and the shear stresses on the valve (and related cell tissues) are measurable.

[0061] The resulting systolic and diastolic modelling of the microfluidic pumps are represented in FIG. 8C. As a reference, FIG. 8D provides exemplary data on the strain placed upon the mitral valve anterior leaflet (Sacks, et al., 2009).

[0062] For the purposes of this example, flow profiles are measured by a highly sensitive flow sensor (Sensorion Inc.) to create the feedback control system. In addition, the displacement of the membrane is a critical value for estimating the biomechanical behavior and the cellular response. A high-speed camera captures the motion of the membrane from the top side of the system of the present invention and performs the stress and strain analyses by calculating the curvature of the membrane indirectly. Once mechanical measurements are made, valvular cells are attached to the membrane to investigate three-dimensional cellular behavior under various biomechanical conditions.

[0063] This Example 1 demonstrates the ability to: 1) mimic the motion of a heart valve, 2) generate a heart-like pumping profile, and 3) create the biomimetic valve model to estimate biomechanical behavior changes. The feedback control system generates various pumping profiles inducing distinct biomechanical responses of the cardiac valves. In addition, *in vitro* models of the present invention provide biomechanical differences between normal and pathological conditions by measuring the deformation of the membrane and volumetric flow rate. Loss of the valving capability due to repetitive pumping under high pressure is overcome by adjusting the valve-seat design with further numerical analyses. Additionally, generating high pumping frequencies due to delayed response of external electrical and mechanical system is addressed by adapting the phase-shifting pumping technique, achieving up to 10 Hz which is high enough for mimicking various cardiac pumping cycles.

EXAMPLE 2: Validation of the presence of degenerative phenotypes and signaling pathways according to different flow patterns.

[0064] Recent evidence indicates a link between shear forces and valvular degeneration. However, the signaling mechanisms converting mechanical stimuli into bio-chemical responses are still unclear. The specific hypotheses here are that increasing levels of shear will increase the expression of degenerative proteins (myxomatous or calcific markers) and that, in turn, pharmacological inhibitors will prevent such degenerative changes in the presence of shear stress. Based on previous work and a recently identified role for serotonin in bone formation, it is believed that autocrine serotonin signaling is a central pathway in valvular degeneration that can be mechanically-regulated. Using the system of the present invention, *in vitro* manipulation of valve constructs are more easily achievable and repeatable.

[0065] Microfluidic channels and membrane are modified by dynamic fibronectin coating or photochemical cross-linking with sulfo-SANPAH (N-Sulfosuccinimidyl-6- (4'-azido-2'-nitrophenylamino) hexanoate) and arginylglycylaspartate (RGD) adhesive peptide based on previously developed methods. Primary porcine valvular cells are obtained locally from slaughtered animals. After PDMS surface modification, interstitial cells, from either aortic or mitral valves, are grown to confluence and later covered with a layer of endothelial cells. Endothelialization is verified by immunohistochemistry with an endothelial cell marker, CD-31. Valvular constructs on the system of the present invention will then be stimulated with the application of various membrane thicknesses, flow rates and pumping frequencies as in Example 1. Standard culture medium – DMEM:F12 supplemented with 10% bovine growth serum and 1% antibiotics/antimycotics - are used for culture and flow experiments. Stimulation regimens are applied for 24 hours, and at the end of tests, the system are disassembled for microscopic imaging of cells and other biochemical analyses (proteome profiling, serotonin enzyme- linked immunoassays, along with other *in situ* immunoassays). Inhibition of autocrine serotonin signaling will follow previous experiments, with two inhibition nodes, i.e., inhibition of the synthetic enzyme tryptophan hydroxylase and inhibition of the main valvular receptor 2B.

[0066] In order to validate the presence of different degenerative phenotypes and serotonin signaling inhibition, we will use established protein expression profiling methods and biochemical assays as follows. First, immunoblotting and immunohistochemistry are used

to determine the presence of myxomatous degeneration (α -smooth muscle actin, embryonic smooth muscle myosin, and matrix metalloproteinases 1 and 13) or calcification markers (bone morphogenetic protein 2, runt-related transcription factor 2 and lipoprotein receptor-related protein 5). Second, markers of serotonin signaling are profiled in all experiments, including the serotonin transporter and major receptor classes (subtypes of receptor 2), tryptophan hydroxylase, and downstream associated transforming growth factor beta signaling. Additional analyses include quantification of serotonin itself by enzyme-linked immunoassays, and assays to evaluate calcification and deposition of glycosaminoglycans. Analysis of variance is used with pairwise comparisons to test the effect of increasing shear and pressure on protein expression in valvular constructs. Additional statistical analyses determine the significance of serotonin signaling on shear-induced degenerative transformation. By conducting parallel studies with aortic and mitral valvular cells, we are able to characterize central differences in degenerative mechanisms leading to myxomatous degeneration versus calcification.

[0067] Example 2 demonstrates the ability to: (1) develop a valve construct with primary porcine valve endothelial and interstitial cells; (2) identify the mechanical conditions triggering degenerative cellular transformation; and (3) confirm the mechanical regulation of serotonin signaling in degenerative valve diseases. Potential problems include the possibilities of valvular cells not attaching or differentiating into other phenotypes in the channel environment, regardless of mechanical stimuli. These are addressed by carefully controlling flow rates and pumping frequencies, and in an *in vivo* study to compare pathway activation, respectively. Another risk relates to the lack of selectivity of pharmacological inhibitors which is circumvented with the inhibition of several pathway nodes and even alternative RNAi inhibition.

