Cell-Laden Hydrogels in Integrated Microfluidic Devices for Long-Term Cell Culture and Tubulogenesis Assays

*Nathan P. Gabrielson, Amit V. Desai, Bhushan Mahadik, Marie-Claude Hofmann, Paul J. A. Kenis, and Brendan A. C. Harley**

Microenvironments found in vivo are complex and dynamic, containing spatio-temporally variable contact and diffusible cues. Developing a deeper understanding of epigenetic and transcriptional regulation of cell fate decisions requires a platform to examine how emergent microenvironmental pressures (matrix, biomolecular, cellular) affect cell responses. Isolated cells in the presence of cocktails of biomolecule supplements do not suitably replicate the complex interactions taking place in vivo. As such, ex vivo culture platforms are needed to replicate elements of the spatially and temporally heterotypic 3D microenvironments found in vivo. Such platforms would facilitate a new generation of studies on how microenvironmental factors shape epigenesis, stem cell differentiation, disease pathogenesis (e.g., cancer metastasis), as well as tissue biosynthesis and regeneration.[1,2] Achieving such detailed analysis, however, requires a 3D cell culture platform capable of three basic functions: (i) the ability to efficiently create libraries of diverse microenvironments that define the landscape/geometry in which cells grow; (ii) the ability to perturb the landscape in defined increments so as to assess cell response to defined levels of stimuli; and (iii) the ability to analyze and trace cell response via readily available laboratory techniques including optical and fluorescence microscopy.

Hydrogel biomaterial platforms to define the chemical and geometric microenvironment surrounding cells have been demonstrated by many groups. The work of Anseth et al., for example, showcased cell-supporting 3D matrices that can be cross-linked with UV light to selectively introduce or remove ligands and structural features.[3,4] While effective, the use of UV light can harm cells and is undesirable when working with limited cell numbers of rare cells. Additionally, changes in matrix properties and patterning must be performed experiment-by-experiment, thereby complicating the high-throughput study of cell behavior. Other cell-amenable hydrogel crosslinking methods based on temperature[5] (exemplified by many collagen-based hydrogels), enzymatic[6] (exemplified by many fibrin hydrogels) or chemical conjugation[7] (exemplified by disulfide bond forming hydrogels) have also been developed for in situ hydrogel formation. However, owing to the lack of spatial control afforded by these methods, the realization of chemically- and geometrically-controlled environments using these systems remains a difficult task.

On the microscale, Gunawan et al. used a microfluidic device to generate linear gradients of substrate-immobilized laminin while Toh et al. employed a benzophenone photochemistry approach to photopattern multiple biomolecules on the surface of glass slides.[8,9] Both systems facilitated analysis of cell motility or rolling under a variety of chemically-controlled conditions, albeit only in 2D. In a separate study, Raghavan et al. explored tubulogenesis in unsealed, open-walled microchannels of varying geometries using a chemically uniform 3D matrix.[10] While benefiting from reduced reagent and material consumption afforded by working at the microscale, none of the systems presented an ideal tool to control cell culture environment; the approaches either failed to capture 3D cell behavior or lacked an efficient means to generate heterotypic environments. A potentially superior platform would merge the 2D and 3D microscale strategies outlined above to allow rapid generation of heterotypic matrix environments that serve as reductionist models of complex in vivo conditions yet also permit region-specific analysis of cell response. Such a platform, however, would require tissue culture within microfluidic channels.

Long-term cell culture within microdevices is non-trivial. Many microfluidics-based approaches make use of translucent membranes to support cell nutrient supply and waste removal via diffusion. The membranes also serve to protect the cells from being swept away during media changes. Unfortunately, these membranes hamper characterization of cell phenotype by optical microscopy.[11] Other designs have bypassed membrane incorporation by including features such as additional chambers or orthogonal flow fields to constrain

Dr. A. V. Desai, B. Mahadik, Prof. P. J. A. Kenis, Prof. B. A. C. Harley
Department of Chemical and Biomolecular Engineering
University of Illinois at Urbana-Champaign
600 South Mathews Ave, Urbana, IL 61801, USA
E-mail: bharley@illinois.edu

