Microfluidic arrays for logarithmically perfused embryonic stem cell culture

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We present a microfluidic device for culturing adherent cells over a logarithmic range of flow rates. The device sets flow rates through four separate cell-culture chambers using syringe-driven flow and a network of fluidic resistances. The design is easy to fabricate with no on-chip valves and is scalable both in the number of culture chambers as well as in the range of applied flow rates. Using particle velocimetry, we have characterized the flow-rate range. We have also demonstrated an extension of the design that combines the logarithmic flow-rate functionality with a logarithmic concentration gradient across the array. Using fluorescence measurements we have verified that a logarithmic concentration gradient was established in the extended device. Compared with static cell culture, both devices enable greater control over the soluble microenvironment by controlling the transport of molecules to and away from the cells. This approach is particularly relevant for cell types such as embryonic stem cells (ESCs) which are especially sensitive to the microenvironment. We have demonstrated for the first time culture of murine ESCs (mESCs) in continuous, logarithmically scaled perfusion for 4 days, with flow rates varying >300 × across the array. Cells grown in the slowest flow rate did not proliferate, while colonies grown in higher flow rates exhibited healthy round morphology. We have also demonstrated logarithmically scaled continuous perfusion culture of 3T3 fibroblasts for 3 days, with proliferation at all flow rates except the slowest rate.

Introduction

Recently there has been increasing interest in culturing cells in perfused microfluidic environments. In conventional static cell culture systems, the composition of the microenvironment varies uncontrollably over space and time as cells interact with the media. In contrast, continuous perfusion offers the ability to control cell-media interactions by controlling the chemical composition of the media that surrounds cells as well as the mechanical forces applied during flow. While perfusion culture can be performed at the macroscale, combining perfusion with microfluidics extends the practical range of flow rates. This is especially true for non-recirculating perfusion culture systems, where new media is continuously perfused through the cell culture and then sent to waste instead of being recirculated back to the cells. Using microfluidics to implement non-recirculating perfusion reduces the culture volume by orders of magnitude, thus enabling high flow rates that would otherwise consume large amounts of costly reagents in macroscale systems. Microfluidic perfusion also enables fluid delivery via laminar flow as well as integration of perfusion with other microtechnologies, such as dielectrophoretic cell patterning.1 Finally, microfluidic systems offer the potential for scalable, massively parallel experimental platforms capable of exploring a large experimental space while consuming small quantities of reagents.

To date a variety of microfluidic perfusion culture systems has been developed, including systems with on-chip diluters for applying a linear concentration gradient across an array of cultures,2–4 perfusion culture combined with cell patterning for investigating differentiation on chip,5 devices applying a linear range of flow rates for studying cell adhesion under shear,6 arrays with programmable flow rates7,8 and perfused flat-plate microfluidic hepatocyte cultures as test beds for exploring favorable growth conditions9,10 or for applying oxygen gradients across the perfused culture.11 Microfluidic perfusion has been used to perform continuous chemical probing and monitoring of islets of Langerhans.12 Several groups have also investigated perfused cell culture on microscale scaffolds to create 3-dimensional artificial tissues.13–16 In these cases perfusion is used to deliver nutrients throughout the volume of the 3-dimensional tissue.

There has also been growing interest in using microtechnology to enhance stem cell culture. Stem cell self-renewal and differentiation fates are particularly sensitive to the microenvironment.17,18 Microtechnology offers the potential for manipulating this microenvironment in ways inaccessible using traditional techniques. Some approaches to incorporating microtechnology with stem cell biology include large arrays of wells for isolating and monitoring single neural progenitor cells,19 monitoring mESC differentiation on arrays of various extracellular matrices,20 and static culture of human ESCs.21 We are particularly interested in the use of microfluidics to control the soluble microenvironment in ESC culture; we define “soluble microenvironment” as the biochemical composition of the liquid media in the cell microenvironment.
While conventional dish-based static culture has greatly advanced the knowledge of ESC biology, there are several limitations. In static culture, cells are constantly interacting with the microenvironment, taking up substances and secreting signals and waste. Instead of a defined, homogeneous mixture, the soluble microenvironment becomes a complex, spatially and temporally varying system. While the work to date has investigated many aspects of manipulating cell-culture microenvironments, the role of perfusion in controlling cell-cell interactions has not been fully explored.

To investigate the effects of perfusion on the soluble microenvironment, we have developed a device to apply logarithmic flow rates to perfusion culture across a single device. The device uses syringe-driven flow combined with a network of fluidic resistances to create a logarithmic range of flow rates through separate cell-culture chambers. Microfluidics offers the potential for massively parallel cell-culture systems; incorporating logarithmic scales, both in perfusion and in reagent concentration, extends the range of parameterized control of the soluble microenvironment. Although logarithmic ranges are often used in biology to explore a large parameter space, to-date they have not been commonly applied to microfluidic perfusion culture. In the area of logarithmic gradients, Pihl et al.\(^2\) have developed a device to generate logarithmic concentration gradients for pharmacological testing on a patch-clamped cell. Gu et al.\(^7\) have developed a microfluidic device that can apply a logarithmic range of flow rates using on-chip peristaltic pumps that recirculate media from a large reservoir. Instead, we have been interested in developing a system for applying logarithmic flow rates across a single chip using non-recirculating perfusion.

Here we describe the design, fabrication, and evaluation of our logarithmically perfused cell-culture system. We can successfully culture 3T3 fibroblasts and mESCs in logarithmic flow rates for several days under continuous perfusion. Finally, we also demonstrate how the logarithmic flow-rate device can be combined with other microfluidic functions, such as a logarithmic concentration gradient, to create a wider range of microenvironments on-chip.

**Theory**

Pressure-driven flow through rectangular channels is a well-known phenomenon. The devices described here operate by connecting a constant flow source to a network of geometrically set fluidic resistances. These conditions determine flow rates through each part of the microfluidic network. To determine the geometry corresponding to these fluidic resistances, we calculated the fluidic resistances, \(R\), using the formula for steady-state pressure-driven flow in a rectangular channel:\(^2\)

\[
\Delta P = QR \tag{1}
\]

\[
R = \frac{12\mu L}{h^3w} \left(1 - \frac{192h}{\pi^2w} \sum_{n=0}^{\infty} \tanh((2n+1)\frac{2\pi}{3h})\right) \tag{2}
\]

where \(P\) is the pressure, \(x\) is the length along channel, \(Q\) is the fluid flow rate, \(L\) is the channel length, \(w\) is the channel width, \(h\) is the channel height, and \(\mu\) is the fluid viscosity. This calculation assumes that the direction of flow is along the channel length and that \(w > h\).