EXAMPLE 3: Development of a Biomimetic Microfluidic Flow Profile Generator (BioMFG) Enabling Mechanobiological Responses of Valvular Interstitial Cell.

[0068] Various microfluidic-based cardiac pumping systems have been proposed recently as tools to study mechanobiology of the cardiovascular system. However, a focus on valvular cardiac flow patterns is lacking. Valvular interstitial cells (VIC) have key roles in cusp structural integrity, but mechanobiological responses of VIC to flow-induced shear stress must still be elucidated. To determine VIC responses to fluid shear stress, the device of the present invention, referred to in this Example 3 as a biomimetic microfluidic flow profile

generator (BioMFG), investigates phenotype changes of VIC under various cardiac-like pumping profiles and shear stresses. The study of shear stress applied directly to interstitial cells is relevant to diseases which result in local endothelium removal and expose VIC directly to blood flow.

5 [0069] FIG. 4 shows the BioMFG system 404, which consists of two sets of PDMS-based pneumatic actuated pumps 405, 406 with lifting gate valves that mimic atrial and ventricular actuation. A bifurcating PDMS microfluidic device containing three different channel widths: 250 μm 401, 300 μm 402, and 500 μm 403 is connected between the pumps to expose VICs seeded within the bifurcating channels 401, 402, 403 to various levels of pulsatile shear stresses. Porcine mitral VICs are seeded into the channels and grown until confluent. The BioMFG simulates mitral inflow hemodynamic conditions (FIG. 5). Shear stresses on each channel are calculated using the equation for wall shear stress (τ). The pump actuations are programmed and performed for 24h at 37 °C and 5% carbon dioxide environment. During pumping with the BioMFG, cell culture medium is circulated from atria to ventricles through the fluidic channels containing VIC layers. Live cell images on cell elongation pattern are obtained by inverted optical microscopy.

[0070] Peak flow rates for early (E) and atrial (A) echocardiogram waves are 1.5 $\mu\text{l}/\text{sec}$ and 0.7 $\mu\text{l}/\text{sec}$, respectively. 22, 17, and 8 dyne/cm^2 shear stress for E wave and 14, 11, and 5 dyne/cm^2 shear stress for A wave are obtained based on the measured flow rates as shown in FIGS. 5 and 6. Additionally, VICs are confirmed elongated along the channel direction in continuous flow (0.02 $\mu\text{l}/\text{sec}$) comparing to static condition (see FIG. 7). The results indicate that VICs are shear sensitive and hemodynamic forces can directly regulate VIC morphological phenotype along the flow direction. Additional studies of VIC response to abnormal patterns of E and A waves are able to detect phenotype changes, characteristic of VIC activation in degenerative valves, based on expression levels of α -SMA and MMP-2.

[0071] The BioMFG system is of physiological and mechanical relevance to mitral valvular systems and can be easily adapted to model other heart valves. In addition, the current version of the BioMFG system serves as a baseline for a higher complexity model which integrates interstitial and endothelial cells.

30 [0072] As a further exemplary system for shear stress, a multi-pumping BioMFG system as set forth in FIG. 10A is designed to mimic dynamic shear range effects on cardiac valvular cells. The system is consisted with four different dimensions of pneumatic actuated

micropumps **1002** integrated with cell culturing bifurcating channels **1003**. Each pump **1002** has 1.37, 1.67, 1.94, and 2.37 mm dimension, respectively. These four different pumps with 85 kPa closing pressures generate 1.875-1.5 $\mu\text{l}/\text{sec}$ of volumetric flow rate that enabling to generate 2.5-40 dyne/cm^2 shear stress on the cells. Each pump can generate 2.5-5 dyne/cm^2 ,
5 5 -10 dyne/cm^2 , 10-20 dyne/cm^2 , and 20-40 dyne/cm^2 of shear stress, respectively as shown in **FIG. 10 B**. The multi-pumping BioMFG system with programmed cardiac sequence makes a cardiac-like pulsatile shear stress in a widely adjustable physiological range within a cell culturing channel.

[**0073**] Those skilled in the art will recognize that the methods and systems of the present
10 invention may be implemented in many manners and as such are not to be limited by the foregoing exemplary embodiments and examples. Furthermore, the embodiments of methods presented and described in this disclosure are provided by way of example in order to provide a more complete understanding of the technology. The disclosed methods are not limited to the operations and logical flow presented herein. Alternative embodiments are contemplated
15 in which the order of the various operations is altered and in which suboperations described as being part of a larger operation are performed independently.