Dr. N. P. Gabrielson, Mr. B. Mahadik, Prof. M.-C. Hofmann, Prof. P. J. A. Kenis, Prof. B. A. C. Harley
Institute for Genomic Biology
University of Illinois at Urbana-Champaign
1206 West Gregory Dr. Urbana, IL 61801, USA

Prof. M.-C. Hofmann
Department of Comparative Biosciences
University of Illinois at Urbana-Champaign
2001 South Lincoln Avenue, Urbana, IL 61802, USA

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cells within particular areas where they are safe from being damaged or flushed away during media perfusion.[12,13] For example, Choi et al. have created concave microwells within microfluidic channels that utilize gravity to passively trap cells along the channel.[14] Cells trapped in these microwells can then be supplied with fresh nutrients via gentle perfusion without dislodging the cells. However, reproducible creation of such microwells presents an additional fabrication and design challenges. Additionally, the design is best suited for liquid culture systems and may not be ideal for 3D hydrogel systems.

Here, we describe a widely applicable and customizable platform for 3D cell culture within a sealed microfluidic device. We chose to utilize an advection-based linear gradient generator first described by Jeon et al.[15] (**Figure 1**a). In studies, such generators have allowed the successful formation of off-chip 3D hydrogels containing gradients of macromolecules.[15] The devices fabricated for this study, illustrated in Figure 1b, include herringbone structures to permit enhanced mixing by chaotic advection as well as a porous, optically clear membrane which serves two functions: (i) it acts as the fourth wall of the microfluidic channels to enable controlled