We assumed the flow profile in the middle of the culture chambers would be well-approximated by parallel-plate Poiseuille flow:\(^2\)

\[
v_x(y) = 1.5U \left(1 - \frac{2y}{h} \right)^2 \tag{3}
\]

where \(y\) is the distance along the channel height (\(y = -h/2, +h/2\) at the walls), \(v_x\) is the velocity in the \(x\) direction, and \(U\) is the mean channel velocity.

We modeled the fluid shear on the cells by assuming that it would equal the shear stress at the wall between parallel plates under parabolic flow:\(^2\)

\[
\tau = \frac{6\mu Q}{h^2w} \tag{4}
\]

Because our devices have rectangular culture chambers, this estimate may differ from the actual shears experienced by the cells, but it should be a reasonable approximation because \(wh\) is large (~15). This estimate does not account for changes in flow due to the presence of the attached cells, but since our goal is to ensure that the shear stress is low enough to have little physiological effect, we are interested in an order-of-magnitude estimate. Gaver and Kute estimated the shear stress on a single cell adhered to a channel wall (no neighboring cells) to be ~3 times greater than the shear stress calculated for an empty channel when \(kh \sim 0.1\), where \(k\) is the height of the adhered cell.\(^2\) Since in our case, \(kh \sim 0.1\), a more conservative estimate for the shear experienced by the cells is:

\[
\tau_{\text{single cell}} = 3\tau \tag{5}
\]

**Experimental**

**Cell culture**

We cultured 3T3 murine fibroblasts in 3T3 media: Dulbecco’s Modified Eagle’s Medium (DMEM, 11960044, Invitrogen, Carlsbad, CA) supplemented with 5% bovine calf serum (SH30072.03, Hyclone), 4 mM L-glutamine (25030081, Invitrogen, Carlsbad, CA), 100 U/mL penicillin and 100 μg mL\(^{-1}\) streptomycin (15140122, Invitrogen, Carlsbad, CA). We maintained the cells in a 37 °C humidified environment with 7.5% CO\(_2\), fed cells every other day with 3T3 media and passaged when 90% confluent using a solution of 0.25% trypsin with 3.8 g/L EDTA-4Na (25200056, Invitrogen, Carlsbad, CA).

We cultured ABJ1 mESCs with a stably integrated GFP reporter for Oct-4 without feeders in ES media: DMEM (11960044, Invitrogen, Carlsbad, CA) supplemented with 15% ES-qualified fetal bovine serum (16141079, Invitrogen, Carlsbad, CA), 4 mM L-glutamine (25030081, Invitrogen, Carlsbad, CA), 1 mM non-essential amino acids, 50 U mL\(^{-1}\) penicillin, 50 μg mL\(^{-1}\) streptomycin (15140122, Invitrogen, Carlsbad, CA), 100 μM β-mercaptoethanol (M7522, Sigma,Sa.
Louis, MO), and 500 pM leukemia inhibitory factor (LIF, ESGRO, Chemicon, Temecula, CA). We cultured cells directly on tissue-culture plastic (430639, Corning, Corning, NY) in a 37 °C humidified environment with 7.5% CO₂. For maintenance of mESCs, we fed cells daily and passaged every other day using 0.25% trypsin with 3.8 g L⁻¹ EDTA-4Na (25200056, Invitrogen, Carlsbad, CA) at a density of ~8 × 10⁶ cells cm⁻².

Microfluidic fabrication: 1 × 4 logarithmically scaled flow-rate device

The 1 × 4 logarithmic flow-rate device consists of a single-layer network of channels molded in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) and bonded to glass using standard procedures as shown in Fig. 1. We created the microfluidic channel layout using AutoCAD (Autodesk, San Rafael, CA), printed the design onto high-resolution transparencies and transferred it to a chrome photomask (Fineline Imaging, Colorado Springs, CO). To create the mold, we prepared a silicon wafer (dehydration and Chemicals, Allentown, PA) for 2 hours in a 65 °C oven. After curing we removed the PDMS from the mold wafer and removed the steel posts, cut out individual devices, and punched output access holes into the PDMS using a 16 gauge blunt-end needle. Since fluid flow was always unidirectional from input to output, the more convenient punching method could be used for the output holes, since any PDMS particles at the output would not be swept back into the device. Meanwhile, we cleaned standard 25 mm × 75 mm glass slides (48300-025, VWR, West Chester, PA) with 70% ethanol, immersed the slides in Nanostrip (Cyantek Corp., Fremont, CA) for 10–15 minutes, then rinsed them in de-ionized (DI) water, dried them with compressed N₂ and placed them in a 65 °C oven for 5 minutes to dehydrate. We cleaned the PDMS surfaces with adhesive tape and exposed the PDMS and glass slide to air plasma for one minute (PDC-001, Harrick Plasma, Ithaca, NY). Then we immediately bonded the PDMS and glass to form a completed device, shown in Fig. 1(d). To strengthen the bond, we placed the bonded device in a 65 °C oven overnight. A photograph of the completed device is shown in Fig. 2(a).

