Published in final edited form as:

Lab Chip. 2009 February 7; 9(3): 417–426. doi:10.1039/b806803h.

A multi-purpose microfluidic perfusion system with combinatorial choice of inputs, mixtures, gradient patterns, and flow rates†

Gregory A. Cooksey, Christopher G. Sip, and Albert Folch

Department of Bioengineering, University of Washington, Seattle, WA, 98195, USA

Abstract

Microfluidic perfusion systems, characterized by deterministic flow, low reagent consumption, small dead volumes, large integration in small footprints, high-throughput operation, and low-cost fabrication, are being increasingly used for cell culture studies in applications such as basic cell biology, molecular biological assays, tissue engineering, and systems biology. We report a multipurpose, pressure-driven and computer-controlled microfluidic perfusion device containing sixteen inlets and a large cell culture chamber. The user can choose, with sub-second temporal resolution, (a) to feed the chamber with one of 16 inlets, all 16 inlets, or one of 64 combinations of 2, 4, or 8 inlets using a binary multiplexer; (b) to introduce into the chamber a heterogeneous laminar flow of the inlets, a smoothed gradient, or a fully homogenized mixture; (c) to bypass the chamber in order to purge the inlet lines so as to minimize the dead volume; (d) to generate asymmetrical and curvilinear flow patterns within the chamber by opening side outlets; and (e) to slow down the flow by combinatorially adding segments of high fluid resistance (sixteen different levels of flow rates are possible using only four valves). All functionalities are combined to create complex gradient patterns and sequential perfusions within the central chamber.

Introduction

Characterization of cellular responses in large numbers of parallel experiments requires automated handling of fluids and cell culture substrates. The current approach to large-scale biological assays involves multi-well plate formats and expensive robotic manipulators for fluid exchange, while continuous perfusion is not possible. Several groups are developing miniaturized cell culture reactors in microfluidic devices, whereby seeding of cells and chemical perfusions are coordinated *via* an array of pneumatic valves integrated into the device. Opening and closing of microvalves, deflectable membranes that either break or enable flow through a microchannel, enable straight-forward routing and pumping of fluids through microdevices;^{1–8} thus, by building up “fluidic circuits” with multiple valves, it is possible to create devices that can sequence perfusions through a single reaction chamber or run a combination of perfusions in parallel through many reaction chambers. While devices of limited complexity (<8 total fluid and valve inlets) are rather straightforward to design and implement, many challenges exist for creating systems that contain multiple functionalities (which can require large spaces and/or large numbers of valves) or greater quantity of fluid inlets (which themselves may require more valves). The incorporation of deflectable microfabricated elements has enabled many other functional modules to be

†Electronic supplementary information (ESI) available: Supplementary Fig. S1 and Video S1–Video S5. See DOI: 10.1039/b806803h

© The Royal Society of Chemistry 2009

Correspondence to: Albert Folch.

incorporated, such as micromixers,^{5,9,10} dynamic gradient generators,^{11,12} and traps.¹⁰ In addition, the demonstration of multiplexers^{13,14} and latched valving schemes⁸ provides new strategies to reduce the number of valves required to control a complex device. Recently, Quake and coworkers¹⁵ have developed a cell culture system incorporating a bank of 16 different inlets that feeds an array of 96 cell-culture chambers (900 $\mu\text{m} \times 1120 \mu\text{m}$ footprint) through an integrated mixer, a pump, and a multiplexer. Here we address the challenge presented by studies requiring the perfusion of large cell numbers (a 5 mm $\times$ 7 mm footprint chamber) with combinatorial choices of cell culture conditions (mixtures, gradients, and flow rates).

In this paper we demonstrate a multipurpose microfluidic perfusion device containing a cell culture chamber fed by 16 inlets. At any point in time and through a custom-made (Lab-View-based) computer interface, the user has the option of choosing (a) which inlets or combinations of 2, 4, 8 or all 16 inlets (81 different choices are possible) feed the central perfusion chamber, or to bypass the chamber; (b) whether to activate a mixer upstream from the chamber: if the mixer is on, the inlets are homogenized, otherwise a gradient (choice of stepwise or smooth) is generated inside the chamber; (c) the flow rate (16 different levels); and (d) the fluid exit, which can be used to create flow patterns. All four capabilities are based on novel design solutions, each with broad applicability.

Materials and methods

Soft lithography

The three-layer microfluidic device was fabricated entirely by micromolding of polydimethylsiloxane (PDMS) using soft lithography techniques.¹⁶ Briefly, clean silicon wafers were spin-coated at 1000 or 2000 rpm for 30 s to achieve a 110- μm or 50- μm (respectively) thick layer of negative photoresist (SU-8 2035, MicroChem) and exposed to collimated UV light through a photomask that contained the desired pattern. Wafers were developed in SU-8 developer (MicroChem) for 15 min at room temperature and derivatized with a fluorosilane (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane, United Chemical Technologies) in a desiccator under vacuum to facilitate removal of PDMS from the wafers. PDMS pre-polymers were poured over the wafers and cured overnight in a 65 °C oven. The PDMS was then cut and peeled from the silicon wafer. Microvalve arrays were made as previously reported¹⁷ by sandwiching a thin PDMS membrane between two microstructured PDMS layers (Fig. 1a); the features on the bottom layer formed the fluid flow network and the features on the top layer formed the pneumatic lines (Fig. 1a). Thin PDMS membranes were made by spin coating a 3 : 1, PDMS : hexane mixture (by wt) at 3000 rpm for 30 s followed by a 4 min cure on an 85 °C hotplate; this procedure results in a 20 μm thick membrane.¹⁸

Bonding

In order to prevent leakage between fluid or valve channels, all layers of the device were permanently bonded together. Prior to bonding, inlet ports were created in the pneumatic layer using either a blunt 18-gauge needle or a 1.2 mm Harris Micro-Punch (Ted Pella, Inc.). To remove debris created from punching, this layer was rinsed with EtOH and water and dried. The micro-channel side of devices was covered with a strip of 2"-wide Scotch™ tape (3M) for storage prior to bonding to keep the surface clear of debris. Next, the PDMS membrane and the pneumatic layer were treated with oxygen plasma (30 s, full power) (Plasma Preen II, Kurt J Lesker Co) and pressed together to promote bonding. After at least 5 min in contact, cuts were made around the pneumatic layer so that the bonded layers could be peeled away from the silicon wafer. Under a stereomicroscope, the membrane over the

inlet ports was manually removed to allow fluids to pass from the pneumatic layer ports into the flow layer.