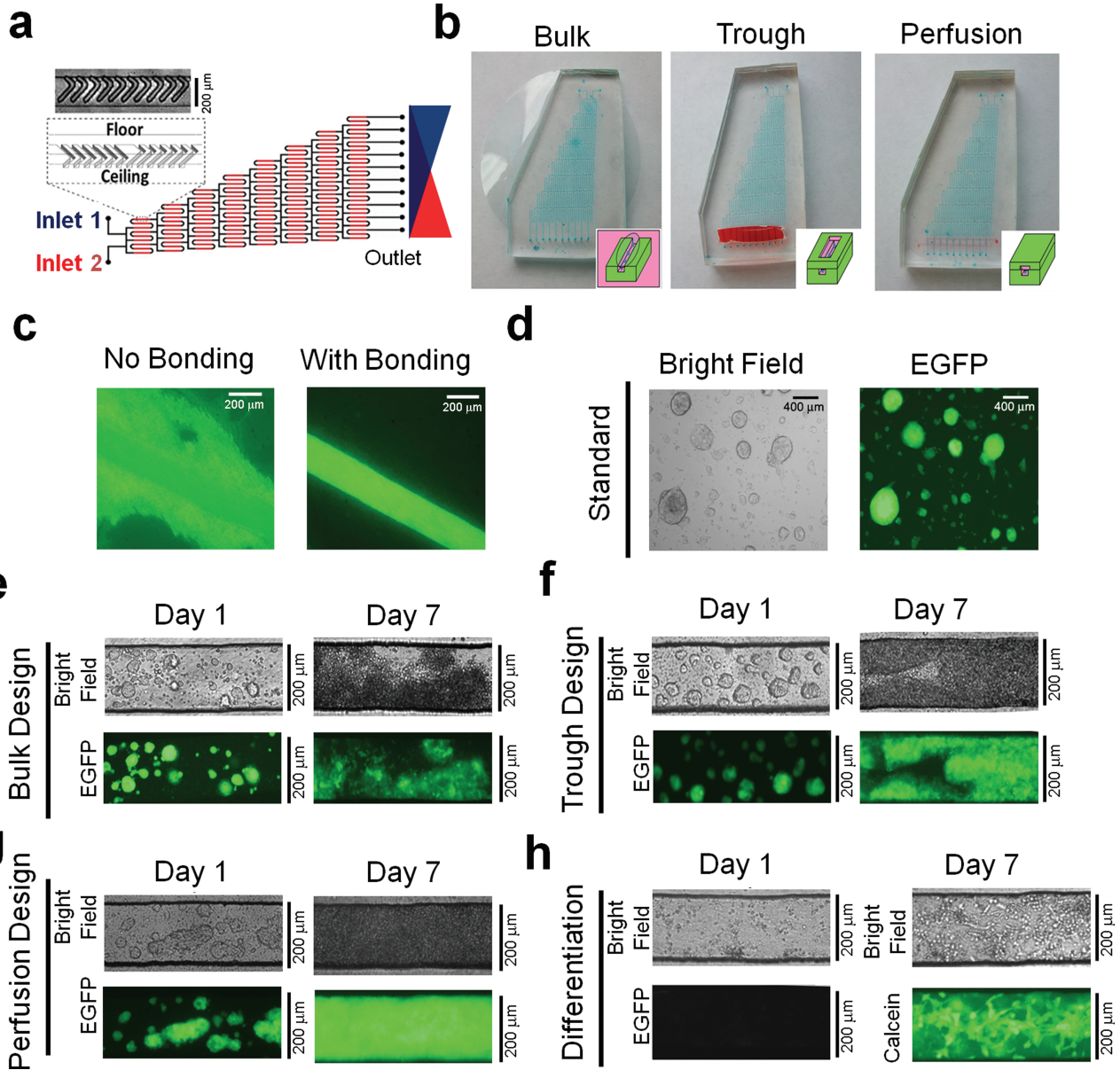

Figure 1. Device design and cell culture. (a) Advection-based linear gradient generator with constant outlet channel geometry. (b) Images of the assembled devices depicting the "bulk," "trough," and "perfusion" supplementation strategies (shown schematically in inset). Cell-laden channels are shown in blue and media channels are shown in red. (c) Devices loaded with a solution of 1 mg/mL collagen I containing FITC-BSA both without and with a chemically bonded membrane. (d) OGR1 cells grown on 0.1% gelatin-coated polystyrene plates. (e) OGR1 cells in 1 mg/mL collagen within a bulk fed device at days 1 and 7. (f) OGR1 cells in 1 mg/mL collagen within a trough fed device at days 1 and 7. (g) OGR1 cells in 1 mg/mL collagen within a perfusion fed device at days 1 and 7. (h) OGR1 cells in 1 mg/mL collagen in a bulk fed device in the absence of LIF.

loading with 3D matrix materials via syringe pump injection; and (ii) it acts as physical barrier to permit cell supplementation without fear of washing away cells or matrix materials. Owing to the membrane porosity, cells can be supplied diffusively with nutrients from a surrounding bulk solution or from troughs or perfusion microchannels overlaid on the cell-laden channels. This setup provides a tractable approach to locally perturb cell growth conditions via the addition or subtraction of biomolecule cues or imaging probes at various times. In contrast with previous approaches that employed membranes, the optically clear membranes employed here allow cell phenotype to be monitored using traditional, readily available optical or fluorescence microscopy. Moreover, the epoxy-amine chemistry used to seal the membrane and the channels results in a robust and leak-free platform (Figure 1c, Supporting Information, Figure 1).[16,17] Overall, the flexibility in channel design, cell supplementation strategy and analysis enabled by the platform offers an unprecedented system to perform microscale 3D cell-biomaterial studies.