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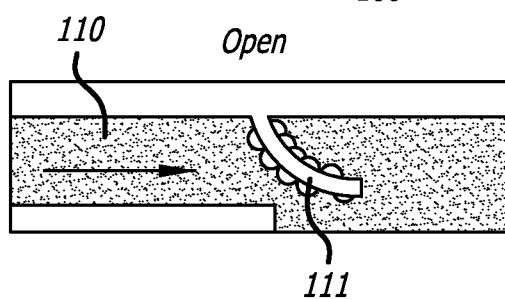
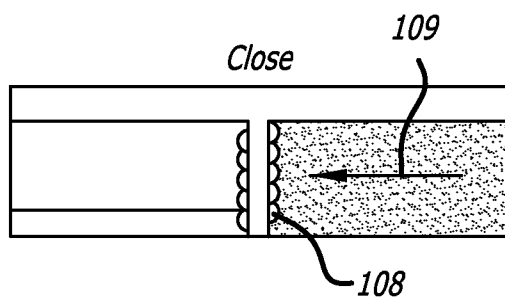
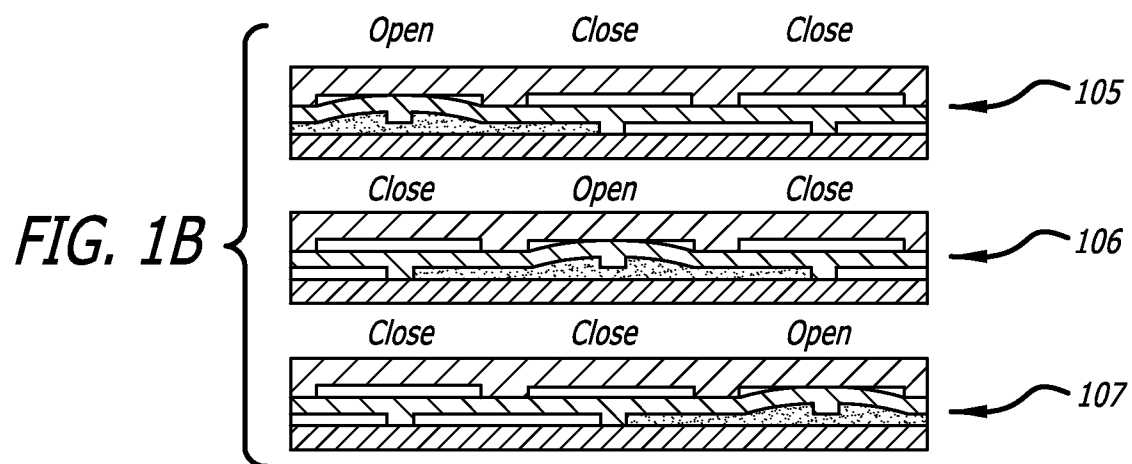
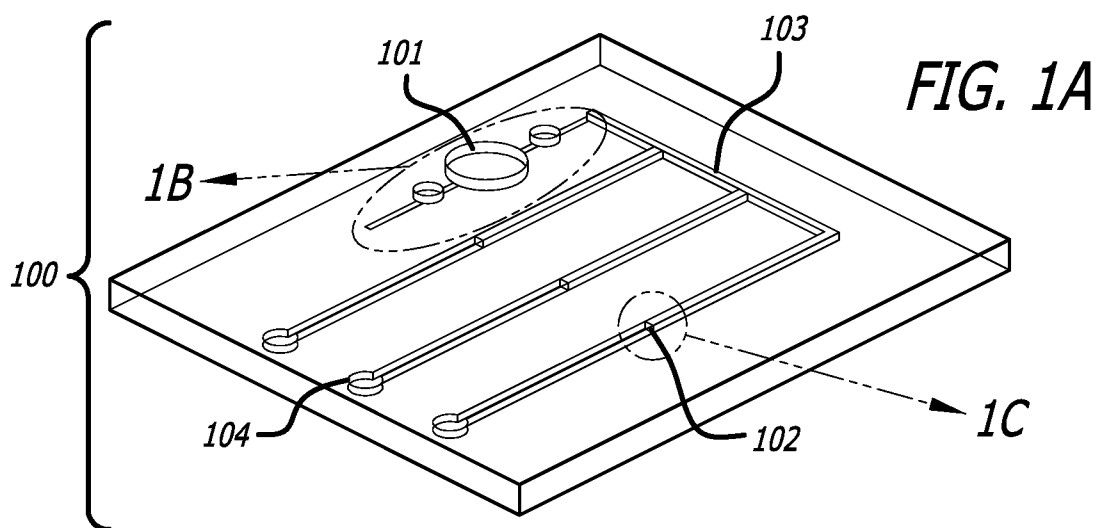
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What is claimed is:

1. A microfluidic device for cardiovascular flow profile generation comprising:
 - a substrate;
 - one or more microfluidic channels;
 - at least one microfluidic pump; and
 - one or more valves comprising a vertical membrane actuatable as a lifting gate valve;wherein the actuated microfluidic pump mimics the pulsatile flow of fluid within a cardiovascular system.
2. The microfluidic device of claim 1, wherein said microfluidic channel is comprised of polydimethylsyloxane (PDMS).
3. The microfluidic device of claim 1, wherein said one or more vertical membrane valves are passive.
4. The microfluidic device of claim 1, wherein at least one of said channel and said membrane surfaces are coated with at least one of valvular interstitial and endothelial cells.
5. The microfluidic device of claim 1, further comprising multiple microfluidic channels of different membrane thickness.
6. The microfluidic device of claim 5, wherein said membrane thickness ranges between 1 and 100 μm .
7. The microfluidic device of claim 5, wherein said membrane thickness ranges between 4 and 51 μm .
8. The microfluidic device of claim 1, wherein the flow rate of the fluid ranges between 0.01 and 50 $\mu\text{L}/\text{sec}$.

