A Microfluidic Platform for the Automated Multimodal Assessment of Small Artery Structure and Function.

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science
Graduate Department of Mechanical and Industrial Engineering
University of Toronto

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Abstract

In this thesis, I present a microfluidic platform that enables automated image-based assessment of biological structure and function. My work focuses on assessing intact resistance arteries from the mouse cerebral vascular bed with a diameter of approximately 120µm in vitro. The experimental platform consists of a microfluidic device and a world-to-chip fluidic interconnect that minimizes unwanted dead volumes and eliminates the need for any liquid-filled peripheral equipment. The integrated platform is computer controlled and capable of fully automated operation once a small blood vessel segment is loaded onto the chip. Robust operation of the platform was demonstrated through a series of case studies that assessed small artery function and changes therein induced by incubation with the drug nifedipine, a dihydropyridine calcium channel blocker. In addition artery segments were stained for L-type calcium channels, F-actin and nuclei, from which structural information about cell alignment and shape was quantified.
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Preface

My thesis is organized in two chapters, each of which will result in a standalone first author publication. Chapter 1 describes an automated platform for staining and probing small blood vessels. Chapter 2 describes a reversible fluidic interconnect for elastomeric devices. In addition I have during my MASc thesis work made contributions that resulted in two second author publications,\textsuperscript{31,50} one poster presentation at an international conference (MicroTAS 2010) and one oral presentation at a North American society meeting (American Physical Society – Division of Fluid Dynamics Annual Meeting, 2010), one filed application for a provisional patent\textsuperscript{70}, and three invention disclosures.
Chapter 1
Multimodal Assessment of Small Artery Structure and Function

1. Introduction

In vivo, biological signals are exchanged across multiple length scales, from intra-cellular signaling cascades\textsuperscript{1-3}, to inter-cellular cell-cell interactions\textsuperscript{4-6}, up to signaling across multiple tissues and organs via hormones or electrically conducted signals in nerves. Along with signaling, biological structure and function at the cellular and tissue scales are dynamically changing and can undergo pathological changes that give rise to diseases such as hypertension,\textsuperscript{7, 8} and numerous other disease states. Thus, there is a fundamental need to comprehensively understand biological structure and function across multiple length scales, i.e., from cells to organs and ultimately to the physiology of an organism, in order to eventually detect and correct pathologies. Gaining a systematic understanding of biological systems across various length scales is challenging due to the variability, and complexity of different interactions across these length scales, and due to technological constraints that limit their systematic investigation in a scalable manner.

In general, \textit{in vitro} approaches used to study biological systems impose an input in the form of the microenvironment and probe a biological output (Fig. 1A). Probes for assessing biological outputs can often be categorized into two forms; real-time techniques such as bright field, DIC, phase contrast or live cell fluorescent imaging, and end-point techniques, such as fluorescent staining, histology, electrophoresis, mass spectrometry, or chromatography. A primary output for biological studies is drawn from staining samples to determine protein expression or localization. In general, staining requires multiple well
defined steps with long waiting periods in between, making it an ideal process for automation.

The ability to control input microenvironment conditions is highly dependent on technological constraints. Discussions of creating physiologically relevant environments started with static culture in dishes,9, 10 to dynamic cultures with flow cells11-13 to microfluidic lab-on-a-chip platforms,14 and at a much larger scale, organ perfusion systems.15 Each platform has specific advantages and disadvantages in their ease of use, scalability, and ability to control inputs at relevant scales.

Microfluidic setups have the specific benefit of providing control over the spatial and temporal microenvironment at scales that correspond with dimensions associated with cells16-18 and small tissue samples. As such, they have been used for cellular mechanobiology19-21, studying liver tissue function,22, 23 recapitulating aspects of mechanical and cellular interactions within organs24, 25, and investigating other complex biological interactions for cell culture,18, 26 3-D cell culture,27 the co-culture of multiple cell types, 28, 29 30 or whole tissue samples.22, 31 Microfluidic systems have also been used to automate cell culture,32, 33 integrate immuno-fluorescence protocols for cells34 in addition the ability to manipulate conditions locally has been used to locally stain histology samples,35 and single cells.17

This work is focused on the investigation of intact small blood vessels, specifically resistance arteries via various optical readouts. In vivo, resistance arteries contain three layers, the adventitia, media, and intima. The percentage of the wall volume made up of adventitia varies significantly within vascular bed, but in general, is made up of connective tissue, specifically collagen and elastin, and contains fibroblast, macrophages, mast cells, nerve
axons, and associated Schwann cells. The media contains a poorly defined external elastic lamina, smooth muscle cells, and a well defined inner elastic lamina. My work primarily focuses on investigation of the smooth muscle cells, which are typically circumferentially arranged in layers of 1-6 cells depending on artery diameter, with typical pitch < 2°. Finally, the inner intima consists of a continuous monolayer of endothelial cells, which also project outwards through the inner elastic lamina of the media to form contacts with the smooth muscle cells. These contacts enable communication between the layers of smooth muscle cells and endothelium.

Dynamically changing environmental conditions result in signal transduction within the artery that cause changes in ion flow, membrane potential and intercellular calcium, often resulting in changes to smooth muscle cells (SMCs) tone. However, in addition to constrictions, changing environmental cues can cause changes in protein expression, phosphorylation level, and their localization within the cell. These changes can also result in structural and functional changes which lead to the development of various cardiovascular diseases in humans. Currently, small arteries have been studied under a wide range of conditions, including in vivo studies, wire myographs, and cannulated setups. The current standard for in vitro physiological studies is the cannulation setup, which is similar to standard static cell culture in such that the artery is in a large fluid bath (~5ml volume) that must be manually exchanged (illustrated in Fig. 1B) according to the experimental protocol. This limits the ability to precisely control the local mechanical and chemical environment over time. More recently, there has been progress towards a microfluidic based platform which makes the direct study of small blood vessels more accessible, but also allows
enhanced environmental control by significantly decreasing fluid volume around the artery segment (~40nl) and through constant superfusing flow, provides the possibility of robust dynamic measurements.