Three separate advection-based linear gradient generator devices, each using a different supplementation strategy, were fabricated to illustrate the potential of the proposed platform. All three approaches allowed cell supplementation to be performed independently of local matrix, cell and biomolecule gradient formation. In the first approach, the membrane was chemically conjugated to PDMS microchannels to provide a ceiling for the channels (Figure 1b, Bulk). In this manner, the device could be loaded with cells and 3D matrix material via syringe and subsequently immersed in a bulk bath of growth media without fear of cell/matrix leakage. While simple, this design required a fairly large (~20 mL) amount of growth media to completely surround the device. In instances of scarce or expensive growth media and supplements, such a strategy may not be suitable. To reduce media consumption, we created a second design, labeled "Trough" in Figure 1b, in which a second PDMS layer forms a trough above the cell-laden outlet channels. Similar to the primary layer, the second PDMS layer can be chemically conjugated to the membrane using epoxy-amine chemistry to provide a leak-resistant bond. Media can then be pipetted directly into the trough to diffusively feed the cells in the outlet channels, effectively reducing reagent consumption 50-fold to approximately 400 μL. In the third design fabricated and tested for this report, the troughs were replaced with perfusion channels overlaid on the cell-laden channels (Figure 1b, Perfusion). Depending on the flow rate and duration of the cell culture experiment, this approach results in an even further reduction in reagent consumption. Moreover, through use of multiple perfusion channels, this design also offers the option to implement locally-defined, multi-factor supplementation schemes.

On-Chip Long Term Cell Growth: We evaluated the three 3D cell culture designs for their ability to support cell growth using OGR1 mouse embryonic stem (mES) cells. OGR1s are pluripotent cells that express enhanced green fluorescent protein (EGFP) under the promoter of Oct3/4.[18] Thus, when OGR1s are cultured in the presence of leukemia inhibitory factor (LIF), a condition that maintains cell pluripotency, they fluoresce. Once the LIF is removed, the cells differentiate and lose their EGFP expression. We chose these cells as a model cell line to evaluate viability within microfluidic channels for three reasons: (i) they represent a cell line that has relevance in many biological studies; (ii) they require expensive and potentially limiting reagents in their culture media; and (iii) their EGFP expression can be used to trace pluripotency. For preliminary tests, OGR1s were diluted in 1 mg/mL collagen I containing LIF and loaded at constant concentration in the gradient device. While the collagen precursor suspension has the potential to lead to channel clogging if the solution is not kept suitably dilute and chilled, no channel clogging was observed in our experiments. Moreover, our use of optically-transparent membranes between PDMS layers potentially allows the integration of photoinitiator-based hydrogels into this system, thereby completely avoiding issues with clogging. Following loading, the cells were subsequently fed in the manner dictated by the device design: "bulk" devices were immersed in a bath of growth media; "trough" devices had their trough filled with media; and "perfusion" devices had a constant flow of media through their perfusion channel (Figure 1b). After one day in the devices, the OGR1 cells formed rounded, EGFP-positive colonies reminiscent of their appearance when cultured in 2D on gelatin-coated polystyrene plates (Figure 1d-g). The cells were able to maintain their pluripotency within the device for at least one week, although by that time they had become confluent. However, if the cells were cultured in the absence of LIF, they ceased to express EGFP as they lost their pluripotency. Nonetheless, LIF-deprived cells remained viable, as evidenced by their staining with the calcein-AM viability marker (Figure 1h).

On-Chip Tubulogenesis: After successful demonstration of long-term cell culture within microchannels, we performed a functional assay by exploring the device's ability to support tube formation in on-chip angiogenesis assays. Current angiogenesis models include 2D cell proliferation and migration assays as well as 3D assays incorporating gels like collagen and Matrigel.[19] Typically, tube formation from endothelial cells (often HUVECs) is evaluated in terms of tube length and number of junctions in the presence of various concentrations of drugs to evaluate their pro- or anti-angiogenic properties.[19] We first evaluated the ability for the device to support tubulogenesis within the microchannels in the absence of any chemical or physical gradients. To ensure that shear stress applied to the cells within the microfluidic device did not alter the cells in such a manner as to prevent tube formation, HUVECs were passed through the linear gradient generator, collected and plated on gels of collagen I containing 200 nM phorbol myristate acetate (PMA), a tumor promoter used to activate the signal transduction enzyme protein kinase C and drive HUVEC tube formation.[20] While flow through the microchannels did not impair HUVEC tubulogenesis (**Figure 2**a), calculations performed subsequent to the experiment revealed that the HUVEC cells experienced elevated shear stress compared to physiological conditions (86 vs. 12 dyne-s/cm^2, see Supporting Information). In future work, this issue could be address via a reduction in the inlet flow rate. Next, to verify that tube formation could occur within the confines of the microfluidic channels, a mixture of 1 mg/mL collagen I and HUVEC cells was loaded in the bulk device. After incubation at 37 °C for 15 min to allow gelation,