Microfluidic fabrication: 4 × 4 flow rate and concentration gradient device

Fabricating the upper and lower PDMS molds. As shown in Fig. 3(g), the finished 4 × 4 flow rate and concentration gradient device consisted of four interconnected microfluidic layers: a 30 μm-high mixer layer, a 100 μm-high delivery layer, a 100 μm-high via layer, and a 100 μm-high chamber layer. An upper PDMS slab contained the mixer and delivery layers, while a lower PDMS slab contained the via and chamber layers. To pattern the upper and lower PDMS slabs, we fabricated two 2-layer SU-8 molds. Fig. 3(a)–(d) summarizes the fabrication of a 2 layer SU-8 mold—we used similar procedures to make the upper and lower molds, the only difference being the heights and masks used for individual layers. To fabricate the mold wafer for the lower PDMS slab, we patterned the 100 μm chamber layer onto a Si wafer using the same procedure described for the 1 × 4 device, except that the SU-8 layer was not developed (Fig. 3(a)–(b)). We then repeated the procedure, coating a second 100 μm layer on top of the patterned but undeveloped layer and aligning the photomask for this via layer with the visible (but undeveloped)
pattern, exposing both layers (Fig. 3(c)). Finally, we spin developed both layers simultaneously for 7–10 minutes (Fig. 3(d)). We repeated this process for the “upper” wafer, substituting a thinner 30 μm second layer using SU-8 2015 (Microchem, Newton, MA. 5 s spread at 500 rpm, 5 s acceleration, 30 s at 1300 rpm), with shorter softbake (95 °C, ~2 min), exposure (1 min) and postbake (95 °C, ~1 min) routines. We coated both upper and lower mold wafers in HMDS for 30 minutes to prevent PDMS adhesion.

Molding the PDMS and assembling the device. We mixed PDMS in a ratio of 10:1, base to curing agent, degassed it in vacuum for 30 minutes, and poured a small volume (~5 mL) onto the lower mold wafer. Next we sandwiched the PDMS-coated wafer between two sheets of transparency film, placed a blank 4º Si wafer on top of the transparency layer that touched the PDMS, and sandwiched the stack between two 1 cm-thick aluminium plates. We placed the entire sandwich, including aluminium plates, on a room temperature hotplate and compressed it using a large C-clamp, creating a ~200 μm-thick layer of PDMS, illustrated in Fig. 3(f). We set the hotplate temperature to 65 °C and cured the PDMS in this setup for 2 hours. After curing we removed the transparency + PDMS + wafer sandwich from the hotplate and peeled away the transparencies, leaving the PDMS on the wafer mold. At this time we inspected the patterned PDMS layer and manually cleared any unopened vias.

Meanwhile we patterned the upper PDMS slab using HMDS-coated 19 gauge steel wire posts to mold input and output holes. We poured PDMS onto the upper mold wafer to a thickness of ~7 mm and cured the PDMS in an oven at 65 °C for 2 hours, shown in Fig. 3(e). We then peeled the cured PDMS off of the mold and cut apart individual devices. Without removing the lower PDMS layer from the mold wafer, we exposed the lower and upper PDMS layers to air plasma for one minute, then aligned by hand and bonded the two PDMS layers. Next we removed the two bonded PDMS layers from the lower mold wafer, cut apart individual devices, and plasma bonded them in the same manner to a standard 25 mm × 75 mm glass slide (48300-025, VWR, West Chester, PA), shown in Fig. 3(g). We placed the entire device in a 65 °C oven overnight under compression to improve bond strength. The completed device is shown in Fig. 2(b).
Optics

In the perfusion culture experiments we used an inverted microscope (Zeiss Axiovert 200, Thornwood, NY) with a SPOT RT Color 2.2.1 camera (Diagnostic Instruments, Sterling Heights, MI). We used an automated upright microscope (Zeiss Axioplan 2 Imaging, Thornwood, NY) with an LA Vision ImagerQE camera in the particle velocimetry and concentration gradient experiments, and also to measure the PDMS layer heights using a 20× Mireau interferometric objective (Nikon).

Measuring microfluidic channel heights

To measure the channel heights for both the 1 × 4 and 4 × 4 devices we used a 20× Mireau interferometric objective (Nikon) to locate planes within the PDMS by visualizing interference fringes. We made measurements on unbonded PDMS pieces patterned from the same molds used to make the devices we characterized. We measured the distance between planes by recording the microscope stage height at each plane. We repeated this at several locations across the device. Channel heights typically varied by ~10% across each chip. For the 1 × 4 device the mean channel height was 83.1 μm with a standard deviation of 3.6 μm over 20 measurements. For the 4 × 4 device the mean height of the chamber layer was 102.9 μm ± 3.3 μm (± 1 S.D., n = 16 measurements). The mean height of the delivery layer was 97.5 μm ± 2.5 μm (n = 19). The mean height of the mixer layer was 32.7 μm ± 1.4 μm (n = 10), and the mean height of the via layer was 116.5 μm ± 5.9 μm (n = 16).

Characterization of logarithmically scaled flow rates: Particle velocimetry

To characterize the flow rates in the 1 × 4 logarithmic flow-rate device we used a particle velocimetry approach. To access the microfluidic channels we press-fit PEEK tubing (1532, Upchurch, Oak Harbor, WA) and Teflon plugs (P-316, Upchurch, Oak Harbor, WA). To remove air bubbles in the device, we filled the chip with deionized (DI) water using a syringe at high pressure (>5 psi) for ~15 minutes (this standard procedure is also referred to as “blind filling” or “dead-end filling”). We suspended 2 μm-diameter red-fluorescent polystyrene beads (19508, Polysciences, Warrington, PA) in DI water with 0.1% Triton X-100 (Sigma, St. Louis, MO) at a concentration such that the areal density at one focal plane was ~1.5 × 10^3 beads μm^-2. We loaded this suspension into a 250 μL glass syringe (Hamilton, Reno, NV) that was mounted into a syringe pump (KD Scientific 200, Holliston, MA). Taking care not to introduce new bubbles, we connected the syringe to the device to inject the bead suspension.

We measured the bead velocities one chamber at a time, starting with the slowest chamber. For each chamber, we set the syringe pump flow rate and allowed the system to reach equilibrium for ~5–10 minutes. Then we focused the microscope halfway through the depth of the chamber and took snapshots of the bead suspension at 5 second intervals, repeating this at three locations along the length of the culture chamber. After taking measurements for each chamber, we flipped the device upside-down to minimize bead settling. Because of the wide range of flow rates across the device, we used different total-chip flow rates to measure each chamber. This scaling ensured that the bead velocities remained within a practically measurable range. We set the total perfusion rates to 0.1 μL min^-1, 0.1 μL min^-1, 3 μL min^-1, and 5 μL min^-1 (slowest chamber to fastest chamber). To calculate the velocities, we used Matlab (Mathworks, Natick, MA) to measure the distances traveled by beads in the 5 second intervals. For each sample, we chose the fastest bead velocity, as it represented the centerline flow velocity.