Bonding the membrane-covered pneumatic layer to the flow layer is a critical step that requires precise control of oxygen plasma conditions, feature alignment, and time. With the microchannel side protected with Scotch™ tape, the top of the flow layer was bonded to a clean glass slide (30 s, full power). The rigid backing prevents deformation of the flow layer during alignment with the membrane-covered pneumatic layer. Next, the tape was removed from the flow layer and the two layers were exposed to an oxygen plasma at reduced power (30 s, 45–55% power). After bonding, the two layers were aligned under a stereomicroscope, using features of the multiplexer region to guide overall device alignment. Most features were functional if alignment was within 50 μm , which we found to be readily achievable with a 1 cm field of view under a stereomicroscope. Once the layers were in contact, pressure was gently applied to remove air between the layers. Care was taken to prevent undue force over valved areas, which could cause the PDMS membrane to permanently seal against the valve seat (*i.e.* locking the valve in the closed state). For about 30 s, the PDMS layers were manually pressed together, so that when inlet lines are inserted, the layers do not pull apart. Finally, the valve ports were connected to negative pressure (approx. -100 kPa) so that valve membranes could be pulled away from the valve seat. Usually less than 2 min had elapsed since removal from the oxygen plasma reactor; variations in time or oxygen plasma power setting may be necessary in order to optimize bonding such that the bonds between the layers in the rest of the device are strong enough to prevent leakage, but at the same time the valve pads do not bond to the valve seats.

Reservoir and valve connectivity

Inlets and pneumatic lines were connected into the device using blunted 18-gauge needles with 90-degree bends connected to 1.14 mm inner diameter (ID) silicone tubing. Inlet lines were connected to ports drilled in the side of 15 mL conical tubes, which were filled with solutions of interest and, to drive flow, were positively pressurized (typically 0.25–1.5 psi) through ports in the caps (Fig. S1).[†] Pneumatic lines were connected to 3-way solenoid switches (The Lee Company) (we could support a bank of 24 switches) controlled by two 12-input USB data acquisition cards with a custom LabView (National Instruments) program (readily available at no cost upon request).

Microscopy and imaging

Micrographs were acquired with a stereomicroscope (Nikon SMZ 1500) fitted with a handheld digital camera (Nikon Coolpix E990). Fig. 1f was acquired with a digital SLR camera (Canon EOS 20D fitted with 50 mm f/1.8 lens and a 25 mm extension tube).

Measurements

Flows within microfluidic devices were visualized with dyes dissolved in water. Flow rate measurements were made by collecting and weighing water that flowed through the device and out into a capped 15mL conical tube with a small hole drilled in the side (see Fig. S1).[†] At least 200 μL effluent was collected (typically over 10 min to 24 h) before weight measurements were made. Since the conical tube reservoir was capped, evaporation was negligible; weight of 5 mL of water in a reservoir with the same configuration (open to the environment) varied by less than 30 μL over a 3 day period.

[†]Electronic supplementary information (ESI) available: Supplementary Fig. S1 and Video S1–Video S5. See DOI: 10.1039/b806803h

Microfluidic resistance calculations

Our basic design principle is that the resistance of a microfluidic channel scales according to the length of the channel, as predicted by the equation to estimate flow resistance R in a rectangular channel with low aspect ratio:

$$R = \frac{12\mu L}{wh^3} \left[1 - \frac{h}{w} \left(\frac{192}{\pi^5} \sum_{n=1,3,5}^{\infty} \frac{1}{n^5} \tanh\left(\frac{n\pi w}{2h}\right) \right) \right]^{-1} \quad (1)$$

where μ is the dynamic viscosity of water, L is the length of the channel and w and h are the shorter and larger cross-sectional dimensions of the channel, respectively.¹⁹ The lengths (L) of the microfluidic resistor (μ FR) channels in our device are 4.1 mm, 8.1 mm, 17.3 mm, and 31.3 mm; their flow resistances are termed R_1 , R_2 , R_3 , and R_4 , respectively, in Fig. 4a. Thus, turning on and off the 4 resistor valves (a total of 16 unique combinations) the total length of this μ FR circuit can be tuned between 0 mm and any of the 15 combinations of channel lengths up to $(4.1 + 8.1 + 17.3 + 31.3) = 60.8$ mm (spanning a factor of ~ 15).

In our simplified model, the flow rate can be calculated by dividing the change in pressure across the device by the total fluidic resistance:

$$Q = \frac{\Delta P}{R_0 + V_{R1}R_1 + V_{R2}R_2 + V_{R3}R_3 + V_{R4}R_4} \quad (2)$$

where R_0 denotes the resistance of the system outside of the μ FRs and V_{R1} , V_{R2} , V_{R3} , V_{R4} denotes the state of the resistor valves (open = 0 and closed = 1). Eqn (2) can be transformed into a linear equation of the form $Ax = B$ (where A is a matrix denoting valve states and B is the pressure drop divided by the measure flow rate for each valve state) and solved to determine the best-fit R values for a particular set of flow rate measurements:

$$\begin{bmatrix} 1 & 0 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 & 0 \\ 1 & 0 & 1 & 0 & 0 \\ 1 & 1 & 1 & 0 & 0 \\ 1 & 0 & 0 & 1 & 0 \\ 1 & 1 & 0 & 1 & 0 \\ & & \vdots & & \\ 1 & 1 & 1 & 1 & 1 \end{bmatrix} \begin{bmatrix} R_0 \\ R_1 \\ R_2 \\ R_3 \\ R_4 \end{bmatrix} = \begin{bmatrix} \frac{\Delta P}{Q_1} \\ \frac{\Delta P}{Q_2} \\ \frac{\Delta P}{Q_3} \\ \frac{\Delta P}{Q_4} \\ \frac{\Delta P}{Q_5} \\ \vdots \\ \frac{\Delta P}{Q_{16}} \end{bmatrix} \quad (3)$$

In an ideal circuit, five measurements of Q using any five different combinations of valves would suffice to solve for any R_0 , R_1 , R_2 , R_3 , and R_4 (including a set of unknown channel resistances); however, because we have made many more measurements of Q , we can estimate all the R_i values (and significance of the errors associated with our above assumptions) with good confidence bounds.