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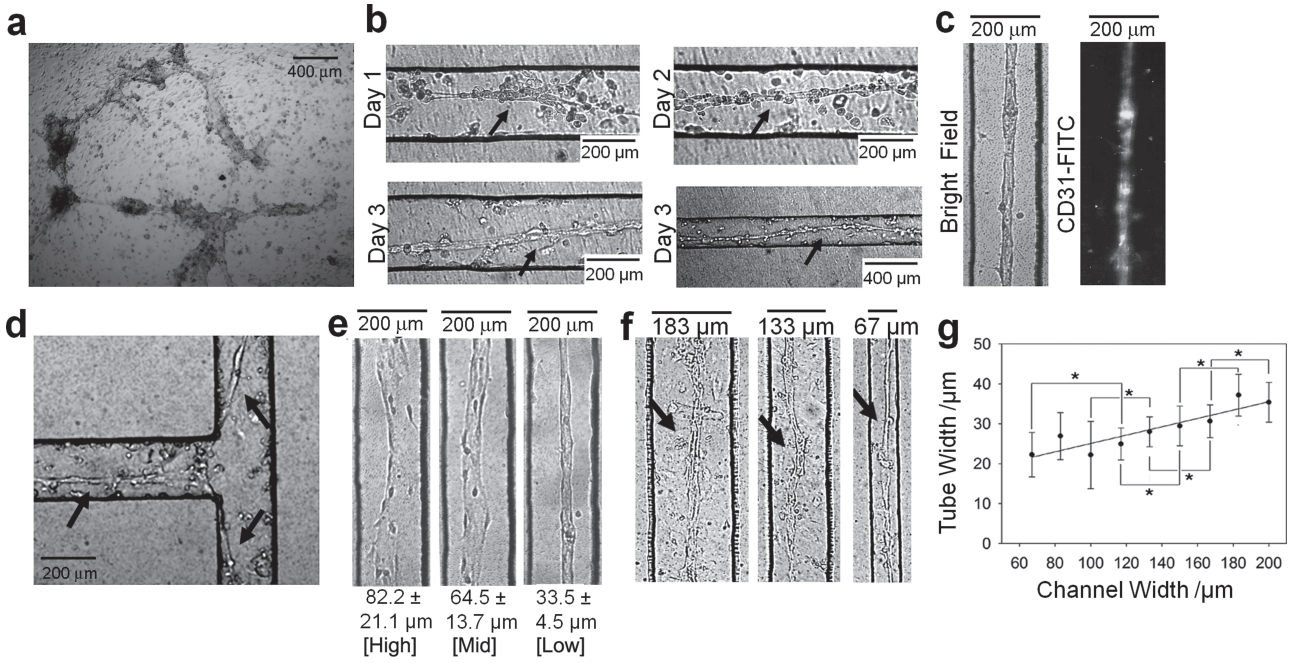

Figure 2. On-chip tubulogenesis. (a) Tube-like network formation with HUVECs growing on collagen after passage through the microfluidic device. (b) Tube formation with HUVECs within microchannels at various times. (c) Tube-like structures formed with HUVECs within microfluidic channels show positive staining for CD31. (d) Tube branching observed along the geometric confines of the microfluidic device. (e) Tubulogenesis in outlet channels of a device loaded with constant cell concentration and a collagen gradient of 2 mg/mL–0.25 mg/mL. The lowest collagen concentrations (~0.25–0.4 mg/mL) did not support tube formation. Representative images for high (~2 mg/mL), mid (~1.4 mg/mL) and low (~0.8 mg/mL) collagen concentrations are shown. (f) Tubulogenesis in channels of varying width. For brevity, only a selection of the total tested channel widths are shown. (g) Measured tube diameter as a function of channel width (* = $p < 0.05$).

the device was immersed in a cell culture dish containing endothelial cell growth media and 200 nM PMA. After one day in culture, cell networks were visible within the device. After three days, clearly defined tubes with positive CD31 staining and lengths on the order of millimeters were present (Figure 2b,c). Interestingly, the tubes that formed adjusted to the contours of the device and branched at channel junctions, probably guided by the physical constraints of the walls (Figure 2d).

