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Cell patterning by surface tension pinning in microfluidic channels

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ABSTRACT

We present a simple method to pattern multiple cell populations inside a microfluidic channel. The microchannel is partially filled with a cell suspension, and the position of the liquid boundary remains pinned by surface tension. Cells then adhere only in the filled portion of the channel, producing a very sharp boundary. The process can be performed in an unmodified microfluidic channel with only a manual syringe and can be repeated multiple times to pattern cocultures or tricultures. We demonstrate the patterning method with two different mammalian cell types, 3T3 fibroblasts and NMuMG epithelial cells, and channel heights of 1.5 mm and 0.5 mm. We anticipate that this method will be useful for studies of cell–cell interactions where precise control of the fluidic microenvironment is required.

INTRODUCTION

Micropatterning of two cell populations into adjoining regions has been a useful experimental tool for applications including the study of cell migration and invasion,[1,2] cell–cell competition,[3] contact-dependent cell signaling,[4] and diffusible morphogen gradients.[5] Cell patterning can be achieved by selective adhesion onto patterned proteins,[6,7] spatial confinement by an elastomeric stencil or microchannel,[5,7] or spatial reconfiguration of the substrate.[3,4] At the same time, cell culture inside of microfluidic channels has proven to be a useful biological tool for imposing shear forces,[8–10] minimizing the media volume,[11] modulating or abrogating autocrine and paracrine signaling,[12–14] rapidly changing the soluble microenvironment,[15] and constructing interconnected organs on a chip.[16] Creating a patterned cell interface within a microchannel could combine the capabilities of both tools, but few examples of this can be found in the literature.

While both cell patterning and microfluidic cell culture are well established biological tools, combining the two has not been straightforward. The most common and robust process for bonding a microfluidic channel to a substrate requires treating both the channel and substrate with a plasma, which is incompatible with cells and proteins, thereby complicating any process flow that aims to pattern proteins or cells prior to introducing the microchannel. On the other hand, it is also challenging to perform cell or protein patterning inside of a sealed microchannel. One effective solution has been to perform protein patterning and plasma bonding simultaneously. Rhee and co-workers used the plasma treatment step of the bonding process to achieve protein patterning by protecting selected regions of the substrate with an elastomeric stamp, allowing the plasma to ablate away adsorbed protein in the unprotected regions.[17] Another effective solution has been to leverage the laminar flow properties of microfluidic channels to pattern stripes of cells within microchannels.[18]

Here, we report a very simple method to pattern cells inside of a microfluidic channel. A dry microchannel is partially filled with a cell suspension and then incubated to allow cell attachment. The liquid boundary is pinned by surface tension at the liquid–air interface, producing a very sharp cell boundary. Non-specific cell attachment beyond this boundary is extremely low and is superior to patterning that depends on selective cell adhesion. Patterning by laminar flow is also subject to cross-contamination due to diffusion between streams or uneven pumping, though this can be reduced by two-phase flows[19] and flow engineering.[20] Our simple method does not require any additional engineering or equipment beyond a standard microfluidic channel and a manual syringe. Whereas the ablation of an adhesive protein is limited to patterning two cell populations, our technique can be repeated to pattern three populations or more. Finally, while laminar flow patterning always creates

cell interfaces that are parallel to the channel, our approach always creates interfaces that are perpendicular to the channel.