Measurement of logarithmic concentration gradient

To characterize operation of the logarithmic diluter in the 4 × 4 device, we used quantitative fluorescence microscopy to measure the concentration gradient. We first blind-filled the with DI water to remove any air bubbles. We then filled one 1 mL plastic syringe (Becton Dickinson, Franklin Lakes, NJ) with 1 mM fluorescein sodium salt (Sigma, St. Louis, MO) and a second with DI water. We selected the fluorescein concentration to enable imaging over a large range of dilutions without requiring excessive exposure times. We then established a concentration gradient with the device in the “loading” mode by setting a flow rate of 5.00 μL min^-1 for the fluorescein solution and 13.02 μL min^-1 for the DI water. After a steady gradient was established throughout the array, we imaged each chamber. We selected exposure times such that fluorescent intensity varied linearly with exposure time at the concentrations of interest (as determined by calibration measurements), allowing us to compensate for differences in exposure times across the 4 × 4 array by linearly scaling the results. For each chamber, we imaged the chamber’s center, fully occupying the field of view. We waited 2 minutes after each exposure before obtaining the next image, allowing the contents of each chamber to turn over ~5 times to minimize the effects of photobleaching. (With the device in “loading” mode, all chambers had equal flow rates.) We used a calibration curve (created via a dilution series of the 1 mM fluorescein solution) to convert fluorescence intensity to concentration.

Logarithmically scaled perfusion culture: fluidic setup

We connected the 1 × 4 logarithmic flow-rate device to a fluidics setup as shown in Fig. 4(b) using connectors, valves, and tubing from Upchurch (Oak Harbor, WA). We used a syringe pump (KD Scientific 200, Holliston, MA) as a constant flow source for the fluidics. We placed the syringe pump outside the incubator (Steri-cycle, Thermo Forma, Philadelphia, PA) and positioned the device and accompanying valves inside the incubator to maintain the device in a 37 °C, humidified, 5% CO2 environment. We connected a plastic syringe (Becton Dickinson, Franklin Lakes, NJ) outside the incubator to ~2 feet of flexible Teflon tubing (1536, Upchurch Scientific, Oak Harbor, WA) that was threaded through an existing access port in the incubator. Inside the
In the incubator, we attached the tubing to a Luertight connector system (P-837, Upchurch, Oak Harbor, WA) which we connected to a large bubble trap (also known as a debubbler) (6115, Varian, Torrance, CA) with a 1 mL total volume. The debubbler allowed the device and fluidics setup to be disconnected and removed from the incubator for microscopy without introducing bubbles. As shown in Fig. 4(b), the fluidics setup inside the incubator consisted of two main paths to the device: a loading path and a feeding path, connected by various Upchurch valves (V-101D, V-101T, Upchurch Scientific, Oak Harbor, WA). Using separate loading and feeding tubes reduced the amount of "backlog" cell suspension fed to the cells after starting perfusion. For this reason we also minimized the length of the tube joining the T-connector to the device. We also included an additional, smaller debubbler and 2 μm filter (P-272X, Upchurch, Oak Harbor, WA) in the feeding path to guard against bubbles during perfusion; even with the larger, upstream debubbler at the input, we still occasionally observed new air bubbles in the device a few days after starting perfusion. For this reason we also minimized the length of the tube joining the T-connector to the device. We also included an additional, smaller debubbler and 2 μm filter (P-272X, Upchurch, Oak Harbor, WA) in the feeding path to guard against bubbles during perfusion; even with the larger, upstream debubbler at the input, we still occasionally observed new air bubbles in the device a few days after starting perfusion. We fabricated the smaller, downstream debubbler by carving a ~200 μL cavity in a ~2 cm × 2 cm × 0.7 cm slab of PDMS, punching two access holes in the cavity, and bonding the PDMS slab to a glass slide, creating a microfluidic chamber. We oriented the small debubbler slide-side up with press-fit input and output tubes pointed downward so that any passing bubbles would collect in the chamber. We connected the output of the small debubbler to a 2 μm filter (P-272X, Upchurch, Oak Harbor, WA) to prevent large particles from clogging the smaller channels in the device. Except for the tubing connecting to the small debubbler chamber, all other fluidic routing used flexible clear Teflon tubing (1529, Upchurch Scientific, Oak Harbor, WA and 10-15-00214 Optimize Technologies).

Logarithmically scaled perfusion culture: general procedure for culturing cells

Each perfusion experiment used the following protocol: We prepared equilibrated media by incubating the media overnight in a 10 cm or 15 cm cell-culture dish in a humidified 37 °C environment with 7.5% CO₂; this media was used throughout the experiment (blind filling and perfusion culture). Next we assembled the entire fluidics system as shown in Fig. 4(b) and flushed the fluidics and chip with 70% ethanol for >30 minutes to sterilize the system. To remove air bubbles from the device and flush the ethanol out of the system, we plugged the loading outputs shut, connected 20 of 1/16"-OD/0.0024"-ID tubing (1560, Upchurch, Oak Harbor, WA) to the logarithmic output, and perfused the system overnight with equilibrated media at 10 mL min⁻¹, creating pressures >5 psi within the channels to drive air out through the PDMS walls. We set the 3-way valves so that all parts of the system were fluidically connected as shown in Fig. 4(a.1).

The next day (designated day 0) we loaded the cells into the microfluidic device. We made a cells-in-media suspension by trypsinizing cells with TrypLE Express (12605-010, Invitrogen, Carlsbad, CA), quenching trypsin with serum-containing media, then counting cells on a hemacytometer. To make the appropriate concentrations we centrifuged the initial cell suspension for 5 minutes at 1000 rpm, aspirated the supernatant, resuspended the cells in equilibrated media, and triturated to produce a single cell suspension at a density of 0.5 × 10⁶ cells mL⁻¹ for the 3T3 cells and 2.2 × 10⁶ cells for the Logarithmically scaled perfusion culture: general procedure for culturing cells.
mL$^{-1}$ for the mESCs. All cells used were passage 21 or lower. After placing the fluidics and device in a sterile biosafety cabinet, we turned the upstream 3-way valve such that the cell suspension would flow only through the loading input tube and the feeding tube was bypassed (no flow through the feeding tube), shown in Fig. 4(a,2). We then plugged the logarithmic output shut and unplugged the four loading outputs. We loaded the cell suspension into a 1 mL syringe (Becton Dickinson, Franklin Lakes, NJ) and attached it to the 4-way valve as shown in Fig. (b). To monitor loading we placed the device on a phase microscope (Zeiss Axiovert) and slowly injected the cell suspension by hand. When sufficient cells were loaded into each chamber, we plugged the loading outputs and removed the syringe containing the cell suspension. We then turned the upstream 3-way valve so that the device was fluidically isolated from the syringe input, leaving the logarithmic inputs plugged. (Thus, all inputs/outputs to the device were blocked to minimize fluid movement during cell attachment.) To flush out any extra cells in suspension in the tubing we flushed the rest of the system with new media. Fig. 4(a,3) shows the valve/plug settings for this step.