Ideal platforms for cell-biomaterial studies allow heterotypic, non-uniform environments to be captured experimentally. To demonstrate the potential of the platform in this respect, the gradient feature of the device was explored (Supporting Figure 2). Cell-laden precursor suspensions of 0.25 mg/mL collagen and 2 mg/mL collagen were injected into the two inlet streams of the device to evaluate the effect of collagen concentration (e.g., matrix stiffness) on tube formation. We observed that more concentrated collagen solutions support the formation of wider tubes as opposed to less concentrated solutions (Figure 2e).

Subsequently we explored the influence of a physical gradient on the resulting tube-like structures by fabricating a device in which the width of parallel outlet channels was systematically reduced from 200 to 50 μm in a channel-by-channel manner (Supporting Figure 3). HUVECs were loaded in the device and supplemented with growth media containing 200 nM PMA for 6 days. Tubulogenesis was observed in all channels with the exception of the narrowest width (50 μm; Figure 2f). Interestingly, we observed a significant effect of device design parameters on tubulogenesis, with statistically significant reductions in tube width occurring for every 33 to 50 μm reduction in channel width (Figure 2g).

The ability to culture cells within microdevices for a week or longer with only simple modifications to existing device design and fabrication (i.e. one-step addition of a clear, porous membrane) opens up a wide range of possibilities for experimental cell biology studies. Here we demonstrated the use of microfluidic-based platforms to replace wellplate-based assays by both increasing throughput and reducing reagent volume and initial cell requirements. More efficient use of materials, in particular, is greatly important when working with stem cells or other rare cells. With the platform described here, the microfluidic hallmark of efficient material usage has been extended to the 3D culture and supplementation of cells. Furthermore, with the ease of use and self-contained nature of the platform, one can envision collecting a patient's cells, reprograming these cells into induced pluripotent stem (iPS) cells, and using the microfluidic cell culture device to study patient-specific response to anti-angiogenic chemotherapeutics. This reprogramming could occur off-chip via established viral infection or protein transduction methods, with the small number of reprogrammed cells being transferred to the device for angiogenesis studies. Alternatively, due to the low efficiency of most iPS cell generation protocols, the reduced material consumption and ability to rapidly screen cells from discrete compartments of this microfluidic platform make it attractive to envision directed supplementation of the reprogramming protein cocktail through the porous

membrane from discrete perfusion channels for on-chip iPS cell reprogramming.

In conclusion, we developed and validated a 3D cell culture platform that allows mammalian cells to be kept viable in microchannels for extended periods (here up to 7 days) and also enables in situ studies of cell response upon exposure to different cues. Moreover, the platform can be readily incorporated into existing single-layer channel designs to enable 3D, on-chip cell culture. This potentially includes designs to explore cell migration, cell culture within microwells of various sizes, or studies exploring cell behavior under a variety of 3D hydrogel niches (e.g. hydrogel concentration/matrix stiffness). In essence, all that needs to be done is replace the hard, non-porous bonding substrate with a covalently bound, optically membrane. Cells can then be supplemented via the strategies (i.e. bulk, trough or perfusion) detailed above. We demonstrated that cells can not only be kept viable and pluripotent, specifically for the case of mES cells, but also that they retain their native functionality, specifically HUVEC-mediated tubulogenesis, under a variety of conditions. The integration of an optically clear membrane allows for changes in phenotype or immunofluorescence associated with specific local microenvironmental cues to be readily captured in situ using traditional microscopy techniques. Critically, the microfluidic approach reported here offers a readily accessible pathway to study a wide range of biological questions in which 3D heterotypic microenvironments are important.