Results and discussion

Device overview

Here we present a multipurpose microfluidic device containing closed-at-rest microvalves (Fig. 1a,b) (see valve operation details elsewhere¹⁷) that includes the following modules (ordered from upstream to downstream): (a) a multiplexer for fluid routing that allows for

combining multiple fluid inputs (from a battery of 16 inlets and one rinse channel) into one microchannel, producing a heterogeneous laminar flow mixture; (b) an on/off chaotic micromixer circuit through which the flow can be diverted to diffusively broaden the mixture concentration profile (if the micromixer is inactive) or for convectively homogenizing the mixture (if activated); (c) a bypass channel to divert the flow to the waste output, thus minimizing dead volume into the central chamber; (d) a central chamber containing three exits; and (e) a novel microfluidic resistor designed to control fluid flow rates through the device over a wide, pre-specified range of flow rates; the design shown here allows for 16 different flow rates over a ~12-fold range, but the principle is applicable to arbitrarily large ranges.

A schematic block diagram of the device is shown in Fig. 1c; each functional module is highlighted on a detailed layout showing both the fluid flow layer and the underlying valve control layer (Fig. 1d). Flow routing within the device was visualized by loading inlet channels with alternating dye colors. Fig. 1e shows the detail of how the inlet channels (which were designed to be of equal flow resistance) merge with the rinse channel (also constructed to have matched resistances) to reduce dead volume during rinsing. A photograph with all inlets open demonstrates laminar flow through the central chamber forming a 16-component gradient (Fig. 1f).

Device construction and connectivity

The three-layer device is constructed from two face-to-face PDMS layers separated by a 20 μm -thick PDMS membrane (see Materials and methods). We use a normally-closed valve design that is compatible with SU-8 photolithography.¹⁷ In the device presented here, all surfaces were made of PDMS; however, it is also possible to mount valves over other substrates, such as glass, if the flow layer is created by exclusion molding.¹² In our design, dead-end flow channels are held closed by applying positive pressure (greater than the pressure driving the fluid flow at that point in the fluid path) through a small pneumatic line to a depression underlying (and larger than) the valve seat between the broken channel (Fig. 1a). The positive pressure can be either applied externally or, if sufficient, by the restoring force of the deflected pad. When suction is applied, the PDMS membrane deflects into the pneumatic layer, providing a connection for fluid to pass (Fig. 1b). Quake's group has demonstrated microvalves that use an orthogonal channel to compress (with positive pressure) a rounded microchannel created by a photoresist reflow technique.² Fabrication of open-at-rest valves requires reflowing the photoresist to create rounded channels that can be fully compressed to closure when positive pressure is applied to the pneumatic line. In contrast with microchannels molded from photoresist-reflowed structures, microchannels molded from SU-8 photoresist patterns have flat faces (making modeling of flow velocity profiles straightforward and easily-scalable), including flat roofs (optically superior to rounded roofs for transmitted light microscopy), and can have $> 10 : 1$ aspect ratios.

The intricate connectivity of the device, in particular the presence of several loops in the design, makes the trapping of air bubbles inevitable the first time the channels are filled with fluid. Removal of air bubbles is facilitated by pressurizing all inlet fluids with 1–2 psi of pressure. Valves, in particular those alongside the chamber (see below), have proven to be very effective in removing air bubbles that form within the device. By applying suction to the valves (and building pressure within the device by closing particular valves or pinching off the exit line), trapped air bubbles adhering to the hydrophobic PDMS walls are efficiently removed into the pneumatic lines through the thin gas-permeable PDMS membranes.

Multiplexer

Controlling a large number of inlets with dedicated microvalves (*i.e.* one microvalve per inlet) requires, for each inlet, a separate line on the pneumatic layer and an additional piece of tubing, thus taking up a large fraction of real estate in the device and adding additional tubes and switches connected to the device. To reduce the number of valves necessary to control many inlets, Quake's group developed a clever binary multiplexer scheme,¹³ which can control N PDMS microchannels with only $X = \log_2 N$ pairs of pneumatic lines (or $2X$ total pneumatic lines). Using this scheme, more than 1000 flow channels could be controlled with only 20 pneumatic lines.¹³ In the Quake "binary" design, each pneumatic line is connected to a group of valves controlling half of the channels, and a "complementary" pneumatic line controls the other half of the valves/channels. (Although the words "valve" and "pneumatic line" are used interchangeably, strictly speaking in a multiplexer a pneumatic line opens a group of valves, each of which controls flow in a different channel.) For example, our device has 16 inlets (named A, B, ... through P) controlled by 8 multiplexing lines of valves (named $V_1, V_2, \dots$ through V_8). Line V_1 must be opened to turn on any of the 8 leftmost channels A through H and line V_2 must be opened to turn on any of the 8 rightmost channels I through P (Fig. 2a); the V_1 and V_2 valves are said to be a "complementary pair" of valves; V_3 (which is necessary to open the first and last 4 channels) is complementary of V_4 (which is necessary to open the middle 8 channels), *etc.* We denote the "state" of the multiplexer by the state of its valves, $V = \{V_1, V_2, V_3, V_4, V_5, V_6, V_7, V_8\}$, with V_i taking the values of 0 (closed) or 1 (open); note that, for clarity, we write a space between each pair of complimentary bits. To open a single inlet, one (and only one) valve from each complementary valve pair needs to be opened. For example, to open inlet M, valves V_2, V_3, V_6 and V_8 need to be opened, but V_1, V_4, V_5 and V_7 are "forbidden" and must be closed ($V = \{01\ 10\ 01\ 01\}$) (Fig. 2b).