I present an automated microfluidic platform for the routine investigation of small arteries. A fluidic manifold was designed and built which integrates off-chip components allowing enhanced functionality while maintaining low dead volumes and maximizing utilization of chip footprint. The resultant platform was used to probe various output signals beyond bright field diameter measurements for mouse cerebral arteries. Signals such as intracellular calcium provide an important link between the vasoactive substance and cellular activation, resulting in observable change in diameter. However, assessing local variations in the calcium concentration as well as other cellular properties (such as protein expressions) requires the routine application of cellular staining protocols. We demonstrate a flowable format for the automated implementation of different staining protocols for SMCs. The composition and temperature of the fluid stream that is flowing over the small blood vessel segment (superfusion) is pre-programmed according to a staining protocol. The approach can be extended to apply different conditions (for instance, varying drugs/vasoactive substances) along a single artery segment to subsequently stain (Fig. 1C), or even to other biologies, cell culture, or probing soft material constructs. The remainder of this paper contains a description of the platform, followed by two case studies showing robustness and demonstrating proof of concept.
2. Experimental

2.1 Microfluidic device design and operation

A schematic of the setup is shown in Fig. 2A. It interfaces large hydrostatic driven external reservoirs (5-10ml) and pressurized smaller wells (max 300µl) with on-chip flow control via computer controlled pneumatic valves (The Lee Company, Essex, USA). The fluidic device at the region of interest (Fig. 2B) contains a microfabricated array of posts that are located at the top and bottom surfaces of the channel, allowing a well defined area for fluid to pass over the artery (Fig. 2D). The resultant artery geometry enables a significant portion of the artery wall to reside within a single focal plane, allowing for high resolution imaging of real-time fluorescent signals within the artery wall (such as intracellular calcium). In addition, for the first time, smaller and more delicate olfactory arteries (outer diameter ~120µm) have been loaded and studied on a microfluidic platform (Fig. 2C). Olfactory arteries isolated from the cerebral vascular bed of mice are ideal for staining protocols since they are not surrounded by a dense extracellular matrix, thus allowing easier loading of fluorescent labels (eg. FURA-2). Microfluidic devices with a feature depth of 70µm were used to investigate mouse olfactory arteries. The posts around the artery segment are 20µm in diameter and 35µm deep, with 40µm center to center spacing at the inspection area (Fig. 2D). Channel width leading into and out of inspection area is 60µm wide.

Due to the cross-flow over the vessel through the narrow region defined by post height, a high superfusing flow results in a large pressure drop across the vessel and high shear. Pressurized fluidic wells in the device manifold (Fig. 2F) are used to drive fluid flow at low flow rates up to 0.5µl/min, producing max flow velocities of 3.1mm/s, with a pressure drop
of 8 Pa (0.06mmHg, or 0.1% of the transmural pressure used) in the inspection area across the vessel, and peak shear stresses at the artery wall of 5.7dyne/cm² determined from finite element models (Fig. 2E) (see Appendix B for details). This level of shear stress on the smooth muscle cells is within the range calculated to be experienced by smooth muscle cells in vivo due to interstitial flow out of the artery, and well below shear levels known to induce constriction,44, 45 or production of factors such as nitric oxide and heme oxygenase.46

Standard microfluidic devices which apply well-based flow schemes often build the well directly into the microfluidic device. However this strategy consumes valuable space within the device footprint. In addition, off-chip connections to external control structures (such as valves) often require lengths of tubing which add dead volumes that can potentially limit response times. Utilizing a novel interconnect strategy for PDMS devices (see Chapter 2), a manifold was developed that incorporates pressurized wells, external fluidic inlets and outlets, and pneumatic valves (Fig. 2F). The fluidic manifold minimizes the amount of liquid-perfused peripheral equipment needed, is robust and easy to use. Sample volumes of up to 300µl that contain vasoactive substances, buffers or staining reagents can be preloaded into the wells, and can be changed/refilled during the experiment by stopping the superfusion. However, at 0.5µl/min there is sufficient volume to have constant flow from a single well for more than 10 hours. Flow from the wells are controlled with on-chip valves47 (Fig. 2G) allowing the selection of fluid from one of the wells to be applied to the abluminal environment. The on-chip valves are liquid filled to prevent the growth of bubbles within the fluid channels, and controlled by pneumatic valves integrated into the manifold as shown in Fig. 2F.
The temperature on chip is controlled via a PID controller (TE Technology, Traverse City, MI, USA), connected to a thermoelectric element (TE Technology) which either heats or cools the attached to a sapphire disk (UQG Optics, Cambridge, UK) in response to a temperature measurement provided by a thermistor (TE Technology) bonded to the disk with thermal epoxy (Omega, Quebec, Canada). The high thermal conductivity of sapphire ensures a uniform temperature distribution across the sapphire disk and therefore, below it, the microfluidic device’s region of interest. However, this configuration requires the heat to be conducted from the sapphire disk through a ~3mm thick layer of the poly(dimethylsiloxane) (PDMS) to the region in which, the blood vessel segment is hosted. Thus, the temperature at the small artery location is measured by lithographically patterned resistors (Fig. 2H). The measured temperature provides the input signal to the PID controller that is operated at a set-point of 37.5±0.5°C (see Appendix A). Temperature, valve actuation and pressure control were programmed in Labview using the DAQ6008 (National Instruments, Austin, TX, USA) for input/outputs. The custom made program and is capable of both manual operation via a user interface, or automated control using input from a text file encoding the sequence of events (see Appendix D).

2.2 Device fabrication

All devices (shown in Appendix E) have been fabricated in PDMS using standard multilayer soft lithography techniques. The fluidic layer was formed by spin coating PDMS onto the SU-8 master to create a thin membrane (~400µm thick) required for the on-chip valves. The thicker valve actuation layer was subsequently bonded to the fluidic layer via partial curing techniques previously described, and the resultant PDMS device was bonded via O₂ plasma
to a thin PDMS slab (<1mm) which contained the bottom organ bath post structure. Non-bonded regions required for the on-chip valves were obtained by selective spotting of CYTOP (Asahi Chemical Company, Japan) in the desired locations under a stereomicroscope. A gold coated (100nm thick) glass slide (EMF, Ithaca, NY, USA) was etched to create a resistance temperature detector (RTD) used to locally measure the temperature at the glass surface. A thin layer of PDMS was spin-coated (2000rpm) on the glass slide, cured and bonded to the PDMS device via O₂ plasma (Harrick Plasma, Ithaca, NY, USA). The actuation lines were liquid-filled to prevent bubble generation during periods of prolonged actuation, by submerging the completed device in de-ionized water (DI) and degassing under a 20 inches of mercury (in Hg) vacuum for 2 hours. The device was subsequently stored in a vial of DI water until use. After each experiment, the device was flushed with DI water and stored in a vial of DI water to prevent drying and to ensure the valves remain liquid-filled until the next use. Devices have been successfully used after storage in this fashion for more than 1 month.