9. The microfluidic device of claim 1, wherein the flow rate of the fluid ranges between 0.09 and 11 $\mu\text{L}/\text{sec}$.
10. The microfluidic device of claim 1, wherein the pumping frequency of the microfluidic pump ranges between 0.5 and 10 Hz.
11. The microfluidic device of claim 1, wherein the pumping frequency of the microfluidic pump ranges between 1 and 5 Hz.
12. The microfluidic device of claim 1, further comprising a flow sensor.
13. The microfluidic device of claim 1, further comprising measurement of membrane displacement.
14. The microfluidic device of claim 1, wherein the pump is actuated using phase-shifted pumping.
15. A method for evaluation of cardiovascular conditions, comprising:
 - utilizing a microfluidic device for cardiovascular flow profile generation having one or more microfluidic channels; at least one microfluidic pump; and one or more valves comprising a vertical membrane actuatable as a lifting gate valve;
 - actuating the microfluidic pump to mimic the pulsatile flow of fluid within a cardiovascular system; and
 - measuring the biomechanical conditions of the fluid flow within the microfluidic device.
16. The method of claim 15, wherein said microfluidic device is comprised of polydimethylsyloxane (PDMS).
17. The method of claim 16, further comprising surface modification of the PDMS for attachment of valvular interstitial cells.

18. The method of claim 17, wherein said surface modification further comprises photochemical cross-linking N-Sulfosuccinimidyl-6-(4'-asido-2'-nitrophenylamino) hexanoate) and arginylglycylaspartate (RGD) adhesive peptide.
19. The method of claim 16, further comprising endothelialization.
20. The method of claim 15, wherein at least one of said channel and said membrane surfaces are coated with at least one of valvular interstitial and endothelial cells.
21. The method of claim 15, wherein said measurement of biomechanical conditions occurs using instrumentation selected from the group consisting of: flow sensors, cameras, high-speed cameras, and force feedback sensors.
22. The method of claim 15, further comprising evaluation of one or more microfluidic devices having at least one of normal conditions and pathological conditions.
23. The method of claim 15, wherein said fluid further comprises a culture medium.
24. The method of claim 23, further comprising using DMEM:F12 supplemented with between 5-15% bovine growth serum and between 0.1 and 5% total of at least one of antibiotics and antimycotics.
25. The method of claim 15, wherein said cardiovascular condition is valvular degeneration.
26. The method of claim 15, wherein said cardiovascular condition is determination of shear stress on cardiac valvular cells.