Respecting the complementarity rule (*i.e.* not opening any of the forbidden valves), as Thorsen *et al.* did in their seminal paper,¹³ is not a design constraint—only an operational constraint to ensure that only one inlet is chosen at a time (*i.e.* maintaining the multiplexer in a "single-inlet state"). If, in any single-inlet state, a previously-closed forbidden valve is opened, then an additional inlet opens. For example, in the single-inlet state shown in Fig. 2b where inlet M is open ($V = \{01\ 10\ 01\ 01\}$), if forbidden valve V_7 is opened (*i.e.* the multiplexer state is changed to $V = \{01\ 10\ 01\ 11\}$) then inlet N opens, too (Fig. 2c). As a rule, there will be 2 open inlets for any multiplexer state that has one complementarity violation. When there are 2 complementarity violations, then 4 inlets open; *e.g.* in Fig. 2d, $V = \{01\ 10\ 11\ 11\}$ opens inlets MNOP. With 3 complementarity violations, 8 inlets open; and so on. Optical micrographs for several multiplexer states are shown in Fig. 2e–h: $V = \{01\ 10\ 11\ 11\}$ (four inlets MNOP) (Fig. 2e), $V = \{01\ 11\ 01\ 11\}$ (four inlets IJMN) (Fig. 2f), $V = \{11\ 11\ 11\ 10\}$ (eight inlets A,C,E,G,J,L,N,P) (Fig. 2g), and $V = \{01\ 11\ 11\ 11\}$ (eight inlets I through P) (Fig. 2h). It is straightforward to see that there are (a) 32 ways of producing (only) one complementarity violation (32 multiplexer states with one forbidden valve open), *i.e.* 32 possible 2-inlet combinations; (b) 24 possible ways of generating (only) two complementarity violations (24 multiplexer states with two forbidden valves open), *i.e.* 24 possible 4-inlet combinations; (c) 8 possible ways of producing (only) three complementarity violations (8 multiplexer states with three forbidden valves open), *i.e.* 8 possible 8-inlet combinations; and (d) only one way of producing four complementarity violations, which is all 8 valves (and consequently all 16 inlets) open (shown in Fig. 1e). Adding the 16 possible ways of producing zero complementarity violations (single-inlet states) equals 81 possible combinations. For more demonstrations of multiplexer operation, see Videos S1a–d.[†]

We emphasize that not all combinations of inlets can be selected and only combinations of $R = 2^n$ open inlets (with n = the number of complementarity violations) are possible with this

multiplexer design (*e.g.* combinations of 3, 5, 6, 7, 9, 10, *etc.* inlets are not possible). For multiplexers having numbers of inlets other than 16 (and thus different requirements for the number of pneumatic lines), here we provide a general expression for the number of possible combinations of R inlets (number of allowed “ R -tuplets”) that can be obtained with a multiplexer. Assume a multiplexer with $2X$ valves (*i.e.* X is the number of pairs of complementary valves), which can control up to $N = 2^X$ (*e.g.* in our device $X = 4$, so $2X = 8$ valves control $N = 2^4 = 16$ inlets). Thus, $R = 2^n$ with $n = 1 \dots X$. Note that each pair of valves, *e.g.* $\{V_1, V_2\}$, has two possible states that respect the complementarity rule, $\{V_1, V_2\} = \{1, 0\}$ and $\{V_1, V_2\} = \{0, 1\}$, and one state that violates it, $\{V_1, V_2\} = \{1, 1\}$. From a set of X pairs of valves, there are obviously X possible ways of producing $n = 1$ complementarity violations ($R = 2$). In general, there are XC_n possible ways of producing n complementarity violations (*i.e.* of choosing n pairs in the $\{1, 1\}$ state), where

$${}^XC_n = \frac{X!}{(X-n)!n!}$$

For the $X-n$ pairs of valves that do not violate complementarity, there are 2^{X-n} possible states. Thus, the number of allowed R -tuplets is

$${}^XC_n \times 2^{X-n}$$

where $R = 2^n$ and $n = 1 \dots X$ is the number of complementarity violations.

A summary of the allowed combinations using this multiplexer design is shown in Table 1, along with the number of combinations that would be possible if each inlet were actuated by a unique valve (*i.e.* no multiplexing). Nevertheless, even with a moderate number of inlets, a large quantity of combinations becomes possible; for cell culture applications where one wants to measure the response of cells to multiple factors (one in each inlet), addressing a large number but incomplete set of combinations is likely sufficient at the present time, given the complexity of analyzing the data from the responses of mixtures of just two factors.

Recently, an even more efficient multiplexing scheme has been reported by Hua *et al.* that uses only M pneumatic lines to address $M!/(M/2)!^2$ flow channels.^{20,21} In this design, however, there is a tradeoff between flexibility and space: the number of potential combinations decreases as the number of pneumatic lines decreases. Using 6 pneumatic lines to select 16 inlets, as shown in Hua *et al.*,²⁰ it is possible to achieve 37 combinations (16 singles, 8 triplets, 6 quadruplets, 2 sextuplets, 4 nonuplets, and 1 with all 16 inlets on) compared to 81 with the multiplexer used here. (Obviously, all possible combinations are possible when each inlet has its own pneumatic line, see Table 1).

We note that the valve layout is designed with complementary symmetry, *i.e.* the valves control alternating valve sets of equal number of valves. Here we have achieved a large number of combinations by releasing the constraint that the binary multiplexer be operated without complementarity violations. Similarly, interesting situations arise when the multiplexer is designed without complementary symmetry. New sets of combinations (without sacrificing the ability to address each inlet individually) are achieved when valves are moved around to different pneumatic lines. For example, moving a valve from the V_5 set of valves to the V_3 set of valves (so that V_5 and V_3 open 7 and 9 valves, respectively, instead of 8) on inlet 5 results in 78 unique combinations: 16 singlets (with 6 additional repeats of inlets E (3), F, G and K with other unique valve sequences), 28 doublets, 4

triplets, 19 quadruplets, 1 septuplets, 6 octuplets, 1 nonuplets, and all 16. We have not yet worked out design rules for manipulating valves on pneumatic lines (or adding additional pneumatic lines) to achieve specific combinations, but we are working on programming algorithms that can aid in determining the placement of pneumatic lines to achieve a desired set of combinations.