2.3 Small artery isolation and testing

The microfluidic device was loaded into the manifold, flushed with 1% Bovine serum albumin (BSA), allowed to sit for 5 minutes and subsequently flushed with a 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, with the bubble traps running to eliminate on-chip bubbles. Cerebral olfactory arteries isolated from C57black 6 mice (Charles River, MA, USA) were loaded on chip as previously described for mesentery arteries, using the fluidic setup shown in Fig. 2A. With this setup, the olfactory arteries were pressurized to 45mmHg via the perfusion line with hydrostatic head. Superfusing flow
of MOPS buffer was kept at a constant flow rate of up to 0.5µl/min via pressurized head supplied by digital pressure regulators (MarshBellofram, WV, USA) on the fluidic wells. Fixation pressure was kept at 45mmHg below atmospheric pressure via hydrostatic head, and vessels were heated to 37.5°C with steps of 3 degrees each held for 5 minutes. Bright field and fluorescent images (model TI Eclipse, Nikon, Japan) were carried out at a total magnification of 200× unless otherwise specified.

Phenylephrine (Sigma Aldrich) was used to evaluate vessel function, using premixed solutions loaded into the appropriate well. In experiments, changes in functionality as a result of a drug were shown by incubating the calcium blocker Nifedipine (Sigma Aldrich) for 30 mins, at a concentration of 1µM. Nifedipine was freshly mixed before each experiment and protected from light throughout experiment until imaging. Artery diameter was evaluated from recorded images using custom software (MATLAB) previously described31.

2.4 Small artery staining

2.4.1 Calcium

Artery segments were stained with 2µM FURA2-AM (Sigma Aldrich) for 90mins at 37°C. Stained arteries were imaged with an EMCCD camera (Evolve512, Photometrics, Tuscon, AZ) and ratio values were extracted in MATLAB by averaging pixel intensities over a selected region of interest which was kept the same for both 340nm and 380nm images over the entire time series. In addition dose responses before and after FURA-2 staining, did not show significant differences, indicating extended periods of superfusion (greater than 2 hours) did not measurably affect artery function.
2.4.2 Cytoskeleton

Artery segments were fixed with 4% methanol free PFA at 4°C for 30mins, permeabilized with 0.5% TritonX (Sigma Aldrich) for 30mins, blocked with 1%BSA for 30min and co-incubated with Alexafluor 594 Phalloidin (Invitrogen, Grand Island, NY, USA) for F-actin and DAPI (Roche Mississauga, Canada) for nuclei for two hours at room temperature, and individually imaged on a Nikon Ti Eclipse at 40x (ELWD NA0.6) with a Qimaging Exi Blue fluorescent camera.

2.4.3 Immunofluorescence

Immunofluorescence for L-type calcium channels was carried out using 1:50 dilution Anti Ca_{v}1.2 (Alomone Labs, Israel) for 10 hours, 1:1000 dilution Alexafluor568 Anti-rabbit IgG (Invitrogen) for two hours, and the same fixing/permeabilizing/blocking protocol as actin staining described above. Negative controls were conducted with preincubation of antibody with antigenic peptide supplied with antibody (details available in appendix B).

3. Results

Two case studies were conducted to demonstrate robust operation of the above described platform to probe biological processes within small arteries.

In the first series of tests, small artery viability and change in functionality in response to a drug was demonstrated.

The artery function was evaluated by observing the degree of constriction in response to chemical stimuli via phenylphrine. Phenylephrine exerts a dose dependant affect on the SMCs wrapping around the artery by binding to the adrenergic receptors, which leads to a
downstream increase in intracellular calcium and arterial constriction. The artery was subsequently incubated with the L-type calcium channel blocker Nifedipine and the effects were observed.

To directly observe calcium activity within the smooth muscle cells, the small artery was stained with FURA-2AM as shown in figure 3A. Plotting the FURA ratios (which is directly related to the calcium concentration) with the diameter shows the direct relationship between calcium within the cells and the final arterial tone as a result of phenylephrine stimulation (Fig. 3B). Phenylephrine binds to adrenergic receptors on the smooth muscle cells, resulting in downstream release of calcium from internal stores and opening of voltage gated calcium channels. The subsequent increase in internal calcium causes the buildup of tension within the cells, which when coupled around the entire artery segment results in the artery constriction. Incubation with a calcium blocker reduces the calcium inflow currents when stimulated (Fig. 3C), resulting in lower smooth muscle cell activation, thus producing smaller constrictions with the same phenylephrine concentrations. The reduced calcium activity resulted in a significant shift in the dose response curve (Fig. 3D), demonstrating the reduced function of the small arteries when incubated with the L-type calcium blocker Nifedipine as expected. This result demonstrates the ability to robustly assess small artery function, and investigate the effects of various drugs in real time.

The second case study set out to demonstrate successful staining of components within the smooth muscles cells of the small artery segment. Remodeling of cytoskeleton proteins have not only been implicated in the constriction response but are also a factor in arterial remodeling. In addition, imaging of entire smooth muscle cell bodies over time can show
cell lengthening, shortening and movement relative to each other. In addition to applications investigating the cytoskeleton, the ability to visualize other proteins can be crucial to the study of functionally pathways, and identifying membrane receptors. Thus, as proof of concept, F-actin and cell nuclei were stained to show that the artery segment has similar morphologies to that found in cannulated setups, and presumably in vivo. In addition, the L-type calcium channels blocked in the first case study were stained and visualized via immunofluorescence.

A quantitative observation of nuclear eccentricity and alignment was conducted from DAPI stained artery segments (Fig. 4A). Eccentricity was quantified by fitting an ellipse to the stained nuclei (see Appendix B). The fitted ellipse was also used to quantify alignment by drawing a vector along the major axis as shown in figure 4b. The results show an average eccentricity of 0.991±0.006 (Fig. 4C), and a pitch of -0.3°±3.4° (n=98 cells from 6 vessels) off the circumferential axis (Fig. 4D) which is consistent with past results. In addition, F-actin staining was demonstrated (Fig. 4E). Qualitative observation of results along with alignment and shape of cell nuclei show no conformational change around the post structures in the organ bath. Thus, at short time scales (experiments up to 4 hours prior to staining) the posts do not appear to significantly affect vessel structure. As expected inspection of individual channels (shown in appendix B) clearly show exclusion of actin stain from nuclei.