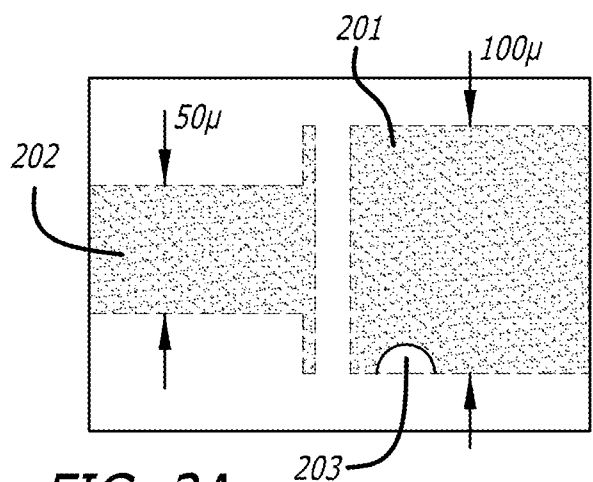


FIG. 2A

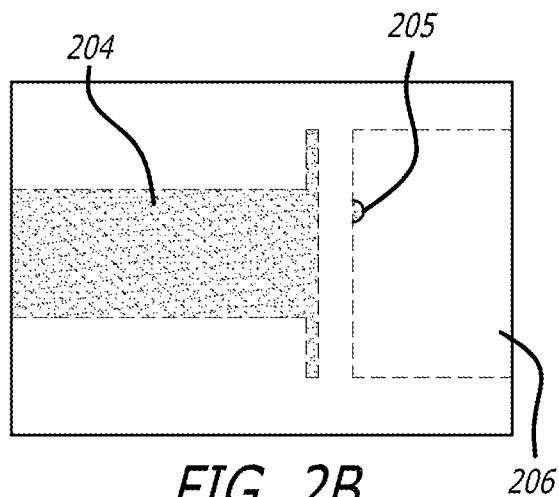


FIG. 2B

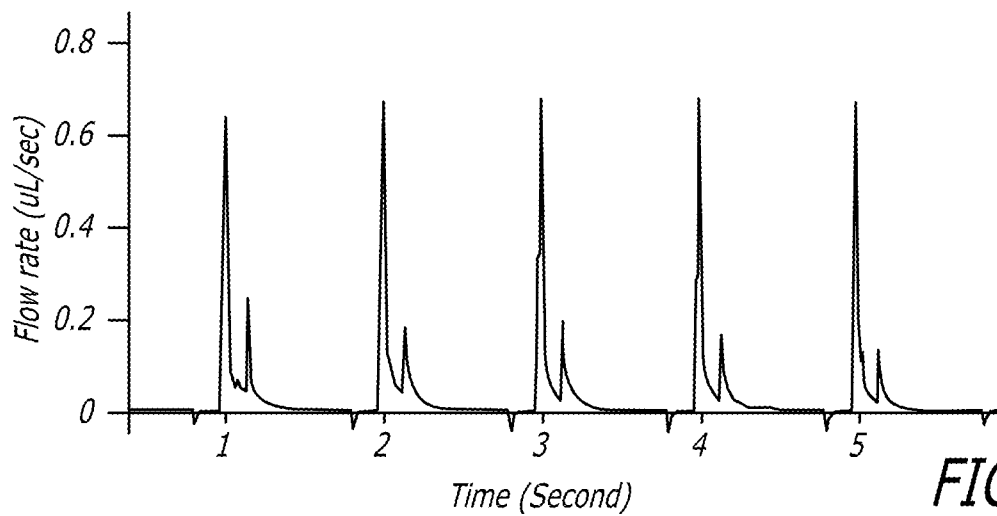


FIG. 2C

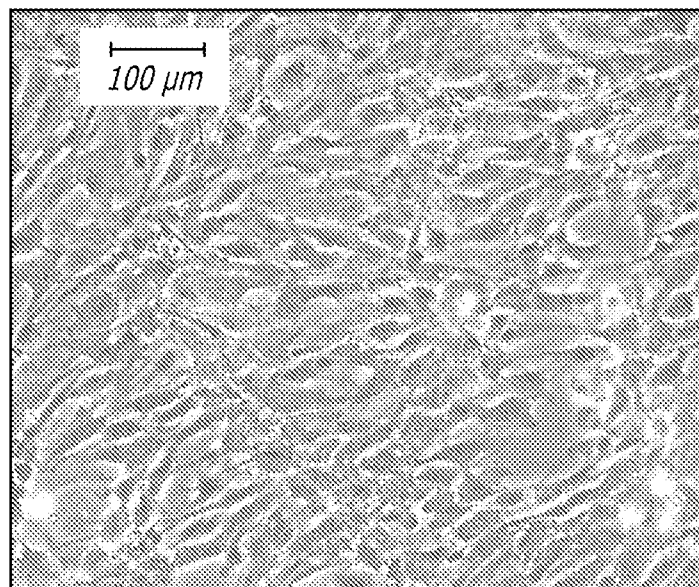


FIG. 3

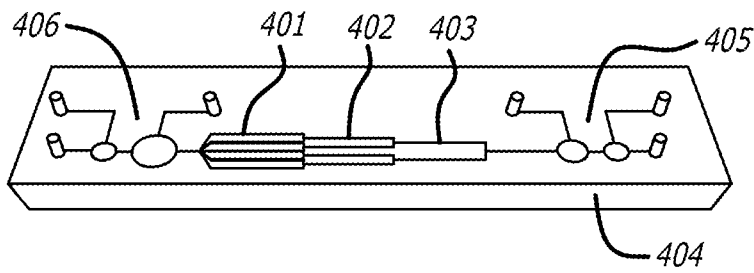


FIG. 4

FIG. 5

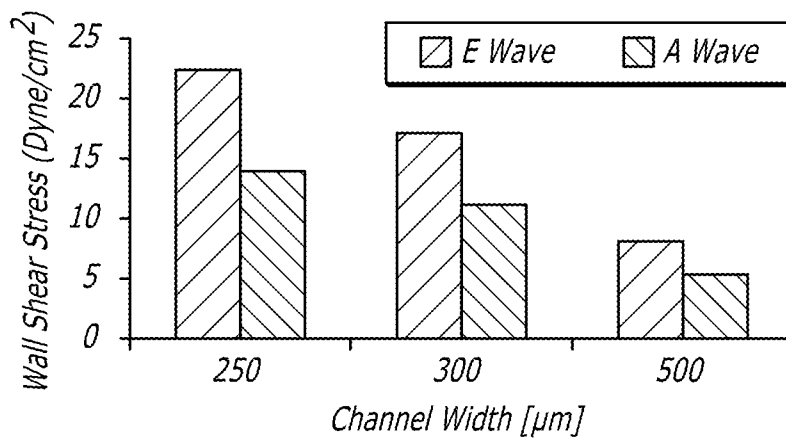
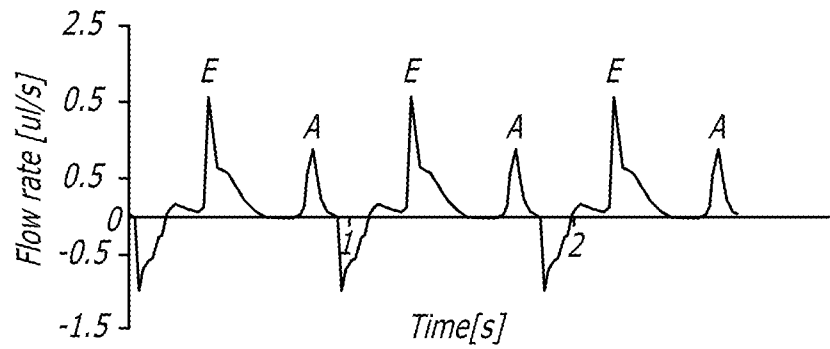