Experimental Section

Materials: Silicon wafers were purchased from University Wafers (Boston, MA). SU8-2050 negative photoresist was purchased from MicroChem (Newton, MA). RTV615 high strength transparent silicone rubber compound (poly(dimethylsiloxane), PDMS) was purchased from Momentive Performance Materials (Columbus, OH). Transparent membranes with 1 μm pores were purchased from AR-Brown (Pittsburgh, PA). Human umbilical vein endothelial cells (HUVECs), medium 200 (M200), low serum growth supplement, DMEM, ES-qualified fetal bovine serum, GlutaMAX, penicillin-streptomycin, MEM non-essential amino acids, MEM sodium pyruvate, Leukemia inhibitory factor, and irradiated mouse embryonic fibroblasts were purchased from Life Technologies (Grand Island, NY). Phorbol myristate acetate (PMA) was purchased from Enzo Life Sciences (Farmingdale, NY). The fluorescent viability dye calcein-AM, collagen I from rat tail and mouse anti-human CD31-FITC was purchased from BD Biosciences (Franklin Lakes, NJ). Gelatin from porcine skin, 2-mercaptoethanol, (3-aminopropyl) trimethoxysilane (APTMS) and (3-glycidyloxypropyl) trimethoxysilane (GPTMS) were purchased from Sigma (St. Louis, MO). All other chemicals and solvents were purchased from Sigma and used without further modification. The NE-300 syringe pump was purchased from New Era Pump Systems (Farmingdale, NY). The G3P-8 spin coater was purchased from Specialty Coating Systems (Indianapolis, IN). The plasma cleaner/sterilizer was purchased from Harrick Plasma (Ithaca, NY). OGR1 mouse embryonic stem cells were the kind gift from the lab of Prof. Ning Wang at the University of Illinois, Urbana-Champaign.

Device Design and Fabrication–Channel Design: The program Freehand MX was used to draw the advection-based linear gradient generator and perfusion channels with widths of 200 and 500 μm, respectively. Freehand MX was also used to draw the herringbone structures with a line width of 30 μm. The perfusion channels were widened with respect to the outlet mixing channel widths to simplify the alignment of the perfusion channel above the mixer outlet channels. Photomasks of the device designs were printed at a resolution of 5080 dpi on transparencies (Facilities and Services Printing Department, University of Illinois, Urbana, IL) and used without further modification.

Device Design and Fabrication–Silicon Master Fabrication: To prepare masters, silicon wafers were first cleaned with acetone, isopropanol and dried with a stream of nitrogen. The wafers were then loaded into the spin coater and SU8-2050 was poured on top of the wafer. For the mixing and perfusion channels, the spin coater was programmed to coat the wafer with SU8-2050 at a thickness of 100 μm. After coating, the wafers were subjected to the manufacturer's suggested pre-bake schedule (7 min at 65 °C, 30 min at 95 °C) and exposed to UV light with the appropriate photomask in place. In the case of the perfusion channel master, the wafer was immediately subjected to the manufacturer's suggested post-bake schedule (5 min at 65 °C, 15 min at 95°C) and developed in a solution of propylene glycol methyl ether acetate (PGMEA). However, for the mixing channel master, a second layer of SU-8 was applied at a thickness of 50 μm using the spin coater. This second layer of SU8-2050 was subjected to the manufacturer's suggested pre-bake schedule (3 min at 65 °C, 10 min at 95 °C), which also served as a post-bake for the initial SU8-2050 layer. Then, the herringbone structures were aligned and the wafer was exposed to UV light. Next, the wafer was subjected to a post-bake schedule (2 min at 65 °C, 7 min at 95 °C) and developed in PGMEA. Following development, both the perfusion channel master and the mixing channel master with herringbone structures were rinsed with isopropanol, dried with a nitrogen stream and passivated via vapor deposition of (trideca-fluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane under vacuum for more than 4 h.