In addition to structural proteins, immunofluorescence of the voltage-gated calcium channel (Ca, 1.2), which were the target of the above discussed calcium blocker, was carried out (Fig. 4F). These results show strong expression of the channel throughout the smooth muscle cells of the small artery.
4. Conclusion

We have developed a robust platform for investigating small artery structure and function using a variety of measurement techniques, including bright field diameter measurements, live cell staining for visualization of ion movement into and out of cells, and end-point staining requiring cell fixation and permeation. The method described above makes use of a custom manifold to integrate off chip components into an easy to use platform using a computer controlled system to fully automate experimental procedures once the artery was loaded on chip. The platform robustness and ease of use was demonstrated by conducting two case studies. The first was to show viability and changes in vessel function as a result of an applied drug. This was demonstrated with cerebral olfactory arteries which have an average diameter of approx 120µm. In addition, FURA staining was carried out to visualize calcium currents in the artery segment. The second case study involved a variety of stains to evaluate small artery structure on-chip. Cell orientation and shape match previous studies and show no changes around post regions, indicating that the post structure does not cause significant effects over short time scales. Finally, immunofluorescence of the calcium channels targeted in the first case study was conducted showing the successful ability to visualize proteins within the artery segment. These experiments were carried out using reagent concentrations similar to those used with the standard cannulation setup. However total reagent consumption was an order of magnitude less (currently max 300µl over a 10 hour period, as opposed to 5ml cannulation dishes) but an increase in fluidic resistance of the superfusion line can significantly reduce flow rates and thus reagent consumption even more. In addition, fluid evaporation over long staining periods is not an issue as a result of constant flow.
In addition to these experiments, the microfluidic platform can be harnessed to take advantage of the enhanced spatial and temporal control provided by the reduced scales to probe the small artery with heterogeneous microenvironment as previously shown with microfluidic devices for cells and embryos. The low dead volume integration of pneumatic valves and on-chip flow control can allow rapid flow actuation to provide dynamic changes. In addition, precise control of fluid flow, allows the investigation of processes which have been theorized, but have never been testable due to technical challenges (such as interstitial flow as a result of transmural pressure).
Figure 1. (A) Generalized experimental process. Exposing a biological system to a modulating environmental conditions and observing resultant behavior in either real time (diameter change of ion movement) or as an end-point analysis (protein expression, phosphorylation level, or localization) at specified time points, revealing information about biological systems. (B) Schematic illustration of experiment. The artery is subjected to varying substances (colour) at specific environmental conditions, and stopped at some state (by fixing, pink) to stain, and subsequently imaged via fluorescence. (C) On chip, small artery is constantly surper fused, allowing well defined changes to environmental conditions. Also enables automated changes to superfusing solution.
Figure 2. (A) Schematic of experimental setup. Superfusion and perfusion pressures are set via computer controlled pressure regulators. Fixation pressure and superfusion outlets controlled via hydrostatic head. Computer controlled pneumatic valves select (6) between high and low pressure for operating on-chip valves (3). Local heating (5) via sapphire disk, TE element in feedback control with a thermistor and on-chip resistors (4) (B) Schematic of inspection area. (1). Scale bar is 200µm (C) Bright field image of olfactory artery loaded on chip corresponding to box outlined in B. Scale bar is 40µm (D) Cross section of inspection area showing post structure above and below artery segment to allow fluid flow across vessel. (E) Numerical simulation of shear rate as fluid superfuses artery segment. (F) Manifold containing fluidic wells, pneumatic valves, and connectors for threaded connectors enabling robust connections and device operation. (G) Image of on-chip valve. Red dye in the fluid layer, green dye in the valve actuation layer, and gas removal region. Scale bar is 40µm (H) Image of inspection area showing location of temperature measurement electrodes. Scale bar is 500µm.
Figure 3. (A) False coloured Fura-2 stained small artery. Scale bar is 40µm (B) Traces of time dependant diameter and Fura-2 ratio to increasing PE concentrations. PE concentration plotted on a Log scale (C) Change in calcium responses before (Blue) and after Nifedipine incubation (red). (D) Dose dependant response to phenylephrine and effect with Nifedipine (* indicate p<0.05). n=5
Figure 4. (A) Artery segment on-chip with stained nuclei. Long thin nuclei aligned perpendicularly to axial axis are smooth muscle cells, larger nuclei aligned parallel to axial axis are endothelial cell nuclei (see appendix B). (B) Zoomed in region of A, showing example best fit ellipse of SMC nuclei used to calculate eccentricity based on ratio of minor and major axis. Red vectors along the major axis indicate pitch of the smooth muscle cell. Scale bar 5µm. (C) Histogram and box and whisker plot with individual data points of nuclear orientation. Vertical line in box and whisker plot indicated median value, with box span distance between 25th and 75th percentiles (interquartile range, IQR). Whiskers (error bars) span distance between the 10th and 90th percentiles. Data points in black indicate outliers, defined by points that lie outside 1.5*IQR. (D) Histogram and Box and whisker plot of nuclear eccentricity. (E) Co-stained F-actin and nuclei of on-chip artery segment. (F) Immunofluorescence of Cav1.2 voltage gated calcium channels. All scale bars are 40µm unless otherwise specified.
Chapter 2
Reversible World-to-Chip Interface for Elastomeric Microfluidic Devices

1. Abstract

We present a robust fluidic world-to-chip interface for elastomeric microfluidic devices suitable for standardization. The reversible interface does not require any additional steps or modifications to the device fabrication sequence, is compliant with variable device thicknesses (tested between 1.5mm and 4mm and up to ±10% variation in height across a single device), and has been tested without failure for inlet pressures of up to 2 bar. We outline the working conditions of the fluidic world-to-chip interconnect. As a case study, we use the world-to-chip interconnect in the context of a microfluidic device for the investigation of small blood vessels. Microfluidic devices have been successfully connected and disconnected more than 50 times over a period of more than 2 months. The robustness, consistent operation and simplicity of the presented fluidic interconnect make it a suitable candidate for a wide range of possible applications of microfluidic devices and allow for standardization and automation.

2. Introduction

During the past decade, microfluidic technologies have contributed to addressing a range of important questions in areas that include analytical chemistry, clinical diagnostics, biology, regenerative medicine, materials science, and energy. Soft lithography and the ability to rapidly prototype elastomeric microfluidic devices in poly(dimethylsiloxane) (PDMS) have played a particularly important role, and its use widespread in academic research laboratories. In the majority of applications, the operation of microfluidic devices requires
peripheral components that are located either upstream (e.g., pumps) or downstream (e.g., analytical equipment), or even an in-series configuration of multiple devices. Many of the solutions for elastomeric devices exhibit limited scalability, require additional device fabrication steps, exhibit inconsistent performance, or require highly customized manifolds (Table 1). Robust, well-characterized interconnects are a well-known challenge for PDMS devices. The majority of previously demonstrated fluidic interconnects are limited to rigid substrate materials (Table 1).