MATERIALS AND METHODS

Microfluidic channel design and fabrication

The microchannel design consisted of a main channel (10 mm length × 2 mm width) connected to circular inlets and outlets. Curved corners were included in the channel design to alleviate bubble capture during media perfusion. Polydimethylsiloxane (PDMS) fluidic channel layers were fabricated as previously described by casting from an acrylic mold.[3] Briefly, laser-cut acrylic pieces (McMaster, USA) were adhered to square polystyrene Petri dishes (Fisher Scientific, USA) using cyanoacrylate glue (Gorilla Glue Company, USA). PDMS was mixed at a base-to-curing ratio of 10:1 and degassed. 25 g was then poured into the mold and cured at 65 °C for 4 h. The molded PDMS was then removed, and individual channels were cut and cleaned by sonication in 10% isopropyl alcohol (IPA) (Fisher, USA) for 20 min. Fluidic inlet and outlet holes were created by punching through the PDMS with blunt needles (No. 23.5, McMaster, USA). Cleaned channels were then irreversibly bonded to clean glass slides (Fisher Scientific, USA) using plasma from room air for 40 s. Assembled devices were then baked at 65 °C for 20 min to increase bond strengthening. To facilitate cell attachment, channels were filled with 5 μg/ml of human fibronectin (Sigma-Aldrich, USA) and placed at 37 °C overnight. Prior to cell seeding, fibronectin was aspirated, and channels were left to dry at room temperature for at least 24 h. In order to demonstrate the quality of the sharp interface achieved by surface tension pinning, 15 μl of a fluorescein solution (Sigma-Aldrich, USA) was added to the channels using a syringe, and the resulting signal was imaged.

Cell culture

NIH 3T3 cells expressing nuclear fluorescent reporters, H2B-mTurquoise2 and H2B-Citrine, and NMuMG cells were kindly provided by Dr. Michael Elowitz (California Institute of Technology, USA). NIH 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% Bovine Calf Serum (BCS) (Gibco, USA), 1% L-glutamine (Caisson Laboratories, Inc., USA), and 1% penicillin/streptomycin (Caisson Laboratories, Inc., USA) at 37 °C and 5% CO_2. NMuMG cells were maintained in DMEM media supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA), 1% sodium pyruvate, 1% L-glutamine, and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Prior to channel seeding, cells were trypsinized and re-suspended at a density of 1.5×10^6 cells/ml.

Cell viability was assessed by the Calcein assay. Channels were washed once with 1× PBS, and 1 μl/ml of Calcein AM (Invitrogen, USA) was added and incubated at room temperature for 15 min. Channels were then washed twice with PBS and imaged by fluorescence microscopy.

Cell seeding into microchannels

A 15 μl mixture of a first cell population was pushed into the inlet channel manually using a 100 μl gastight syringe (Hamilton, USA) and incubated for 3 h (NIH 3T3 H2B-mTurquoise2 cells) or 24 h (NMuMG cells). Non-adherent cells were washed away by aspirating the liquid from the inlet. A 40 μl suspension of a second cell population (NIH 3T3 H2B-Citrine cells or NMuMG cells) was then pushed into the inlet and incubated overnight to establish confluence. After overnight incubation, media were aspirated and replaced with fresh culture media.

Cell patterning by plasma ablation

A clean glass slide (Fisher Scientific, USA) was submerged in 5 ml of 0.01% poly-L-lysine solution (Sigma-Aldrich, USA) for 5 min. The liquid was then aspirated and the slide was left to air dry. A rectangular PDMS piece was placed in the middle of the slide and placed under air plasma for 30 s. Following plasma ablation, the PDMS piece was removed and the glass slide was placed inside a Petri dish and incubated with a cell suspension of NIH 3T3 cells (500 000 cells/ml) for 1 h. Non-adherent cells were washed away by aspiration and fresh culture medium was added to the dish and incubated prior to imaging.

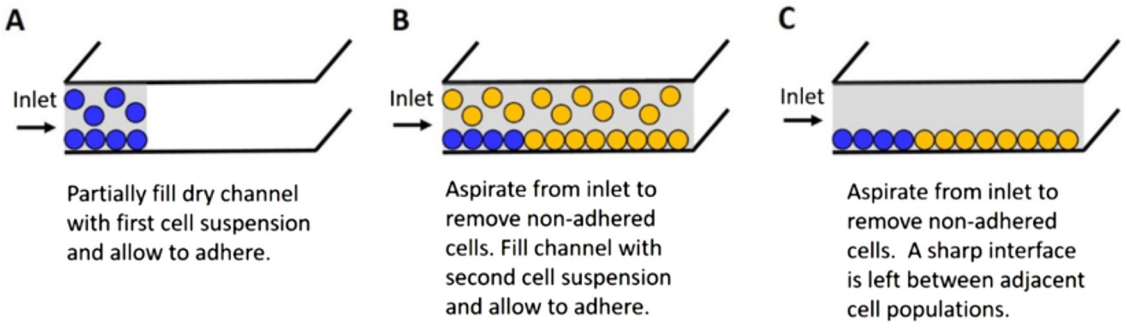

FIG. 1. Coculture patterning steps. The border of the first cell suspension is pinned by surface tension (a). After allowing the second cell population to adhere (b), a sharp cellular interface is created (c).