FIG. 6

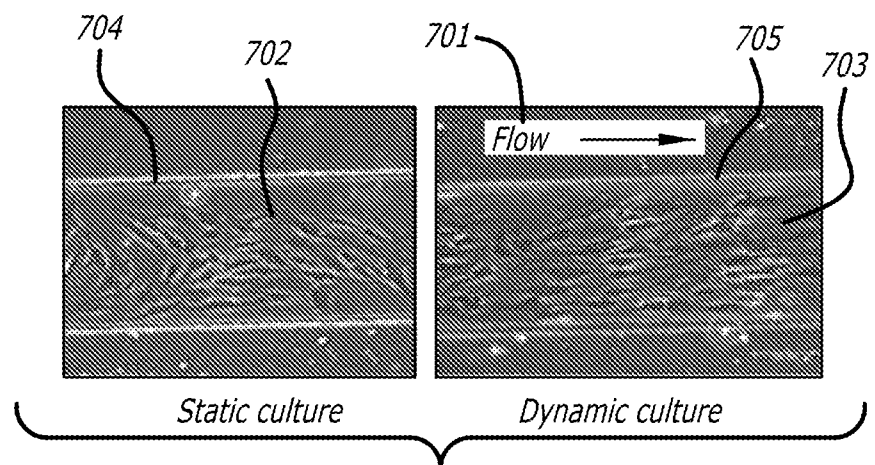
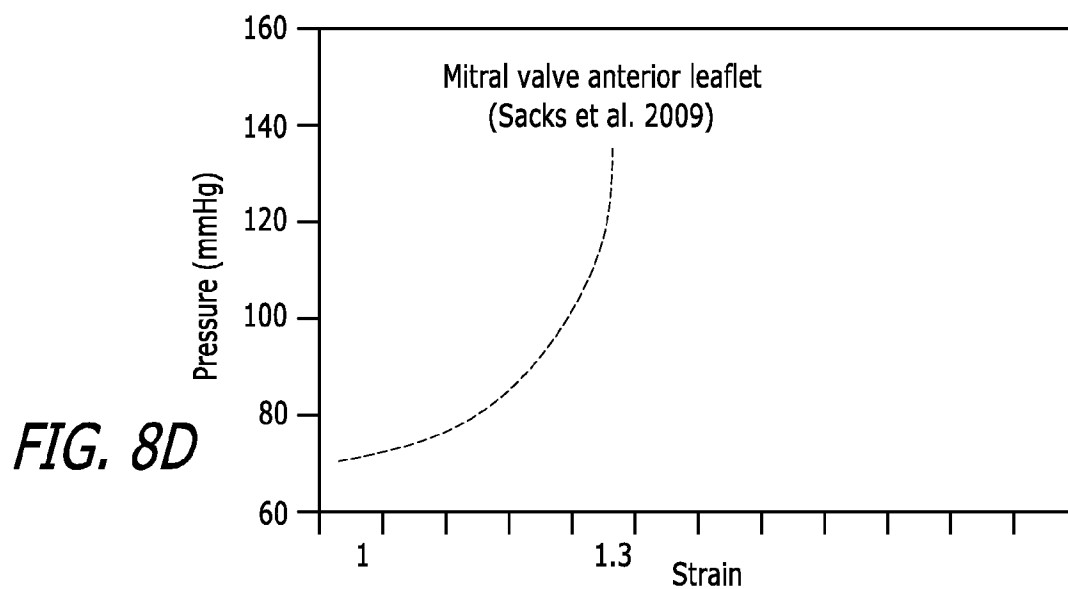
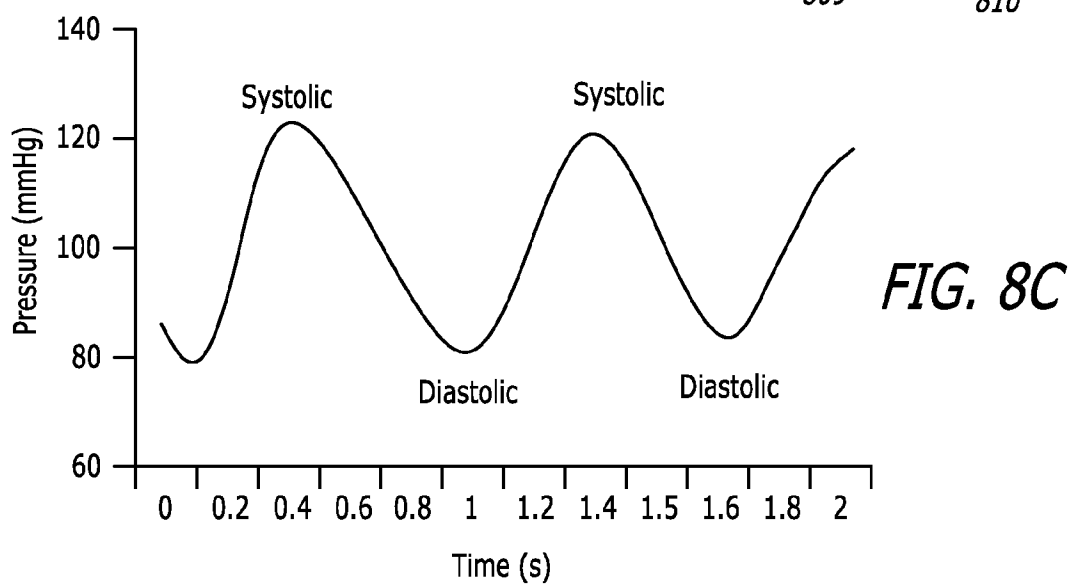
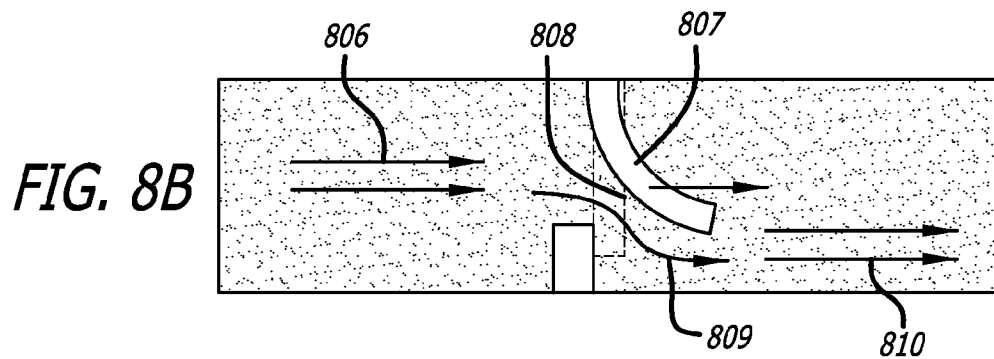
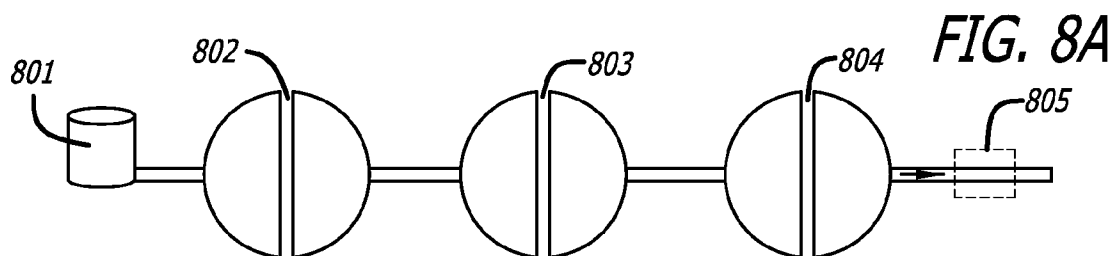