The current lack of a robust interconnect method capable of handling the large tolerances associated with manually replica-molded PDMS devices (as exemplified by varying device thicknesses and, to some extent, thickness uniformities) presents a significant hurdle to transition from proof-of-concept solutions to laboratory processes or even commercial products with well-defined performance requirements.

Figure 1A outlines requirements for a fluidic interconnect for elastomeric devices where they would arise in a typical process flow starting with device fabrication to usage in the context of a biological experiment. Specifically, the interconnect should (1) be scalable without the need to manually connect individual inlets or outlets, (2) be reversible, (3) exhibit robust and consistent performance over time and over multiple sealing cycles, (4) be easy to use, (5) have a small footprint, (6) allow for integration with various upstream and downstream components via standardized components, (7) not impose any limitations to the device design or fabrication process and (8) be compatible with different imaging modalities.
3. **Sealing mechanism**

The proposed interconnect produces robust seals by focusing an externally applied force, $F_A$, locally around the proximity of a fluidic inlet or outlet. Local application of the force is achieved by compressing a cylindrical rim, with contact area $A_{rim}$, positioned coaxially with respect to a fluidic inlet or outlet. Figure 2A shows a cross-section of the configuration and Fig. 2B shows the corresponding stresses that were obtained from a numerical simulation (Comsol 4.2, Burlington, MA, USA).

The edges of the cylindrical rims compressing against the device surface causes stress concentrations in the elastomeric device resulting in peak normal stresses approximately 2.5 times larger than the average stress applied to the rim surface, $\sigma_{seal}=\frac{F_A}{A_{rim}}$ (Fig. 2C), and therefore forming a robust seal. In addition, localizing $F_A$ to a small fraction of the total device surface purposefully confines the deformation of the elastomeric material to the sealing region.

The seal fails if the internal fluid pressure, $P_i$, increases to a level where it deforms the PDMS surface within the cylindrical rim to an extent that the normal stress at the interface between the device and the rim vanishes. Increasing $F_A$ will therefore be required to consistently seal at increased pressures. Note that for $P_i>200$ kPa (gauge pressure), plasma bonded PDMS devices (using plasma generated under high vacuum) show a decreased yield and start delaminating.\textsuperscript{48, 58} Unnecessarily high values of $F_A$ might compress the device to an extent that internal channel networks (partially) collapse, especially for device thicknesses $<2$ mm. Understanding how the obtained seal is related to $F_A$, $A_{rim}$ and $P_i$, is therefore an important requirement for the design and consistent application of the proposed fluidic interconnect.
4. Experimental

4.1 Design and fabrication

4.1.1 Interconnect

The fluidic interconnect was designed in Autodesk Inventor (Autodesk, Inc., CA, USA), CNC milled in aluminum and anodized to protect from corrosion. The sealing rim had a surface of 20 mm² (rectangular with inner dimensions 8 mm × 11 mm, outer dimensions of 9 mm × 12 mm and 1 mm tall).

4.1.2 Microfluidic Device

PDMS microfluidic devices (2-4 mm thick) were fabricated using standard soft lithography and plasma bonded (Harrick Plasma, NY, USA) to standard 1mm thick 1” × 3” glass slide (VWR). Holes for fluidic inlets were manually cored with 20 gauge blunt needles producing holes approximately 1mm in diameter. The hole quality does not impact sealing performance as long as the hole itself or cracks in its proximity do not extend beyond the rim’s outer perimeter.

4.2 Sealing validation

The sealing mechanism was quantitatively evaluated by applying a constant force $F_A$ up to 30 N, corresponding to $\sigma_{\text{seal}}$ of 1.5MPa. The force was established using a pneumatic cylinder (McMaster-Carr, NJ, USA) which was attached to the interconnect co-axially with the sealing rim via threaded connector. The pressure applied to the pneumatic cylinder was measured with a 100psi pressure gauge (McMastercarr, USA). Compressed air and de-ionized water were used as the working fluid and applied through the interconnect to a microfluidic device containing a single meandering channel. A pressure transducer (30PSI
full scale, Honeywell, NJ, USA) was connected to the outlet of the microfluidic device as shown in Fig. 3a to determine \( P_i \). The sealing force \( F_A \) was subsequently decreased until a leak was detected by monitoring \( P_i \).

5. Results

Figure 3b shows the obtained experimental results. In line with our expectations, we found no difference between the results obtained for compressed air and distilled water. A linear trend \( y = a \cdot x + b, \ a = (176 \pm 13) \times 10^{-3}, \ b = -(24 \pm 10) \times 10^3 \text{ Pa} \) was observed between the average applied stress \( \sigma_{\text{seal}} \) and maximum fluid pressure resulting in seal failure \( (P_{i,\text{max}}) \). Measurements were also performed for higher applied stresses, maintaining seals beyond 200kPa. However beyond 200kPa the PDMS-glass bonds were inconsistent and are thus of limited relevance for plasma bonded PDMS devices.

5.1 Application

The simplicity of the interconnect design allows for easy integration with various upstream and downstream components via standard fluidic connections (e.g. standard Upchurch connectors). During the characterization experiments, pneumatic cylinders were used to apply a uniform force \( F_A \) coaxially with one fluidic connection. However, microfluidic devices typically require a number of fluidic inlets and outlets. Methods other than pressurized cylinders including springs,\(^{59}\) or screws may also be employed to provide the required sealing force, \( n \cdot F_A \).