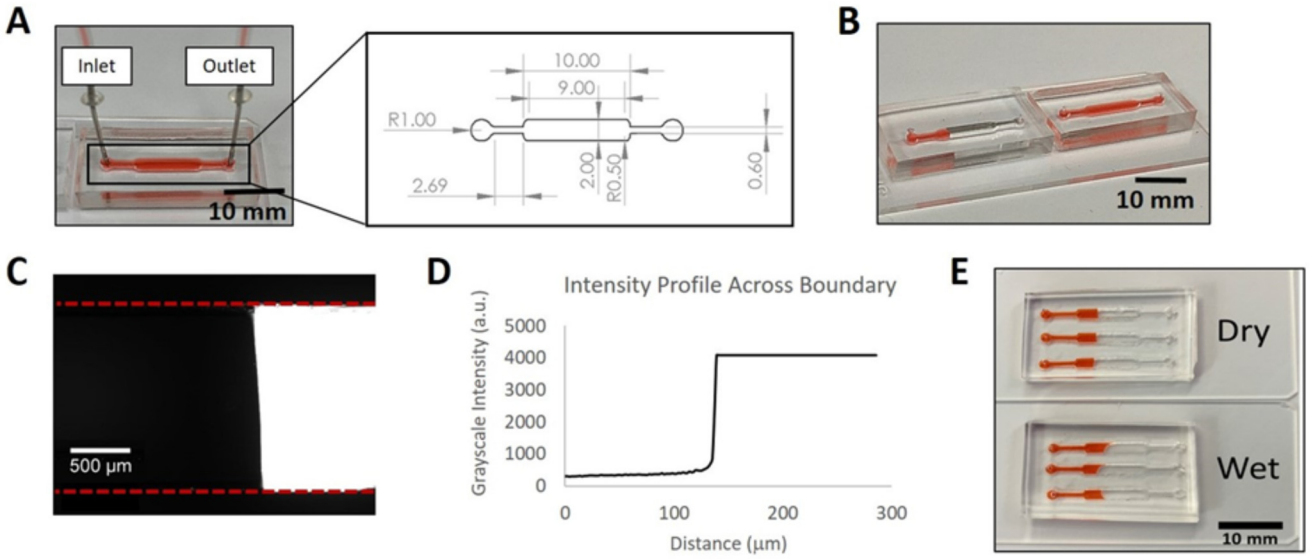

FIG. 2. Device overview. (a) Microchannel is designed with a circular inlets/outlets and curved edges to minimize bubble formation during filling. Dimensions are given in millimeters. PDMS is molded from laser-cut acrylic and bonded to glass slides using air plasma. Tubing is connected to the inlet and outlet to permit perfusion and collection of waste. (b) Partial (left) and complete (right) filling of channels with dye. (c) Fluorescent image illustrates the sharp liquid–air interface after partial filling of a microchannel with fluorescein. Red dashed lines indicate the channel edges. (d) Fluorescence intensity data demonstrate the sharpness of the liquid–air interface. (e) Partial filling of the colored dye into dry (top) and wet (bottom) microchannels, demonstrating that sharper boundaries can be achieved in dry channels.

Cell imaging

Imaging was performed using an inverted epifluorescence microscope (Eclipse, Nikon, USA) equipped with Cyan Fluorescent Protein (CFP), Fluorescein isothiocyanate (FITC), and Tetramethylrhodamine (TRITC) filter cubes for the visualization of nuclear expression of mTurquoise, Citrine, and mCherry, respectively. Fluorescein and Calcein AM were also imaged through the FITC cube. Bright-field and fluorescence images were taken of the same field of view without adjusting the focus. ImageJ (NIH, USA) was used for all image processing. Linear filters were applied to improve image clarity.