We allowed the cells to attach for 4 hours in static culture (no perfusion) by placing the setup in the humidified incubator at 37 °C and 5% CO$_2$, with valve/plug settings as shown in Fig. 4(a,4). For both 3T3 and mESC culture we observed that minimizing fluid movement during the attachment period was helpful for cell attachment. After cell attachment we reconnected the setup to the syringe input, as shown in Fig. 4(a,5). We set the syringe pump outside of the incubator to a fixed flow rate. Each day perfusion was interrupted for a brief time (<30 min) while we removed the setup from the incubator to take images at the microscope. After imaging we replaced the setup inside the incubator and resumed perfusion.

### Results

**Design of 1 $\times$ 4 logarithmic flow-rate device**

We have designed a microfluidic device that generates a logarithmic range of flow rates through separate cell-culture chambers. The design is easy to fabricate, with no on-chip valves, and operates as a simple, single-layer fluidic resistance network. An off-chip syringe pump feeds constant-current flow to the fluidic resistance network in the device, creating a logarithmic range of flow rates. The device layout is shown in Fig. 2(a) and contains four cell-culture chambers, each 1.25 mm$^2$ and chamber volume of 0.32 mL. The chambers are connected to a joint input via low-resistance channels designed to have the same fluidic resistance.

Each chamber has two output channels, a “loading” output and a “logarithmic” output. The loading output channels have the same geometry and fluidic resistance, whereas the logarithmic output channels have logarithmically varying fluidic resistances. The device operates either in “loading mode”, where the logarithmic output is temporarily plugged, or in “logarithmic mode”, where the loading output channels are plugged (Fig. 5). In “loading mode” the flow rates through each channel were designed to be the same, whereas in “logarithmic mode” the flow rates through each channel were designed to span a logarithmic range across the device. The logarithmic output channels all connect to the same tube off of the chip, whereas the loading output channels each lead to a separate tube off of the chip.

We set the logarithmic flow rates by choosing the appropriate fluidic resistance for each logarithmic-mode output channel ($R_i$, where $i = 1, 2, 3, 4$ in Fig. 6(a)). To illustrate, consider a hypothetical isolated Y-shaped tubing junction, with a constant input flow rate from the bottom tube of the “Y”. If the fluidic resistances of the left and right branches are the same, there will be equal flow through each branch. However, if the fluidic resistance of the right branch is 6$\times$ larger than that of the left branch, the flow rate through the right branch will be 6$\times$ smaller than the flow through the left branch according to eqn (1) and Kirchhoff’s current and voltage laws. Using these principles, we designed a logarithmic range of flow rates across four parallel “branches”, where the flow rate of each branch, $Q_i$, is determined by eqn (6).

$$Q_i = Q_{total} \left( \frac{1}{(R_{ch} + R_i) \sum_j \frac{1}{R_{ch} + R_j}} \right)$$

where $i$ is the branch index, $j$ is a dummy index over all branches, and $Q_{in}$, $Q_{total}$, $R_{ch}$, $R_i$, and $R_j$ refer to flow rates and fluidic resistances as shown in Fig. 6(a). We assume that in logarithmic mode, $R_L \rightarrow \infty$ and there is no flow through the $R_L$ branches.

For both devices presented here, we designed the flow rates to vary by a factor of 6 from one chamber to the next when in logarithmic mode. We chose the factor of 6 to allow the entire device to be of a size that it could be extended to a 4 $\times$ 4 array that would fit on a standard microscope slide. In addition, we...
designed the smallest channel dimension to be >40 μm to reduce channel clogging during operation. To avoid bulging of the PDMS or bursting the PDMS/glass bond, we designed the channel geometries so that the pressure drop in the device was small (<1 psi) under normal operation. To allow easy connection to off-chip fluidics, we designed large areas for input/output tubing.

To choose the device geometry, we first started with the desired flow rates, $Q_i$ and worked back to calculate the necessary channel geometries using eqn (1)-(2) and basic circuit theory. We show a schematic of the design process for the 1 x 4 device in Fig. 6(a) along with an image of the completed device in Fig. 2(a). We designed the layout of the device as follows: (1) Choose logarithmic mode flow rates $Q_1$–$Q_4$. (2) Determine all fluidic resistances $R_{ch}$, $R_L$, and $R_i$, where $i = 1,2,3,4$: (a) Choose $R_{ch}$, $R_L$. (b) Choose an arbitrary, low pressure difference $P_{in} - P_{out}$. (c) Assume $Q_{L1} = Q_{L2} = Q_{L3} = Q_{L4} = 0$ in logarithmic mode. (Loading outputs plugged closed.) (d) Using Kirchhoff’s current law: $Q_{ch1} = Q_{ch2} = Q_{ch3} = Q_{ch4} = Q_L$. (e) Calculate $P_L = P_{in} - Q_iR_{ch}$, where $i = 1, 2, 3, 4$. (f) Calculate $R_i = (P_L - P_{out})/Q_i$, where $i = 1, 2, 3, 4$. (3) Draw an initial network layout of lines to determine $L_i$ = channel length for each resistor $R_i$. (4) Given $R_i$, $L_i$, and $h_i$ = channel height, calculate $w_i$ = channel width using eqn (2). (We used the “fsolve” nonlinear solver in Matlab with n = 2.) (5) “Fill out” the network layout using $w_i$’s calculated in step 4. (6) Repeat steps 3–6, adjusting the network layout until channels fit, with acceptable spacing between channels. Also iteratively adjust $P_{in} - P_{out}$ as necessary to create practical fluidic resistances.