We have observed that the multiplexer can act like a pump if multiple valves on a single inlet line are alternately switched, which can lead to some fluid leakage into the active flow stream. One solution to the problem is to rinse after every step or keep the valves closest to the chamber (*e.g.* V_7 and V_8 in Fig. 2a) closed until after other valves have been switched. Because our device has rinse lines between every inlet, and all inlets are rinsed at once, a rinse between each inlet change removes the possibility of downstream contamination. Quake *et al.* have similarly updated their multiplexer layout to reduce dead volume between inlet junctions, but also with a significant increase in required space on the chip.²¹

Integrated on/off mixer

As gradients play an increasingly important role in many cell culture assays,²² we incorporated into the device the capability to turn mixing on and off, such that the same device could be used to generate gradients as well as homogenized mixtures. To achieve mixing, our device has a separate channel whose floor contains deflectable herringbone-shaped PDMS membranes, which become grooves that induce chaotic mixing²³ when the membranes are deflected downward with negative pressure (a previously developed “on/off chaotic mixer”¹¹). The herringbone pattern significantly reduces the length required for mixing, which scales roughly as $\lambda \times \ln(Pe)$, where λ is the characteristic length determined by the trajectories of the chaotic flow in the herringbone and Pe is the Peclet number ($Pe = UD_h/D$, where U is the average flow speed, D_h is the hydrodynamic radius, and D is the molecular diffusion coefficient).²³ We conservatively designed the mixing channel to be 2-fold longer than measured for 95% mixing for $Pe \sim 9 \times 10^5$,²³ which is considerably larger than conditions presented here ($Pe < 1 \times 10^5$).

The operation of the on/off chaotic mixer is demonstrated in Fig. 3. Schematics of the fluid routing under three mixing conditions are shown in Fig. 3a–c. When fluid access to the on/off chaotic mixer is blocked and the bypass valve is open, the flow proceeds directly into the central chamber with no mixing (Fig. 3 column 1). Magnified views of the on/off chaotic mixer (Fig. 3g) and of the chamber entrance (Fig. 3j) demonstrate the case of no mixing (*i.e.* a heterogeneous laminar flow is clearly discernible) when the mixer is bypassed. If the valve at the entrance of the mixer is opened (and the direct path to the chamber closed), fluid enters the mixer but does not mix fully (Fig. 3b column 2), unless the grooves are fully deflected (applied pressure -50 kPa) (Fig. 3 column 3). To assess the completeness of mixing (or lack thereof) in the three different mixing scenarios presented above, we operate the device using a yellow stream (inlet N) and a blue stream (inlet P) (Fig. 3m–o) and plot the linescans of intensity in the green and blue color channels (Fig. 3p–r), respectively. Note that the plots do not reflect the actual dye concentrations (*e.g.* the green color intensity plot contains information on the blue dye distribution as well as on the yellow dye distribution) and are only used to assess whether mixing is complete (yellow and blue, yielding green mixture, are convenient colors because they are well separated in the spectrum). If the on/off chaotic mixer is bypassed (Fig. 3m), very little diffusive mixing occurs between the two colored fluids, so the green and blue color channel intensity plots appear like a step function (Fig. 3p). If fluid goes through the chaotic mixer but the grooves are not actuated, then there is a wide gradient from yellow to green to blue (Fig. 3n,q); if the grooves are turned on, the streams are completely mixed by the time they enter the chamber (Fig. 3o,r). For dynamic operation of the mixer in real-time, see Video S2.[†] Overall, the on/off chaotic mixer adds flexibility to fully homogenize or partially mix (thus creating gradients) any combination of

inlets enabled by the multiplexer. Furthermore, using moderate herringbone membrane deflections (*i.e.* shallow grooves) should yield incomplete mixing manifested as non-monotonic gradients,¹¹ which we have not explored yet.

Modulation of flow resistance

We have recently developed elastomeric “microfluidic resistors” (μ FRs),²⁴ a method to dynamically adjust the flow resistance of microfluidic channels; the constrictions are made of PDMS membranes that span across a channel and that can be inflated to partially constrict the flow (*i.e.* a sort of “leaky valve”). However, predicting the flow resistance requires cumbersome finite-element simulations, which in practice implies that each resistor design needs to be characterized before being used. Here we report a novel μ FR design that uses microvalves to selectively divert flow through a series of microchannels (termed “ μ FR channels”) of various lengths, allowing for straightforward design translation/scalability and for great flexibility in the possible values of flow resistance.

The μ FR design presented here is based on the concept that, for pressure-driven flow of water in a microchannel at low Reynolds numbers, the steady-state flow rate (Q) can be approximated to be proportional to the pressure drop (ΔP); the proportionality constant can be attributed to a “flow resistance” (R) ($Q = \Delta P/R$) that is directly proportional to the length of the channel,²⁵ and is analogous to Ohm’s law in electrical circuits ($I = V/R$). Thus, we may represent the system with a circuit diagram (Fig. 4a). For simplicity, we assume that the cross-section of each resistor valve is identical, that pneumatic lines overlapping the channels have a very small impact on the flow rate, and that the length of a channel is taken to be the length of the path through the center of the channel (even if the channel turns; we have as many as 4 turns in one channel). We also assume that the μ FR channels, which are always open and run in a parallel circuit to the shorter (and much wider) bypass lines (R_v in Fig. 4a), have a negligible impact on the resistance value when the valves are open. Thus, we chose the path length of each successive μ FR channel to be approximately twice as long as the previous channel (though other factors could be chosen for different applications); thus a large range of flow rates (2^n) can be selected by valving n μ FR channels. The minimum flow rate is achieved when all the μ FR valves are closed, forcing flow through every μ FR channel (Fig. 4b,c). When all valves are open, the high resistance paths are “shorted” and the flow rate is maximum (Fig. 4d,e). A video showing a striking transition from 3 to 36 μ L/min is available online (see Video S3).[†]

The average $Q = \Delta P/R_T$ (where R_T is the total resistance of the entire microfluidic circuit) is easily measured by weighing the water volume collected at the output. We demonstrated flow control for a series of inlet driving pressures (0.5, 1, 2, and 3 psi) by measuring flow rates for all the combinations of μ FR valve settings (Fig. 4f). The plots of Q vs. $1/L$ were fit to the equation $Q = a(P)/L + Q_0$, and found to have $Q_0 \sim 0$ and strong R -squared values (R -squared > 0.996 in all cases), showing that flow rate is indeed inversely proportional to the total channel length - despite the presence of the deflecting elastomeric elements that act as capacitors and add unaccounted volumes, the irregular valve geometries that deviate from eqn (1), and the R_v segments and channel turns that were not counted in the estimates of length (which had all, indeed, been assumed to have a negligible effect in the analysis). A plot of the slopes of the best-fit lines, $a(P)$, of Q vs. $1/L$ as a function of driving pressure, indicates that the change in flow rate due to changing the fluid driving pressure is also linear over the range of pressures tested (Fig. 4g). The deviations from linearity in Fig. 4f and 4g, likely attributable to the above assumptions, are relatively small, suggesting that in the future the flow resistance introduced by each μ FR channel can be predicted using simple analytical formulas. We did not measure Q for highly resistive combinations at $\Delta P = 0.5$ psi due to requirement of long measurement times (many hours). Though we expect the linear

trend to continue below the range tested, determining the full range of linearity merits further exploration.