FIG. 7



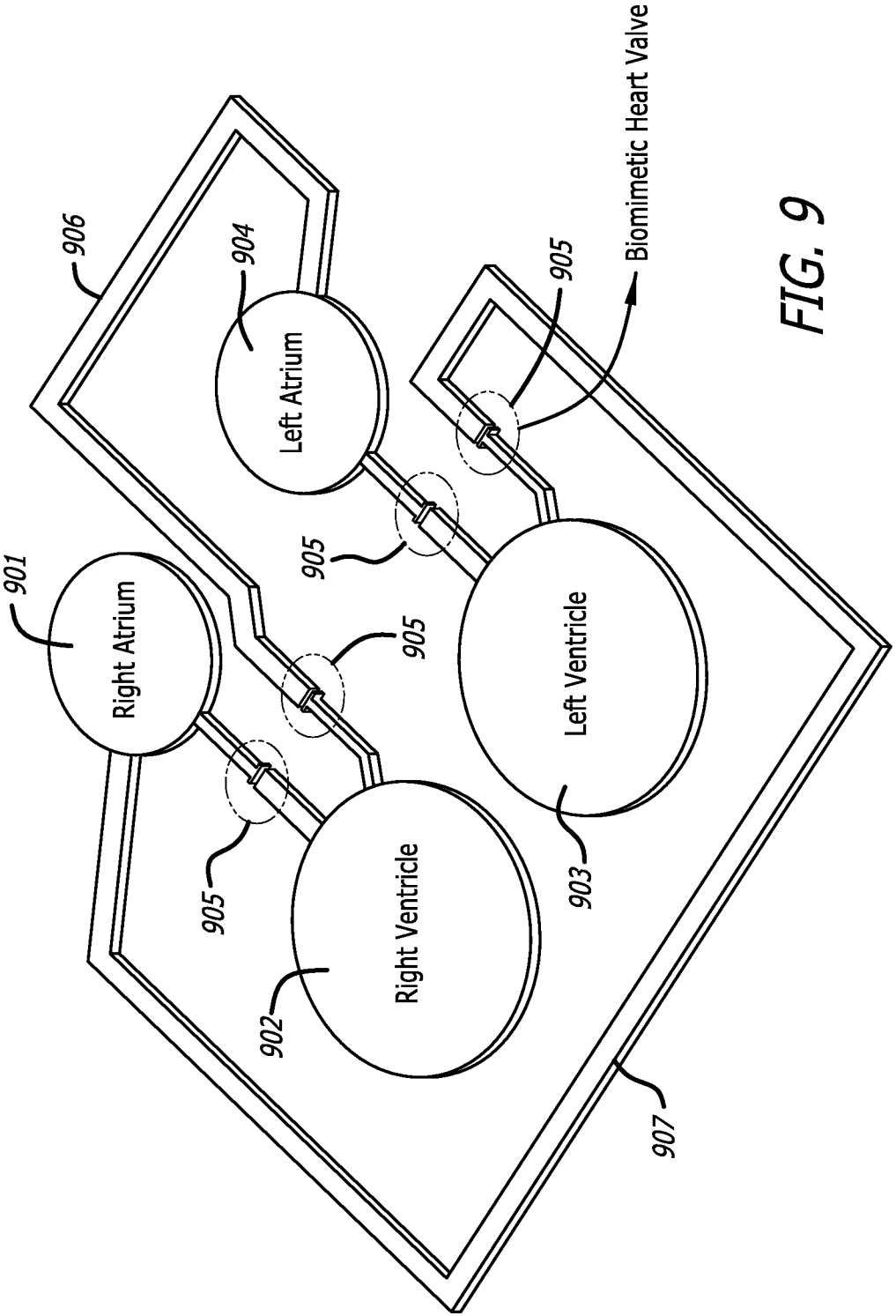


FIG. 10A

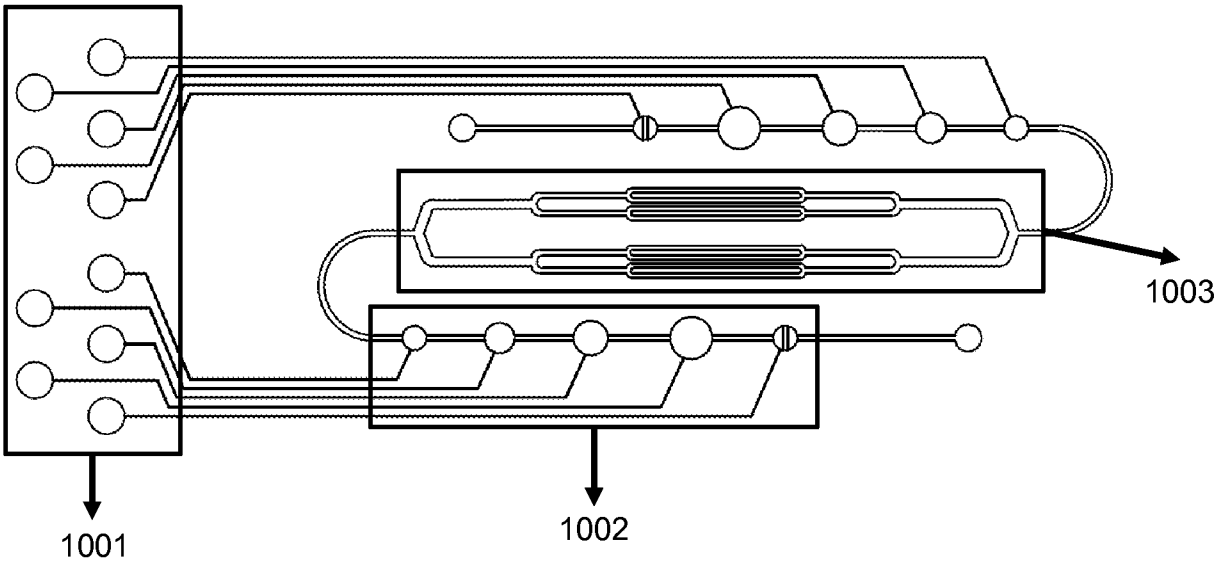
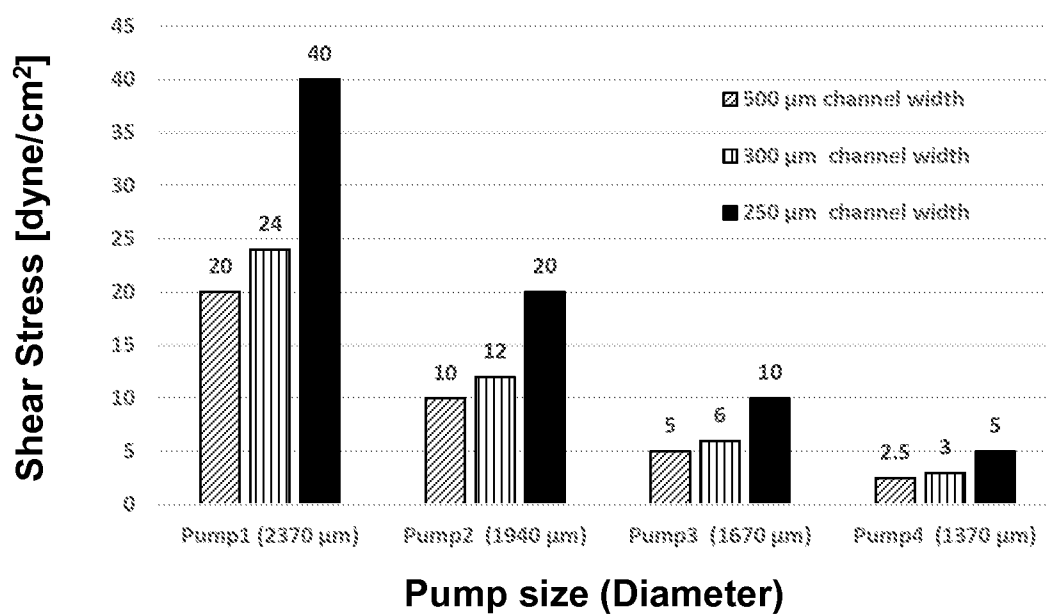


FIG. 10B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/039119

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12M3/06 B01L3/00 F16K99/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12M B01L F16K

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YO TANAKA ET AL: "An actuated pump on-chip powered by cultured cardiomyocytes", LAB ON A CHIP, vol. 6, no. 3, 1 March 2006 (2006-03-01), pages 362-8, XP055212560, ISSN: 1473-0197, DOI: 10.1039/b515149j the whole document	1-26
Y	KIM, J.; KANG, M.; JENSEN, E. C.; MATHIES, R. A.: "Lifting gate PDMS microvalves and pumps for microfluidic control", ANALYTICAL CHEMISTRY, vol. 84, 2013, pages 2067-2071, XP055212539, cited in the application figures 1,5 ----- -/-	1-26



Further documents are listed in the continuation of Box C.



See patent family annex.

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

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"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

16 September 2015

Date of mailing of the international search report

22/09/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Cubas Alcaraz, Jose

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/039119

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUAYING CHEN ET AL: "Cardiac-like flow generator for long-term imaging of endothelial cell responses to circulatory pulsatile flow at microscale", LAB ON A CHIP, vol. 13, no. 15, 1 August 2013 (2013-08-01), pages 2999-3007, XP055212563, ISSN: 1473-0197, DOI: 10.1039/c3lc50123j the whole document -----	1-26
A	EP 2 520 368 A1 (RHEONIX INC [US]) 7 November 2012 (2012-11-07) paragraph [0050] - paragraph [0053]; figures 7A-7F -----	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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