Device Design and Fabrication–PDMS Molds and Device Assembly: PDMS molds of the silicon masters were prepared by first combining RTV615A and RTV615B at a 10:1 weight ratio followed by degassing of the mixture. The viscous solution was then poured over the silanized silicon masters described above and placed in a 65–70 °C oven. After a minimum of 2 h, the molds were removed from the oven and the PDMS layer was peeled off the silicon master. Inlet and outlet ports were punched in the PDMS layer using a 19-gauge blunt syringe needle. For the devices using the trough design, a scalpel was used to cut out a block from a blank slab of PDMS corresponding to the size of the outlet channels. Next, the PDMS devices as well as the transparent membranes were exposed to O_2 plasma for 1 min. The PDMS devices were then immersed in a 1% solution of GPTMS while the membranes were immersed in a 1% solution of APTMS. After a 20-min incubation at room temperature, the PDMS slabs and membranes were removed and dried using a lint-free cloth. Next, the APTMS-treated membrane was brought in contact with the GPTMS-treated PDMS channels. In the case of trough and perfusion designs, the second PDMS layer was then immediately aligned and placed on top of the membrane, creating a PDMS-membrane-PDMS sandwich. The assembled devices were allowed to incubate at

room temperature for a minimum of 2 hours, after which excess PDMS and overhanging membrane was trimmed from the device. Chemical bonding of the layers was determined to form a leak-resistant seal by examining devices loaded with FITC-BSA. Assembled devices were then were autoclaved (to remove low molecular weight material that may cause cell viability and/or protein sticking issues) before use.

Cells: Human umbilical vein endothelial cells (HUVECs) were cultured according to ATCC protocols at 37 °C and 5% CO_2 in M200 supplemented with low serum growth supplement, 10% fetal bovine serum, and 1% penicillin-streptomycin. OGR1 cells were cultured at 37 °C and 5% CO_2 in high-glucose DMEM supplemented with 15% ES-qualified fetal bovine serum, 1% penicillin-streptomycin, 2 mM glutamine, 0.1 mM modified Eagle's medium nonessential amino acid solution, 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate and 1000 U/mL LIF. Frozen stocks of OGR1 were thawed onto a feeder layer of mitotically inactivated murine embryonic fibroblasts before passage onto plates coated with 0.1% gelatin via differential separation.

Device Loading, Cell Culture and Tube Formation: pH-neutralized collagen solutions were prepared on ice according to the manufacturer's recommended protocol and diluted in the appropriate growth media for the particular cell type studied. Cells growing on standard polystyrene culture dishes were then trypsinized, centrifuged, counted and diluted in the collagen solution to achieve a final cell density of approximately 100 000 to 200 000 cells per mL. The cell solution was then loaded in syringes and injected into the device either by hand (for constant concentrations) or using a syringe pump (for gradient formation). After visual confirmation that the channels had been filled, the device was disconnected from the syringe, placed in a covered petri dish and incubated at 37 °C for 15 min to allow the collagen solution to solidify. After incubation, "bulk" designs were immersed in a bath of growth media. For "trough" designs, fresh growth media was pipetted into the trough. To retard media evaporation from the trough, a small dish of water was placed alongside the device within a large petri dish. For "perfusion" designs, the perfusion inlet port was connected to a syringe pump containing fresh growth media set to a rate of 0.1 μL/min. The cells were then allowed to grow undisturbed in an incubator. In experiments evaluating tube formation with HUVECs, the growth media was supplemented with 200 nM phorbol myristate acetate. The diameters of the tubes were quantified using Image J.

Fluorescence Microscopy: Devices were imaged on a Leica DM4000 fluorescence microscope equipped with a 10x objective and a digital camera (Buffalo Grove, IL). EGFP expression in OGR1 cells was visualized without any additional cell treatment. To measure cell viability in differentiated OGR1 cells, calcein-AM was added to the cell media at 5 μM for approximately 30 min prior to visualization. To perform immunohistochemistry experiments, cell-laden devices were first blocked in a reconstituted solution of dry, non-fat milk for two hours. After, the devices were washed and incubated in a solution of CD31-FITC for two hours. After one final round of washing, the devices were fixed overnight and visualized the following day.

Statistical Analysis: Student T-Test (one-tailed) comparisons at 95% confidence interval were used for statistical analysis. The results were deemed significant at $p < 0.05$.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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