Using the Q_i obtained for $\Delta P = 2$ psi, solving eqn (3) in terms of multiples of R_I (with 95% confidence bounds) gives $R_0/R_I = 1.8 \pm 0.3$, $R_1/R_I = 1 \pm 0.2$, $R_2/R_I = 2.1 \pm 0.2$, $R_3/R_I = 4.1 \pm 0.2$, and $R_4/R_I = 7.9 \pm 0.2$. These estimates are in good agreement with the ratio of the actual lengths of the channels: $R_2 = 2.0 \times R_I$ (i.e. the design-predicted value is smaller than the measured one by 4.7%, a “design-prediction error” of -4.7%), $R_3 = 4.2 \times R_I$ (design-prediction error $+5\%$), and $R_4 = 7.6 \times R_I$ (design-prediction error -3.8%). In summary, the designed lengths of the μ FR channels can be used to predict the resulting flow rates during operation within a few percent.

A previous report by Kim *et al.*²⁶ demonstrated the ability to design logarithmically scaled flow rates using chambers with increasing pressure drop across the chamber’s output port. Contrasted with the network of increasing microchannels presented in this paper, the design by Kim *et al.* is not tunable and requires that each flow output be passed to a separate experimental chamber. Thus, we have shown that it is straightforward to design a system of μ FRs to control fluid flow rates over an arbitrarily large range, according to the length of channel, the number of channels (with logarithmic increasing channel lengths, each additional μ FR channel doubles the number of possible flow rates achievable) and fluid driving pressure (eqn (2)). Though it was not explored in the paper, we note that tuning μ FR channel width and height could lead to more dramatic (nonlinear) changes in flow resistance and thus more design options, as predicted by eqn (1).

Central chamber gradients

The broad central chamber (5 mm $\times$ 7 mm) spreads out the stream and provides a large compartment for adsorption of reactive material or indicators, deposition of cells, and/or delivery of reagents, depending on the application. We added an inlet/outlet port on the left/right of the chamber (orthogonal to the main flow path) to enable pre-loading of the device with cells and surface treatments. This port provides a direct entry point for cells that significantly reduces the potential to plug upstream valves or damage the cells under valves. Additionally, the port can be utilized to redirect some of the main flow out the sides of the chamber (see below). Fluidic access from the side is facilitated by a pair of “strip” valves running along each side of the central chamber. The strip valves are connected through a single control line so that they are either both open or both closed.

In combination with the bank of μ FRs to regulate flow through the bottom of the central chamber (see above), a wide variety of unique spatial patterns can be created by actuating the strip valves (Fig. 5). Because the streams are at steady state and under continuous flow, patterns can be maintained for many hours, yet can be switched or rinsed in seconds (e.g. ~ 20 s for a flow rate of 10 μ L/min). Fig. 5a shows laminar streams tightly confined in parallel through the chamber when all 16 inlets (loaded with alternating sequence of red, yellow, green, and blue dyes) are flowed at a rate of 44 μ L/min. However, closing all of the μ FR bypass valves reduces the flow rate to 4 μ L/min, which allows the streams to laterally diffuse into one another (Fig. 5b). Opening the valves along the side of the chamber permits much of the flow to fan out the sides of the chamber (Fig. 5c). When the inlet port on the left side of the chamber is stopped and the side valves are open, fluid exits the chamber to the right and bottom; the balance between the two choices can then be shifted by actuating the resistance network (Fig. 5d–f). Similarly, when an additional dye is injected into the chamber from the left inlet, the flow exiting down or to the side can be precisely controlled by the μ FR network (Fig. 5g–i). Videos S3–S5[†] further demonstrate how the μ FR valves and valves along the side of the chamber can be manipulated to tune the gradient patterns formed in the central chamber in real time.

Conclusions

The perfusion system presented here offers great opportunities for spatiotemporally modulating the biochemical microenvironment of cells in culture (*e.g.* gradients over a large population of cells) and for analyzing cell behavior in well-defined conditions (medium compositions, flow shear, *etc.*). We have demonstrated several microfluidic tools integrated into a single perfusion system that have wide applicability in the automation of multi-parameter cell culture experiments. Combinatorial mixing strategies could benefit high-throughput studies needed in toxicology or drug development, where real-time assays of competition or synergy between multiple compounds could be easily automated. The switchable, discrete μ FR design provides a robust method to control fluid flow (and thus, the shear forces exerted over the cells) over a wide range of flow rates using only a small number of channels; this design, unlike our previous microfluidic μ FR design,²⁴ does not require recalibration of the device for different fluid driving pressures and is easily scalable to wider ranges of flow rates. Last but not least, we have demonstrated complex, dynamic gradients not achievable before by combining inlets, controlling mixing, and regulating flow rate using variable flow resistances in the flow path.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Eric Lam for implementing a LabView program to control the pneumatic valving sequences. This work was funded in part by the National Institute of Biomedical Imaging and Bioengineering (Grant # EB003307) and the National Institute of Deafness and Other Communication Disorders (Grant # DC006564).