To illustrate the utility of the fluidic interconnect, two implementations were applied to a microfluidic device that allows the structure and function of small blood vessels to be
investigated (Fig. 4A) as previously described. Briefly, all channels of the device were initially primed with a physiological buffer. Bubbles were removed using on-chip bubble traps located upstream of the artery segment and supplied with a vacuum of 20 inches Hg. An artery segment dissected from C57 black 6 mice (Charles River, Montreal Canada) was manually inserted into the loading well, and drawn up the loading channel to the inspection area with fluid flow. Upon reaching the desired position in the inspection area, the artery segment was reversibly held in place by applying a subatmospheric hydrostatic pressure of 6 kPa at the fixation outlets e1 and e2. Once the loading well is sealed, the pressure at the perfusion inlet (a) is increased to set the pressure across the arterial wall \( P_{\text{luminal}} - P_{\text{abluminal}} \) to 8 kPa. The artery segment was then heated to 37\(^\circ\)C and a vasoconstrictor (phenylephrine, PE) was applied abluminally at stepwise increasing concentrations. The concentration changes were achieved by diffusively mixing two initially separate streams, a buffer solution (b2) and drug containing solution (b1), at a location upstream of the artery segment such that a constant flow rate of 3 ml/h was maintained (inlet pressure ~75 kPa). A pressurized and heated artery segment on-chip is shown in Fig 4B. The observed constriction of the artery was recorded in a sequence of brightfield images and a custom MATLAB (Mathworks, MA, USA) based program extracted the time-evolution of the outer arterial diameter, shown in Fig. 4C and indicative of small artery function.

Prior to the development of the presented fluidic interconnect, standard pin and epoxy techniques were used and lead to inconsistent performance and a significantly reduced the experimental yield. To increase device performance, a requirement that is particularly important when working with intact tissue from animals, a manifold was designed that is
based on the described sealing strategy. Figure 4C shows a first-generation design where the sealing force is provided by four manually operated screws. While this provides consistent sealing performance, the imprecise force application (both force distribution across manifold and total force applied) can initially lead to device cracking until the user develops a feel for how tight the screws should be. This problem can be completely eliminated by taking the user out of the equation, and developing a method for consistent and automatic application of the sealing force. However, it must also retain an independence of device thickness, eliminating the option of a spring based method. Figure 4C shows a second-generation design where the sealing force is applied via pneumatic cylinder according to the results discussed above. This implementation allows precise and repeatable sealing forces to be evenly applied which enable robust operation by completely eliminating device cracking due to over tightening of the manifold with screw based mechanisms. In addition, the pneumatic actuators provide constant forces for any device thickness within the stroke length of the cylinder (selected cylinder allows ±2mm tolerance in device thickness). In both versions, a base plate holds the microfluidic device with a 1” × 3” footprint, while providing visual access for an inverted microscope. The two separate sealing brackets are connected to syringe pumps or external fluid reservoirs via standard connectors (either adhesively connected Nanoport or threaded connectors) (Idex Scientific, OR, USA). The fluidic connectors can be pre-assembled and do not need to be routinely removed or replaced. In both cases, the device has two regions to be separately sealed. One region contains 8 fluidic inlet and outlets, the second contains a single large well (~8mm diameter) for artery loading which must be separately sealed to enable pressurization of the artery segment.
The demonstrated manifold based approach has significantly increased the overall experimental yield previously dominated by failure of the pins and epoxy based interconnects. As a result, device lifetimes increased and experiment failure due to leaking interconnects was completely eliminated, allowing routine investigation of small blood vessels. In addition, the pneumatically actuated manifold demonstrates sufficient consistency to enable computer control for applications involving complete automation. In such a situation, robotics can be used to position devices in the base. The interconnect piece can be automatically lowered and sealed using pneumatics (as demonstrated above). Computer controlled pumps can purge and run devices. In addition fluidic inlets can be replaced with fluid wells which can also be robotically addressable.

6. Conclusion

We have developed and characterized the working parameters of a robust reversible world-to-chip interconnect for elastomeric devices. The interconnect works by localizing the applied forces to generate peak normal stresses 2.5 times larger than the average seal stress as a result of stress concentration from the edges of the sealing rim around the input/output holes. A relationship between the applied stress and the maximum fluid pressure was obtained. Our fluidic interconnect meets all the criteria established above. (1) The manifold design is scalable to allow for uniform well defined seals across multiple in/outlets with a single interconnect. (2) Sealing/unsealing does not damaging the microfluidic device. It (3) maintains consistent sealing performance after >50 seal/unseal cycles involving complete disassembly, cleaning and reassembly. Also allows the same PDMS microfluidic device to be used daily for more than 2 months without any special precautions. (4) The manifold does
not require any dedicated fabrication steps for the fluidic device and requires no specialized skill or handling to use. Current manifolds in use (5) have a small footprint, allowing centre-to-centre spacing of 5mm between adjacent inlets/outlets (6) allow for integration with a variety of upstream/downstream components, and (7) does not interfere with bright field or fluorescent imaging within the visible regions.
<table>
<thead>
<tr>
<th>Interconnect type</th>
<th>Description</th>
<th>Max pressure [kPa]</th>
<th>Reusability</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS gasket</td>
<td>PDMS device compressed between two flat rigid surface(^6(^1)(E))</td>
<td>463</td>
<td>Yes</td>
<td>• Elastomeric surface compressed against rigid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• High forces required generate seal</td>
</tr>
<tr>
<td>Press Fit</td>
<td>Pins inserted into manually cored holes(^6(^2)(E))</td>
<td>11 – 700</td>
<td>Limited</td>
<td>• Elastic surface against rigid.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Seal strength inconsistent, dependant on PDMS coring</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Manual assembly</td>
</tr>
<tr>
<td></td>
<td>Rigid tube inserted into embedded O-ring (^6(^3)(R))</td>
<td>750</td>
<td>Yes</td>
<td>• Elastic surface against rigid surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Sealing force not defined</td>
</tr>
<tr>
<td></td>
<td>Soft tubing compressed against device (^6(^4)(R))</td>
<td>200</td>
<td>Yes</td>
<td>• Elastic surface against rigid</td>
</tr>
<tr>
<td></td>
<td>Pins inserted into manually cored holes, reinforced by expoy(^6(^0)(E))</td>
<td>6.8-500</td>
<td>No</td>
<td>• Adhesion of epoxy inconsistent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Manual assembly</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Auto-fluorescence associated with epoxy</td>
</tr>
<tr>
<td></td>
<td>Molded, micro-scale luer lock connector (R)</td>
<td>&gt;300</td>
<td>Yes</td>
<td>• Modular microfluidic breadboard(^6(^5))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Implemented commercially &gt; Microfluidic Chip-shop</td>
</tr>
<tr>
<td></td>
<td>Device molded into rigid casing (^6(^6)(E))</td>
<td>N/A</td>
<td>Yes</td>
<td>• Entire device must be fully encased, requires highly customized manifolds</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Increases cost of single device</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Implemented Commercially &gt; GeSim</td>
</tr>
<tr>
<td>O-Ring</td>
<td>Compression between o-ring rigid tubing, manifold, and elastomeric device(^6(^7)(E))</td>
<td>&gt;1000</td>
<td>Yes</td>
<td>• Large footprint</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• O-rings must be removed to clean/sterilized</td>
</tr>
<tr>
<td></td>
<td>Spring-loaded o-ring seals against rigid surface with well defined force(^5(^9)(R))</td>
<td>2070</td>
<td>Yes</td>
<td>• Need 4 components to form seal</td>
</tr>
<tr>
<td></td>
<td>PDMS molded rings forming O-ring style seal (^6(^8)(R))</td>
<td>&gt;600</td>
<td>Yes</td>
<td>• O-rings must be removed to clean/sterilize</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Dedicated fabrication steps required to apply to PDMS devices</td>
</tr>
</tbody>
</table>