RESULTS AND DISCUSSION

Our goal was to develop a simple and robust method for cell patterning in microchannels. PDMS was chosen as the channel material due to its low cost, biocompatibility, gas permeability, and optical transparency.[21] Microchannels were fabricated by molding PDMS on laser-cut acrylic, allowing for rapid prototyping of different channel designs. PDMS channels were then bonded to glass slides using air plasma, creating an irreversible seal that prevents leakage. Standard microscope glass slides ($75 \times 25\ mm^2$) were chosen as the substrate material to ensure compatibility with common microscope stages.

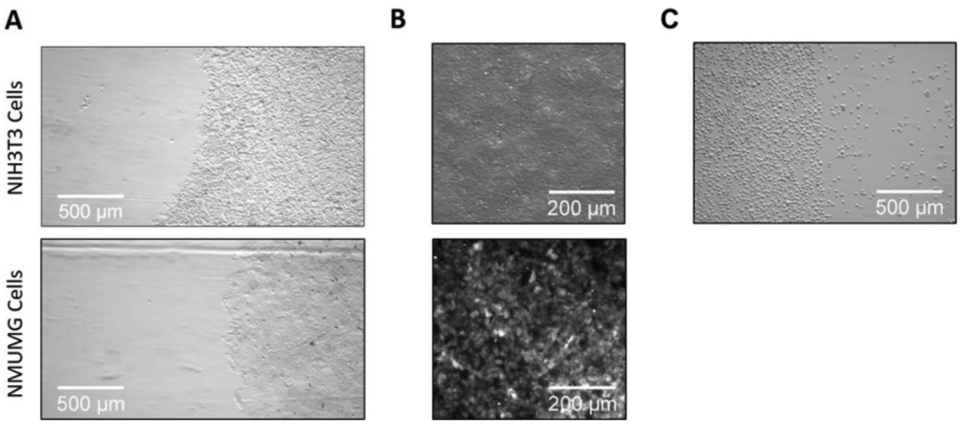

FIG. 3. Cell border patterning. (a) Initial cell seeding step produces sharp transitions at the patterned cell border. Images are representative of the sample-to-sample variation in the border angle. (b) Bright-field (top) and Calcein AM staining (bottom) of NMuMG cells seeded within microchannels for 72 h, confirming good cell viability. (c) Cell patterning by selective plasma ablation of poly-L-lysine results in greater non-specific cell attachment of NIH 3T3 cells than with our method shown in (a).

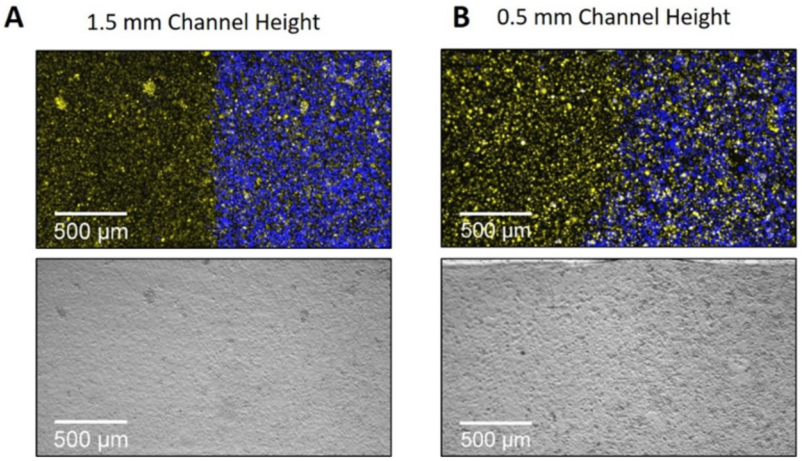

FIG. 4. Sharp coculture interface. Fluorescence (top) and bright-field (bottom) images of patterned 3T3 cells inside microchannels at 16 h post-seeding in (a) 1.5 mm height channels and (b) 0.5 mm channels. Blue represents first seeded cells (NIH 3T3 H2B-mTurquoise2). Yellow represents second seeded cells (NIH 3T3 H2B-Citrine).