References

1. Hosokawa K, Maeda R. J. Micromech. Microeng 2000;10:415–420.
2. Unger MA, Chou HP, Thorsen T, et al. Science 2000;288:113–116. [PubMed: 10753110]
3. Beebe DJ, Moore JS, Bauer JM, et al. Nature 2000;404:588. [PubMed: 10766238]
4. Gu W, Zhu XJ, Futai N, et al. Proc. Nat. Acad. Sci. (USA) 2004;101:15861–16866. [PubMed: 15514025]
5. Li N, Folch A. Exp. Cell Res 2005;311:307. [PubMed: 16263111]
6. Sundararajan N, Kim DS, Berlin AA. Lab Chip 2005;5:350–354. [PubMed: 15726212]
7. Weibel DB, Lee A, Mayer M, et al. Langmuir 2005;21:6436–6442. [PubMed: 15982051]
8. Grover WH, Ivester RHC, Jensen EC, et al. Lab Chip 2006;6:623–631. [PubMed: 16652177]
9. Hansen CL, Skordalakes E, Berger JM, et al. Proc. Natl. Acad. Sci. USA 2002;99:16531–16536. [PubMed: 12486223]
10. Hsu CH, Folch A. Appl. Phys. Lett 2005;86:023508.
11. Hsu CH, Folch A. Appl. Phys. Lett 2006;89:144102.
12. Irimia D, Liu SY, Tharp WG, et al. Lab Chip 2006;6:191–198. [PubMed: 16450027]
13. Thorsen T, Maerkl SJ, Quake SR. Science 2002;298:580–584. [PubMed: 12351675]
14. Bolea S, Sanchez-Andres JV, Huang XY, et al. J. Neurophysiol 2006;95:552–561. [PubMed: 16177178]
15. Gomez-Sjoberg R, Leyrat AA, Pirone DM, et al. Anal. Chem 2007;79:8557–8563. [PubMed: 17953452]
16. McDonald JC, Duffy DC, Anderson JR, et al. Electrophoresis 2000;21:27–40. [PubMed: 10634468]
17. Li N, Hsu C-H, Folch A. Electrophoresis 2005;26:3758–3764. [PubMed: 16196107]
18. Hoffman JM, Shao J, Hsu CH, et al. Adv. Mater 2004;16:2201.

19. Nguyen, N-T.; Wereley, ST. *Fundamentals and Applications of Microfluidics*. Artech House, Boston, MA: 2002. p. 471
20. Hua ZS, Xia YM, Srivannavit O, et al. *J. Micromech. Microeng* 2006;16:1433–1443.
21. Melin J, Quake SR. *Annu. Rev. Biophys. Biomol. Struct* 2007;36:213–231. [PubMed: 17269901]
22. Keenan TM, Folch A. *Lab Chip* 2008;8:34–57. [PubMed: 18094760]
23. Stroock AD, Dertinger SKW, Ajdari A, et al. *Science* 2002;295:647–651. [PubMed: 11809963]
24. Lam EW, Cooksey GA, Finlayson BA, et al. *Appl. Phys. Lett* 2006;89:164105.
25. White, FM. *Viscous Fluid Flow*. Third ed. New York: McGraw-Hill; 2006. p. 629
26. Kim L, Vahey MD, Lee HY, et al. *Lab Chip* 2006;6:394–406. [PubMed: 16511623]

Fig. 1. Overview of device architecture and fluid routing

(a and b) Cross-sectional schematic of microvalve operation (not to scale). The microvalves are actuated by air pressure application *via* dedicated “pneumatic lines”. (a) Positive pressure in the pneumatic line prevents flow through the fluidic channel, whereas (b) application of negative pressure through the pneumatic line pulls the PDMS membrane into the pneumatic layer, permitting flow through the fluidic channel. (c) Block diagram of the device’s modules, which include a multiplexer (“MUX”) for inlet selection, an on/off micromixer, a central chamber, and flow resistance modulators for flow-rate control. The mixer and chamber can be shorted *via* bypass valves (indicated by switches in gray blocks), which also facilitates pre-loading of the device without contaminating the central chamber. The total number of valves connecting to each module is indicated next to a pneumatic line (e.g. the multiplexer, indicated with “ $\times 9$ ”, is controlled by 9 valves: 8 valves to switch the inlets and 1 additional valve to simultaneously switch all the rinse lines intercalated between

each inlet). The pneumatic lines approach from both left and right. (d) Overlay of the design layouts, with the four device modules highlighted in gray boxes. The blue-gray lines represent the pneumatic lines, red lines are the inlet channels, and green lines show the intercalated rinse channels, which merge with the inlet channels right after the multiplexer (box “I”). Note that the rinse stream, starting at the rinse inlet (green circle) is divided into a total of 16 branches (green lines), one per inlet channel, that are balanced in length to provide uniform flow down each branch. Equal-length paths from each of 16 inlet ports (red circles) enter the multiplexer, after which they merge to form a single channel (each branch of the rinse line merges past the inlet valves, such that operating the rinse efficiently eliminates any compounds that may have diffused upstream of the multiplexer during inlet perfusion (e)). Next, fluid can then either be diverted through a long channel containing an on/off chaotic micromixer (box “II” in (d)) or bypass it and proceed directly to the central chamber (box “III”). After exiting the chamber, fluid passes through a segment of selectable resistor channels (box “IV”) that act to control flow rate through the device. (f) Photograph of the device loaded with alternating colored inlets when all inlets are open to flow into the central chamber. Inlets and pneumatic lines were connected to the device using 18-gauge needles having 90-degree bends.

Fig. 2. Chemical combinations with sixteen-inlet multiplexer

(a–d) Schematic of the combinatorial operation of the multiplexer. The pneumatic lines are illustrated as horizontal gray strips; open and closed valves are illustrated as light and dark gray horizontal rectangles, respectively. The 16 inlets (labeled A–P) are colored to represent the dyes loaded in each inlet; the part of a channel downstream of a closed valve is illustrated in light gray to signify absence of flow. Pneumatic lines to control valves are labeled V₁–V₈. The state of the multiplexer can be collectively characterized by a set of eight binary bits $V = \{V_1, V_2, V_3, V_4, V_5, V_6, V_7, V_8\}$, with bit values $V_i = 0$ (for positive pressure or “closed”) or 1 (for negative pressure or “open”). Virtual LEDs indicate whether a valve is open (green) or closed (red). (a) No inlets are open when $V = \{00\ 00\ 00\ 00\}$. (b)