E- Elastomeric substrates (e.g. PDMS); R- Rigid substrates (e.g. Silicon, Glass, Thermoplastics etc.)
Figure 5. (a) Typical experimental process for biological experiments outlining interconnect requirements at its relevant time positions.
Figure 6. (A) Schematic illustration of the proposed sealing mechanism with cross-section of fluidic interconnect (black) at fluidic inlet with internal pressure $P_i$. (B) Simulation of local displacements of region outlined in box in (A), from an un-deformed reference state (contour lines, with numbers indicating deformation in microns) and $y$ component of the stress tensor for a 4 mm thick PDMS substrate and $\sigma_{\text{seal}} = 0.5$ MPa, $P_i = 0$ Pa. (C) Normal stress for various positions across the sealing surface of the rim according to results in (b).
Figure 7. Sealing test (a) Schematic of experimental setup for characterization of interconnect operation. Fluidic input into the manifold is via threaded ports machined into manifold and standard fluidic connectors. (b) Experimental validation of sealing mechanism showing relationship between fluid pressure and applied seal.
Figure 8. (A) Schematic of microfluidic approach for the investigation of intact small blood vessels.31 (B) Artery segment loaded on chip. Arrows indicate flow directions, with letters corresponding to labeled inlets in (A) Scale bar is 200µm. (C) Dose dependent constriction of artery segment in response to phenylephrine (PE) \((n = 6)\). Best fit line shown assuming an underlying sigmoidal behaviour. (B) Fluidic device in assembled manifold with manual force application. Tubes connected via standard nanoport connectors attached to the top surface. Force to seal device applied manually with screws Scale bar is 25mm. (E) Manifold with pneumatic actuators to provide a well defined sealing force. Threaded ports are machined into manifold for fluidic connections.
Chapter 3
Conclusion and Future Direction

The work described in this thesis presents a complete platform for automated analysis and probing of biological systems. In the context of the presented application, the platform not only allows for probing small arteries under dynamic conditions never before possible, it also presents a tool to make the current study of small arteries more efficient. The set-up process to load the artery on chip requires less manual manipulation compared with cannulated setups, which markedly increases as it is applied to even smaller and more delicate arteries, veins or lymphatic vessel. In addition once the artery segment is loaded on chip, all protocols can be fully automated, eliminating the need for human intervention. The manual nature of cannulation and significant training time required has previously been a significant barrier to entry. Thus, the significant reduction of manual processes required in the presented platform can be leveraged to introduce direct probing of small blood vessels into various settings. For example, the platform can be utilized in clinical settings to test artery segments from patients for applications in personalized medicine for hypertension, diabetes and various cardiovascular related diseases. In addition it can enable direct testing of tissue samples ex vivo for drug discovery.

In basic research, the platform can be used to investigate biophysical responses, such as calcium dynamics\textsuperscript{69}, or the confined fluid volume would allow for introducing collagen matrix on chip\textsuperscript{27} to embed the artery segment in an extracellular matrix. However the platform outside the presented microfluidic device is not limited to the study of small
arteries. The platform can be interfaced with other devices to investigate a wide array of systems, from cell cultures to soft material handling and testing.

Finally, the presented interconnect can be implemented in any application for elastomeric devices. It provides a robust and reversible seal, which does not destroy devices and in my experience has significantly increased device lifetimes, allowing a single PDMS device to be used daily for more than a month. The described interconnect requires a manifold to be implemented which allows for integration of sensors and various off-chip components into a single component. A well designed manifold can eliminate the need for fluid filled peripheral equipment, and reduce end user complexity while also being generally usable for a large variety of microfluidic devices with the only requirement being consistent inlet and outlet arrangements.
References


35. R. D. Lovchik, G. V. Kaigala, M. Georgiadis and E. Delamarsh, *Lab on a chip*.


70. US Provisional Patent 61/482,925 “Connector for Microfluidic devices”. May 2011
Appendices

A. On-chip Temperature

The technique used to provide a uniform temperature field over a well defined region in elastomeric devices while allowing visual access to the area has been previously described. However, briefly, a thermoelectric element (TE tech) in feedback control with a thermistor is used to either heat or cool a sapphire disk ($k=32\text{Wm}^{-1}\text{K}^{-1}$) which acts as a heat spreader. This creates a uniform temperature distribution at the device surface. However, heat must subsequently diffuse through the device. As such, temperature sensitive metal electrodes were introduced to provide feedback control at the fluid plane.

Figure A1 – Simulation of temperature field at glass surface through 3mm PDMS device. \(^{31}\)

A.1. Thin film temperature sensor

As a result of thermal diffusion from the controlled sapphire disk to the fluid plane, measurement of the temperature at the artery segment was required. Electrodes were etched out of 100nm thick gold coated glass slides with 10nm Titanium adhesion layer. Thin film
electrodes show a temperature dependant resistance which can be used to accurately measure local temperature. Measurements were made using a four point sensing mechanism in which current lines leading to the electrode were separated from voltage sensing across the region to be measured (figure A1A). Constant current of 5mA was created using the current source circuit shown in figure A2B.

Each device was calibrated by submerging the device in a 30ml Eppendorf tube, which was placed in a circulating water bath (SC100-A10, Thermo Scientific, USA). Upon each temperature change, 10 minutes was allowed to let the temperature stabilize before a voltage measurement was taken. The resultant plot (figure A2C) showed a linear relationship between temperature and voltage \( T = aV + b \) with coefficients of \( a = 0.72 \text{ mV}^{-1}, b = -383^\circ \text{C} \)

**Figure A2** (A) Zoomed in view of electrode showing current flow and voltage measurement branches. (B) Schematic of circuit (5mA current source) used to power on-chip temperature sensitive electrode. Voltage drop measured using a National Instruments data acquisition board. (C) Calibration curve of temperature as a function of the voltage drop across electrode with a constant 5mA supply current.
A.2. Control system

A PID controller (TE tech) is used to control the thermoelectric element with feedback from a thermistor which is attached to the sapphire disk. However, data from the on-chip electrodes is not compatible with the controller. Thus, the difference between the thermistor and on-chip electrodes is used to adjust the set point sent to the controller as shown in figure A3A.