Although we designed the device to experience constant flow through the chambers during culture, in some cases the flow might be so slow that diffusion could become important. This was a concern since the four cell-culture chambers were interconnected through the inputs and the logarithmic outputs. To ensure that diffusion would not be relevant, we designed the interconnections between the channels to be much longer than the ~1.5 mm that a typical 20 kDa molecule would diffuse in 4 days.

Using eqn (4) and a velocity estimate based on particle velocimetry measurements described below, we calculated the shear stress in the device to be $\tau = 0.10$ dyn cm$^{-2}$ at the fastest flow rate used over all the perfusion cultures (both mESC and 3T3 fibroblast). Even the more conservative estimate $\tau_{\text{single cell}} = 0.31$ dyn cm$^{-2}$ (eqn (5)) is well below typical arterial shear stresses of ~15 dyn cm$^{-2}$ 30 and in the same range as in other microfluidic cell-culture devices. 33 Recently Fok and Zandstra demonstrated that the proliferation and developmental potential of mESCs grown in a stirred suspension bioreactor under continuous shear stresses of 6.25 dyn cm$^{-2}$–9.86 dyn cm$^{-2}$ was comparable to that of controls. 32 Our highest estimated shear stress is more than an order of magnitude lower than the shears used, 32 suggesting that it should have minimal effect on the cell cultures.

Flow rate measurements vs. modeling

Since our goal was to develop a device that could easily create a large range of flow rates from a single input, it was important to ensure that the flow rate ratios across the chambers
corresponded to our predictions. As opposed to concentration, flow rate is more difficult to measure in an array of microfluidic devices. However, since our chambers have rectangular cross-sections and thus a known parabolic flow profile (eqn (3)), we used a simplified version of particle velocimetry to measure the actual flow rates in each of the chambers. Essentially, we know that the highest fluid velocity is at the midpoint of the chamber height, and that combining this velocity with the chamber dimensions allows us to back-calculate the volumetric flow rate. Thus, we measured the centerline flow velocity by finding the velocity of a set of beads in two imaged fields (separated in time) and then used the highest velocity as the centerline.

Fig. 7(a) shows a comparison between measured vs. predicted maximum velocities for each channel in logarithmic mode. We calculated the predicted velocities using a combination of Matlab and FEMLAB modeling of as-fabricated fluidic resistances. This shows that we indeed are generating logarithmically varying flow rates across the device. Although the actual range of velocities/flow rates (>300 ×) was larger than we designed for (216 ×), this is due to a modest (~43%) difference in the lowest measured flow rate; the resulting range is extremely sensitive to variations in the smallest flowrate. This difference is likely due to a combination of experimental error, differences between designed-for and fabricated geometries, and simplifying assumptions in the model.

3T3 perfusion culture

To demonstrate the use of the 1 × 4 logarithmic flow-rate device with cells, we performed perfusion culture of murine 3T3 fibroblasts for 3 days in the device at a total flow rate of $Q_{total} = 10 \mu L \cdot h^{-1}$ ($Q_{total} = Q_1 + Q_2 + Q_3 + Q_4$). We estimated the flow rates applied to the four individual cell-culture chambers to be $1.6 \times 10^{-4}$, $2.9 \times 10^{-3}$, $1.7 \times 10^{-2}$, and $0.17 \mu L \cdot min^{-1}$ using the particle velocimetry data. During cell loading we achieved a roughly uniform seeding of cells throughout the chamber. Fig. 8 shows qualitative results on days 1 and 3 at each of the four flow rates. Over several days of culture, cells exhibited a well-adhered, healthy morphology comparable to static culture. Cells proliferated in the fastest 3 chambers but not in the chamber with the slowest flow rate. In the chamber with the fastest flow rates, cells proliferated more than in the other chambers and were more densely packed on day 3, while for the middle two flow rates, the proliferation was qualitatively similar. After day 3 we stopped...
the experiment because the cultures were confluent. These results are consistent with several 3T3 perfusion culture experiments we have run at various cell seeding densities and flow rates on multiple devices.

Although the device was designed for one-time use, we have observed the ability to remove all adhered 3T3 cells from the device by flowing trypsin through the device. We were also able to successfully reseed the surface with new 3T3 cells (data not shown).

Murine ESC perfusion culture

Unlike 3T3 fibroblasts which can tolerate a wide variety of culture conditions, murine ESCs are known to have more specific culture requirements. To demonstrate the capability of 1 × 4 logarithmic flow-rate device for culturing more demanding cell lines, we performed 4 day perfusion culture of murine ESCs in the logarithmic flow-rate device. We applied a total flow rate \( Q_{\text{total}} = 65 \ \mu\text{L h}^{-1} \) to the entire device, and the flow rates through each chamber were estimated using the particle velocimetry data to be \( 1.0 \times 10^{-3}, 1.9 \times 10^{-2}, 0.11, 1.1 \ \mu\text{L min}^{-1} \). Fig. 9 shows the results on days 1 and 4 at the four applied flow rates. The faster flow rates produced larger but fewer colonies. We used MATLAB image processing tools to threshold the image and segment colonies. Once colonies were identified, the largest colony was discarded, as were colonies that were smaller than 0.1 times the second-largest colony. The mean of the remaining colony areas was found for each flow condition and is shown in Fig. 9(i), plotted against flow rate and shear stress estimated using the particle velocimetry data and eqn (5). We also assayed Oct-4 GFP on day 5 of the assay.

![Image](https://example.com/image.png)

**Fig. 9**  Perfusion culture of ABJ1 mouse ESCs over logarithmically scaled flow rates. (A)–(D) show cell cultures after one day of perfusion at various rates. (E)–(F) show resulting cultures after 4 days of perfusion at various rates. Each column (A/E, B/F, etc.) displays results for a different flow rate. Calculated flow rates for each column are shown above in bold. The average time to replace one culture-chamber volume is shown in italics. By day 4, chambers with high flow rates (G)–(H) display large, round colonies, suggesting a favorable growth environment, however, the chamber in E with the lowest flow rate has poor proliferation. All photos are at the same scale. (I) Mean colony area vs. flow rate and estimated shear stress for ABJ1 mouse ESCs grown under continuous perfusion (data from same experiment as Fig. 9). The mean colony areas are larger at higher flow rates (>\( 10^{-2} \ \mu\text{L min}^{-1} \)) while the mean colony area is smaller at the lowest flow rate. Each data point shows the mean value and standard deviation over all colonies from three images at each flow condition.
(data not shown), which indicated that the majority of cells remained undifferentiated.