Actuating one (and only one) pneumatic line from each “complementary pair” (*e.g.* V_1 and V_2 , V_3 and V_4 , *etc.*) is necessary to turn on a single inlet. Inlet M, for example, is open when $V = \{01\ 10\ 01\ 01\}$. Note that the bits in each pair have complementary values. The valves set to 0 can be thought of as “forbidden”. (c) Opening any additional valve (*e.g.* setting the “forbidden” V_7 open, which makes V_7 and V_8 violate the above “complementarity” rule) releases a second inlet: $V = \{01\ 10\ 01\ 11\}$ opens inlets M and N. (d) Four inlets are opened by violating two complementarity conditions (*i.e.* releasing two “forbidden” valves from any single-inlet state). (e–h) Optical micrographs of the device operating under (e,f) two and (g,h) three complementary violations (violation bits are underscored): (e) opening inlets M,N,O,P ($V = \{01\ 10\ 11\ 11\}$) and (f) opening inlets I,J,M,N ($V = \{01\ 11\ 01\ 11\}$). Three complementarity violations (*i.e.* releasing three “forbidden” valves from any single-inlet state) results in 8 open inlets, as shown in (g) for inlets A,C,E,G,J,L,N,P ($V = \{\underline{11}\ \underline{11}\ \underline{11}\ 10\}$) and in (h) for inlets I–P ($V = \{01\ \underline{11}\ \underline{11}\ \underline{11}\}$). In total, the device supports 81 unique chemical combinations: 16 single inlets, 32 doubles, 24 quadruplets, 8 combinations of 8 inlets, and one configuration with all inlets open.

Fig. 3. Demonstration of on/off micromixer

(a–c) Schematics depicting how the device allows flow to enter the chamber by (a) bypassing the 3.5 mm-long herringbone mixer, (b) going through the mixer channel with herringbone membranes undeflected (no grooves), or (c) going through the mixer channel with activated grooves (50 μm channel depth). (d–l) Nine images of laminar flow through the device for the three states of the mixer (bypass/off/on); the three rows show overviews (d–f), mixer details (g–i), and chamber details (j–l) for each of the three states. Flow through the mixer without actuating the herringbone pattern is insufficient for complete mixing (e,h,k) at 44 $\mu\text{L}/\text{min}$. Activation of herringbone pattern results in complete fluid mixing at 44 $\mu\text{L}/\text{min}$ (f,i,l). (m–o) Mixing of two streams (yellow from inlet N, and blue from inlet P)

is demonstrated when the mixer is bypassed (m) and when the flow goes through the mixer with (n) and without (o) actuating the herringbone grooves. (p–r) The effectiveness of mixing in each condition is illustrated in plots of the green and blue color channel intensity from the chamber region (caliper line in (m)) of images (m–o), respectively.

Fig. 4. Flow rate control

Flow rate control is achieved by selectively closing valves to divert fluid through paths of defined lengths (termed “resistor channels”). The valves and resistor channels are modeled as switches (e.g. V_{R1}) and electrical resistors (e.g. R_1), respectively, in a circuit diagram, shown in (a) with all valves open, i.e. $\{V_{R1}V_{R2}V_{R3}V_{R4}\} = \{0000\}$; note that an “open” valve is equivalent to a “closed” switch. The circuit also includes a resistor (in series) R_0 , the equivalent resistance of the device excluding μFRs when all inlets are opened. The resistance of the short channel through an open valve, R_v , is shown for completeness, though its contribution to the overall resistance is so small that it is neglected in the analysis. Each downstream path is designed to be twice as long (and thus twice as resistant) as the upstream path. Thus, combinations of the 4 resistance channels enable 16 discrete flow rates. Valve states are represented as 0 = open/bypass and 1 = closed. Resistor values are shown as multiples of the length of channel R_1 . (b,c) The highest-resistance setting has all valves closed $\{1111\}$ as shown in (b) overview and (c) close-up images of resistors R_1 and R_2 . Arrows denote direction and relative volume of fluid flow. (d,e) All valves are open for the lowest resistance setting as demonstrated in (d) overview and (e) close-up images. (f)

Measurements of flow rates are plotted against the inverse equivalent resistance path length (normalized to include the contribution of R_0) for various valve states at inlet driving pressures of 0.5, 1, 2, and 3 psi. Linear fits at each driving pressure demonstrate that the system behaves as predicted over the ranges of driving pressures and channel lengths tested. (g) The values for each fit's slope, a , are plotted against the driving pressure P and fit to a linear curve. Standard deviations of the flow rate measurements were less than 3 $\mu\text{L}/\text{min}$.

Fig. 5. Gradient flow patterns in the central chamber

Fluid gradient patterns can be created by selecting different combinations of valves around the central chamber. Fine-tuning of gradients is achieved by modulating the resistance network and/or allowing flow in or out the additional inlet to the left of the chamber. (a) At a flow rate of $44 \mu\text{L}/\text{min}$ all inlets were opened into the chamber. (b) Closing all of the resistor valves (and thus forcing flow through the resistor channels), the flow rate was reduced to $4 \mu\text{L}/\text{min}$, which allowed more time for diffusion between the streams. (c) Opening both “strip valves” on the sides of the chamber allowed flow to exit on either side (via a much less resistive path than through the resistor channels). (d) If flow out the left side of the chamber is blocked and the resistor channels are bypassed, flow exits through the right and bottom. (e) Actuating valve R_1 reduces the flow exiting the bottom, diverting a great fraction of the flow out the right. (f) Closing all the resistor valves forces nearly all of the flow out the right. (g) With the resistor valves open and blue fluid injected through the inlet on the left side (same driving pressure), the gradient is split across the chamber. (h) Closing all of the resistor valves forces the blue dye out the right. (i) If the chamber bypass valve is also opened (see Fig. 1c), much of the gradient flow is diverted before it enters the chamber and more blue dye exits to the right.

Tabulation of the number of required pneumatic lines and allowed combinations for a given number of inlets. C denotes the mathematical symbol for combinations: ${}^aC_b = a!/[(a - b)!b!]$

Table 1

# Inlets	# Required pneumatic lines (= 2X)	# Allowed pairs	Total # possible pairs	# Allowed quadruplets	Total # possible quadruplets	# Allowed octuplets	Total # possible octuplets
N	$2X = 2 \log_2 N$	$X \times 2^{X-1}$	NC_2	$X C_{X-2} \times 2^{X-2}$	NC_4	$X C_{X-3} \times 2^{X-3}$	NC_8
2	2	1	1	N/A	N/A	N/A	N/A
4	4	4	6	1	1	N/A	N/A
8	6	12	28	6	70	1	1
16 (this device)	8	32	120	24	1 820	8	12 780
32	10	80	496	80	35 960	40	1.05×10^7
64	12	192	2 016	240	635 376	160	4.43×10^9
128	14	448	8 128	672	1.07×10^7	560	1.43×10^{12}