Figure A3 (A) Modified control system for maintaining artery at the desired temperature (B) Response to step change increases in temperature following heating protocol for artery segments, showing clear offset between on-chip RTD and sapphire thermistor. Solid blue line indicates the temperature set point, solid red line is the on-chip temperature (as measured by RTD), and dotted red line is the temperature of the sapphire disk. (C) Heating protocol using modified control system. On-chip temperature reaches required set point. The additional blue line indicates setpoint sent to TE controller (SPTE), while solid blue line is the actual set point.
B. Staining

Automated staining of artery segment with Phalloidin (F-actin) and DAPI (nuclei) allows visualization of smooth muscle cell bodies. No remodeling is observed around posts at short time scales (less than 4 hours on-chip), longer time scales not investigated. In addition, imaging shows exclusion of actin from smooth muscle cell nuclei as expected.

Nuclear eccentricity was calculated using the equation below.

$$ e = \sqrt{1 - \left( \frac{\text{minor axis length}}{\text{major axis length}} \right)^2} $$

**Figure B1** - Zoomed view of stained artery segment. (A) Actin staining, unstained region labeled (1) are locations of the posts. Complete staining of these regions can be achieved by longer staining periods as shown in Fig. 4f. (B) Nuclear staining of the same region showed in (A). (1) Smooth muscle cell nuclei, (2) Endothelial cell nuclei. (C) Actin and nuclear channels merged. Showing exclusion of phalloidin stain from nuclei as expected.
B.1. Immunofluorescence

Negative control for L-type calcium channel was conducted by preincubating with peptide antigen corresponding to amino acid residues 848-865 of rat Ca\textsubscript{v}1.2 provided with the antibody (Alomone Labs, Israel) at equivalent concentration by weight (16µg/ml).

\textbf{Figure B2-} (A) Negative control for immunostaining (B) Artery segment used for negative control, also DAPI stained.
C. COMSOL modeling

Superfusing shear

Simulation of superfusing flow around artery segment was performed in COMSOL v4.2. The geometry and location of boundaries used is shown in Figure C1 below. The physics is only solved for region in dark blue. Inlet boundary condition was laminar inflow at 0.25µl/min (corresponding total superfusing flow rate of 0.5µl/min), outlet boundary was laminar outlet at 0 Pa.

![Figure C1](image)

**Figure C1** – Geometry containing post structures and artery segment used for comsol model. (1) Inlet boundary (green outline), (2) Symmetry boundary (light blue outline), (3) Outlet boundary (red outline). All other surfaces are walls with no slip boundary condition.

Table C1 below summaries mesh independence study. The largest difference between the two meshes was in peak shear which showed a 0.5% difference. This was deemed acceptable, thus showing the mesh density is sufficient to not affect the returned solution.
<table>
<thead>
<tr>
<th>Property/Calculated value</th>
<th>Mesh 1</th>
<th>Mesh 2</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesh elements</td>
<td>231,386</td>
<td>1,113,752</td>
<td>-</td>
</tr>
<tr>
<td>Average Shear rate across artery</td>
<td>204.60 s(^{-1})</td>
<td>204.64 s(^{-1})</td>
<td>0.02%</td>
</tr>
<tr>
<td>Peak Shear rate</td>
<td>573.61</td>
<td>570.68</td>
<td>0.5%</td>
</tr>
<tr>
<td>Outlet Flow rate</td>
<td>(4.1667\times10^{-12}) m(^3)/s</td>
<td>(4.1667\times10^{-12}) m(^3)/s</td>
<td>&lt;0.01%</td>
</tr>
</tbody>
</table>
D. Automation

The table below shows the contents of an example text file used to automate small artery heating and administer the wake up dose of PE. The Labview code (Fig. D1) reads each row and parses the columns to the appropriate actions. The first column contains the time spent at each step. The second column is the decimal representation of an 8-bit binary number, with each bit controlling the on/off state of a specific well (bits 7 and 8 are ignored since there are only 6 wells hooked in the current chip designs). In manual mode, the code (Fig. D2) automatically detects when a change is made and closes the previously open well to minimize fluid feedback and pressure fluctuations between wells when actuated. To accommodate this in automatic mode, the binary representation of the well to be open must also account for this. Between states where a change is occurring, the common bit is the well that closes, and the different bit opens (an XOR operation). For example, in table B1, between rows 5 and 6, well 1 closes, and well 4 opens. Between rows 6 and 7, the wells switch back from well 4 to well 1 (since just before the switch, the state of the wells is [0001000]. However, a row that is the same as the current state (row 8) results in no change. The third column is the temperature set point. The final two columns are pressure set points for the pressure heads controlling the external perfusion reservoir, and superfusion wells.
Figure D1- Image of Labview code responsible for parsing text file input for automated control of valves, temperature, and pressure regulators.
Figure D2- Labview code responsible for actuating valves based on commands for which well should be opened. Listbox contains ability to configure well-to-valve connections for various chip designs. It also contains a calculation to estimate fluidic resistance used to calculate estimated flow rates based on applied superfusion pressure.
Table D1 - Example of the contents of a tab delimited text file used to conduct automated sequences. The binary representation of the numbers in column two (in square brackets) are shown for the purpose of understanding, typical text files will include just the decimal value.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Well selection</th>
<th>Temperature (°C)</th>
<th>Perfusion Pressure (mmHg)</th>
<th>Superfusion Pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1 [00000001]</td>
<td>25</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>1 [00000001]</td>
<td>28</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>1 [00000001]</td>
<td>31</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>1 [00000001]</td>
<td>34</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>1 [00000001]</td>
<td>37.5</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>9 [00001001]</td>
<td>37.5</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>9 [00001001]</td>
<td>37.5</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>1 [00000001]</td>
<td>37.5</td>
<td>45</td>
<td>5</td>
</tr>
</tbody>
</table>
E. Chip designs

Figure E1- (A) Fluid layer mask design (B) Gas layer mask containing valve actuation channels and vacuum channels to prevent bubble generation via pressurized valves. (C) Overlay of the two layers.