**Extension to 4 × 4 array with logarithmic concentration gradient: Design**

In addition to characterizing the logarithmic flow-rate device, we are also interested in exploring how logarithmic flow rates can be integrated with other microfluidic functions. To demonstrate, we designed and fabricated a microfluidic device to combine the logarithmic flow-rate functionality with a logarithmic concentration gradient in a 4 × 4 array. As shown in Fig. 2(b), a logarithmic concentration gradient is applied along one axis of the device while logarithmic flow rates are applied along the other axis. Each column of the device is a replica of the 1 × 4 logarithmic flow-rate device, however, each column receives a different reagent concentration from an on-chip logarithmic diluter. Thus, each cell-culture chamber can experience a unique combination of perfusion and reagent concentration. Like the 1 × 4 logarithmic device, the 4 × 4 concentration/flow-rate device operates in “loading mode” and “logarithmic mode” so that cells may be seeded at uniform flow rates across the chip. The diluter design also enables a logarithmic cell density gradient across the device. During loading, it is possible to flow low and high density cell suspensions into the two diluter inputs—these cell suspensions would be mixed in logarithmic ratios, generating a logarithmic cell density gradient across the device. However, a uniform seeding density across the device can also be easily achieved by perfusing the same density cell suspension into both inputs of the diluter. This design allows the user to flexibly and independently choose uniform or logarithmic operation for cell seeding density, reagent concentration, and flow rates.

To implement the logarithmic concentration gradient, we designed an on-chip diluter to produce dilutions of 0 : 1 : 10 : 100. To conserve space on the chip, this diluter uses 9 : 1 mixes at each junction (instead of 1 : 1 mixes as are commonly used in linear diluters). A schematic diagram of the diluter is included in Fig. 6(c), where resistors $R_{1d} - R_{10d}$ comprise the diluter.

We designed the diluter using a similar method to that used for the 1 × 4 flow-rate device, again using the fluidic resistance calculation for rectangular channels in eqn (2) with $n = 2$. To conserve space, we incorporated staggered herringbone mixers into the diluter design to promote faster mixing for a given channel lengthossip for channels $R_{ad}$ and $R_{bd}$ (Fig. 6(c)). We followed published design rules and designed the mixer to have a fluid rotation per channel length comparable to published devices. Then using empirical data we estimated the number of mixer cycles necessary for thorough mixing at typical flow rates used in this device.

To combine the logarithmic concentration gradient with the diluter, we attached slightly modified copies (Fig. 6(b)) of the 1 × 4 flow-rate device to each of the four diluter outputs. Thus, each created reagent concentration led to four cell-culture chambers that spanned a logarithmic range of flow rates. The difference between the flow-rate block in the 4 × 4 system (Fig. 6(b)) compared with the 1 × 4 device (Fig. 6(a)) is that the four loading resistances $R_L$ are joined.

We then combined all the loading outputs from each 1 × 4 flow-rate-block (Fig. 6(b)) into one joined output off of the chip, connecting them with fluidic resistances $R_{m1} - R_{m4}$. Using a design method similar to that used for the 1 × 4 flow-rate device, we chose each $R_i$ (where $i = 1, 2, 3, 4$) and $R_{m1} - R_{m4}$ such that the pressure at the node between $R_{m}$ and $R_i$ would be the same for each block. We similarly joined the logarithmic outputs from each 1 × 4 flow-rate-block to form a single joint logarithmic output off of the chip. Because this 4 × 4 design contains 16 culture chambers, having 16 separate loading outputs would have been unwieldy. This joint-loading-output design eliminates the necessity of plugging each output individually, and also eliminates the hardware needed for 16 loading outputs. However, the flexibility of being able to control the individual loading outputs is lost—cells can not be extracted separately from separate chambers in this design. Finally, merging the loading outputs to a common loading output and the logarithmic outputs to a common logarithmic output made it topologically necessary to use two layers of interconnected microfluidics.

We assumed that each logarithmic flow rate block would operate identically (but with a different reagent concentration). Because this 4 × 4 system contained many interconnected cell-culture chambers, we modeled the system as a resistor network in SPICE in both loading and logarithmic modes to ensure that there was no undesired backflow due to communication between chambers. In addition the fluidic resistance of the loading outputs had to be high enough so that in logarithmic mode the proper flow rates were still generated (to prevent the fluid from taking a lower resistance path through the loading output resistor). We estimated the fluidic resistance of individual resistors using eqn (2) with $n = 2$.

Because this device spanned several centimetres, the PDMS thermal shrinkage was ~500 μm over the length of the device. After curing at 65 °C, we cooled both upper and lower PDMS pieces to room temperature and removed the upper layer of PDMS completely off the mold, leaving the lower PDMS layer on the mold wafer. The lower layer remained the same dimensions as the mold, whereas the upper layer shrunk ~1% (linear coefficient of thermal expansion $= 310 \mu m \cdot m^{-1} \cdot ^{\circ}C^{-1}$, Dow Corning product information sheet for Sylgard 184). We compensated for this shrinkage by expanding all features in the upper layer by ~1%.

**Extension to 4 × 4 device: Concentration measurements**

Since we had already measured the flow rates on the 1 × 4 flow-rate device, we assumed that flow-rates in the 4 × 4 device would be similar and instead concentrated on characterizing the operation of the logarithmic diluter. We fed DI water into diluter input A and a fluorescein solution into diluter input B (Fig. 6(c)) and measured the fluorescence intensity for each diluter output. To calibrate the intensities with concentration, we measured the fluorescent intensities of known concentrations of fluorescein. These calibration intensities were used to convert intensities to normalized concentration, shown in Fig. 7(b). These results demonstrate that we have successfully attained logarithmic concentration dilution across the array.
Although measurements of the concentration gradient verified the logarithmic operation of the on-chip diluter, these results differed from the designed normalized concentrations of 0, 0.01, 0.1, and 1. Instead of the designed dilution factor of 10 between columns one and two and between columns two and three, dilution factors of ~18 were measured. These differences are likely due to errors in estimating the fluidic resistances, since the resistances at channel intersections were not taken into account, and the heights of the as-fabricated channel layers were not exactly the same as designed and varied over the device.

Discussion

Our perfusion culture experiments have shown the ability to culture mESCs and 3T3 fibroblasts on chip under a wide range of flow rates for several days, and shown that these conditions have significant effects on mESC colony morphology. We have also demonstrated the design, fabrication, and testing of a 4 × 4 array which simultaneously applies a logarithmic flow rate gradient and logarithmic concentration gradient. In contrast with previous work on perfused cultures, our devices exploit logarithmic scales to explore a large range of biological conditions simultaneously. These devices offer new levels of control over the soluble microenvironment as well as the potential for massively parallel experiments combined with automated microscopy; such tools may enable biologists to explore a wider range of more precisely defined conditions during cell culture.

1 × 4 logarithmic flow-rate device design and characterization

The simplicity of design of the 1 × 4 logarithmic culture device offers many advantages for perfusion experiments. First, the simple SU-8/PDMS fabrication enables the design layout to be easily tailored for specific experiments—customizing the culture chamber size, flow-rate range, etc. The design itself is also simple, with no on-chip valves, and could easily accommodate other procedures such as staining on chip or culture of ESCs on a layer of feeder cells. Second, because the culture chamber volumes are small—0.32 μL each—the total fluid consumed is minimal. Even a 7 day experiment running at 65 μL h⁻¹ would only consume ~11 ml, while a similar experiment in even a 384 well plate would consume ~683 ml (assuming a well volume of 20 μL), never mind that changing the media every ~17 s (as in Fig. 9) in such a system would be tedious.

Our design is also scalable both in the range of applied flow rates as well as the number of different flow rates applied. First, our design could easily accommodate a much larger range of flow rates simply by changing the resistor dimensions. This is in contrast to the programmable flow-rate device of Gu et al., that generates flow by pumping fluid on-chip using piezoelectric pins on a Braille display. While it can also generate a logarithmic range of flow rates (500 ×), the dynamic range is limited by hardware issues such as pumping frequency, elastomeric/fluidic mechanical time constants, and pump design—for example, the large pump design reaches a plateau in flow rate as pumping frequency increases. We can also easily scale up the number of flow rates that we apply by increasing the number of chambers in our devices. A 1 × n device could easily be fabricated by adding more channels to the layout, only limited by space.

The device controls flow rates by setting the fluidic resistance of individual channels in the microfluidic network. One potential challenge is that if any one element of the network changes its resistance (gets clogged with particles), the operation of the entire device will change. This can be minimized by using off-chip filters, by molding inlets to the PDMS device, and by limiting the smallest channel dimension to prevent clogging, as we have demonstrated in experiments. In practice we rarely observed clogging, and we observed no clogging in the experiments presented here. Because we are applying a logarithmic range of flow rates, slight alterations in the individual flow rates due to the presence of cells/colonies in the culture chambers should not have a large effect on the biological outcome when compared with the overall logarithmic flow rate scaling.

Our results show a dramatic difference in cell culture when perfused over a wide range of flow rates, especially for mESCs. In the fibroblast culture, qualitative observations of the proliferation implied increasing proliferation with flow rate. This is consistent with previous studies on C2C12 myoblasts in perfusion culture. This is also consistent with previous results that show that perfused cultures grow poorly at lower flow rates. The 3T3 fibroblasts required lower flow rates than the mESCs to maintain a healthy culture, which is consistent with the static culture requirements of the two cell types since typical 3T3 fibroblasts require feeding once every few days, whereas mESCs require daily feeding. In the mESC culture, it remains unknown why the colony sizes and shapes varied so much at different flow rates. At higher flow rates, not only were the colony areas larger, but there were fewer colonies. Fok and Zandstra demonstrated that the proliferation and developmental potential of mESCs grown under shears ≥6.25 dyn cm⁻² were comparable to that of static controls, and they cited a threshold of 6.5 dyn cm⁻² for removing adherent cells from surfaces. Because the maximum shear stress we applied was comparatively low (0.31 dyn cm⁻²), it is unlikely that shear had a significant effect on colony size, morphology, or the number of colonies. More likely mechanisms behind the flow-rate dependent effects include altered nutrient delivery, waste removal, and concentration of secreted factors. For example, larger colony areas at higher flow rates could have been due to increased nutrient delivery, increased waste removal, and increased removal of proliferation-inhibiting secreted factors. Fewer colonies at high flow rates could have been due to increased colony aggregation or biologically-induced colony detachment.

4 × 4 logarithmic concentration gradient and flow device design and characterization

The 4 × 4 logarithmic flow rate and concentration gradient device demonstrates the ability to apply a wide variety of conditions simultaneously. While this design shares many aspects of the 1 × 4 device, there are important differences. Because the loading outputs are joined into a single loading output tube off of the chip, the design is more scalable.
Although the 4 × 4 design is multi-layered and more challenging to fabricate, it offers greater options for controlling experimental conditions. The user can independently choose uniform or logarithmic operation for the initial cell density, flow rates during culture, and reagent concentration during culture. These options allow increased flexibility in experimental design.

Conclusions

As biology becomes more quantitative, more control over the cell-culture microenvironment is necessary to reduce the number of uncontrolled variables, bringing modeling and experiments closer. We have developed a microfluidic device for culturing adherent cells over a logarithmic range of flow rates and have demonstrated an extended version of the device that integrates a logarithmic concentration gradient capability. These devices set the stage for studying the effects of continuous media exchange on the soluble microenvironment. To demonstrate operation of the flow-rate device with cells, we cultured mESCs and 3T3 fibroblasts in continuous perfusion over logarithmically-scaled flow rates and observed qualitative perfusion-dependent differences in proliferation and morphology. The flexible design enables easy customization, scalability, and potential integration with other devices so that a wide range of conditions may be tested in one experiment. The microfluidic implementation consumes minimal reagents while enabling culture in non-recirculating perfusion over several days. These devices serve as enabling tools for investigating aspects of the ESC microenvironment, including the roles of flow rate, reagent concentration, and cell density.

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