Aqueous solutions demonstrate partial wetting on most microfluidic channel materials, including PDMS and glass. In a partially filled channel, the contact line will experience pinning on surface defects, producing hysteresis between the advancing and receding contact angles. This can be sufficient to anchor the contact line in place despite small perturbations in volume and pressure.[24] Here, we take advantage of this pinning effect to perform cell micropatterning within microfluidic channels. It is of particular importance that the channel be completely dry prior to cell seeding, as this will affect pattern fidelity. This was demonstrated by adding a colored dye to channels where the fibronectin had been aspirated only (wet) and channels that had been aspirated and left to dry for 24 h (dry). Sharper interfaces are achieved for the dye in the channels that were left to dry, with the wet channels experiencing some dye leakage along the channel edges [Fig. 2(e)]. Therefore, we allowed channels to dry for at least 24 h prior to cell seeding. Patterned cocultures were achieved in three steps (Fig. 1). First, the channel was partially filled with a first cell population, establishing a well-defined region for the cells to adhere. Next, the unattached cells were aspirated, and the channel was filled with a second cell population and incubated overnight. Finally, the unattached cells were aspirated again and replaced with fresh media, leaving a sharp interface between the plated cells. The sharpness of the liquid border during the first cell seeding was quantified by loading with fluorescein (Fig. 2). The cell attachment time varied by the cell type. While 3–5 h was sufficient for NIH 3T3 fibroblasts, overnight incubation was preferable for NMuMG epithelial cells. Figure 3(a) shows cell patterning after the first round of seeding, illustrating the sharp border. In comparison to cell patterning by selective plasma ablation [Fig. 3(c)], our patterning approach produces markedly lower non-specific attachment.

After plating both cell populations, the procedure results in a sharp interface between the two populations with minimal cross-contamination of the first population into the second (Fig. 4). There is significant contamination of the second population into the first, however. This is a common issue with all cell patterning techniques that employ sequential seeding (including patterning by selective adhesion or stencils), but many cell patterning experiments can tolerate such contamination.[1,2,5,6] Deep channels (1.5 mm height) and shallow channels (0.5 mm height) both produced good patterning, but the interface was consistently a bit sharper with the deeper channels (Fig. 4). Furthermore, we found that approximately 1.7× higher cell density was necessary to achieve similar confluency in the deep channel compared to the shallow channel. The ability to modify the channel height may be important to produce a particular amount of shear stress at a particular perfusion rate. Patterning was attempted with additional channel heights. Very shallow channels (50 μm height) were not compatible with our patterning method as capillary forces prevented partial channel filling. In very deep channels (6 mm height), the pinning of the first cell suspension was possible, but we found that bubbles often formed in the channels above the cells during cell seeding, decreasing cell viability. Additional precautions to prevent bubble capture are suggested if patterning in deeper channels is performed.

To verify cell health, NMuMG cells were seeded into the channels, and media were exchanged daily. Cells were imaged at 72 h post-seeding, and cell viability was assessed using Calcein AM,

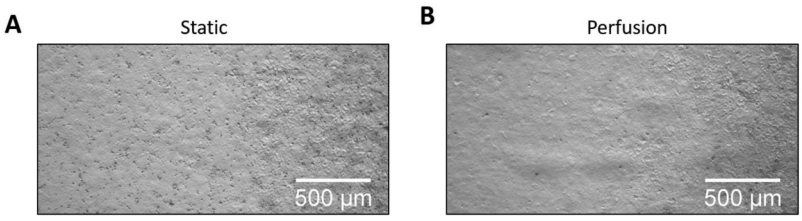

FIG. 5. Bright-field images of NMuMG coculture after 18 h of culture inside 0.5 mm microchannels under static culture (a) and constant media perfusion at 2.1 ml/h (b).

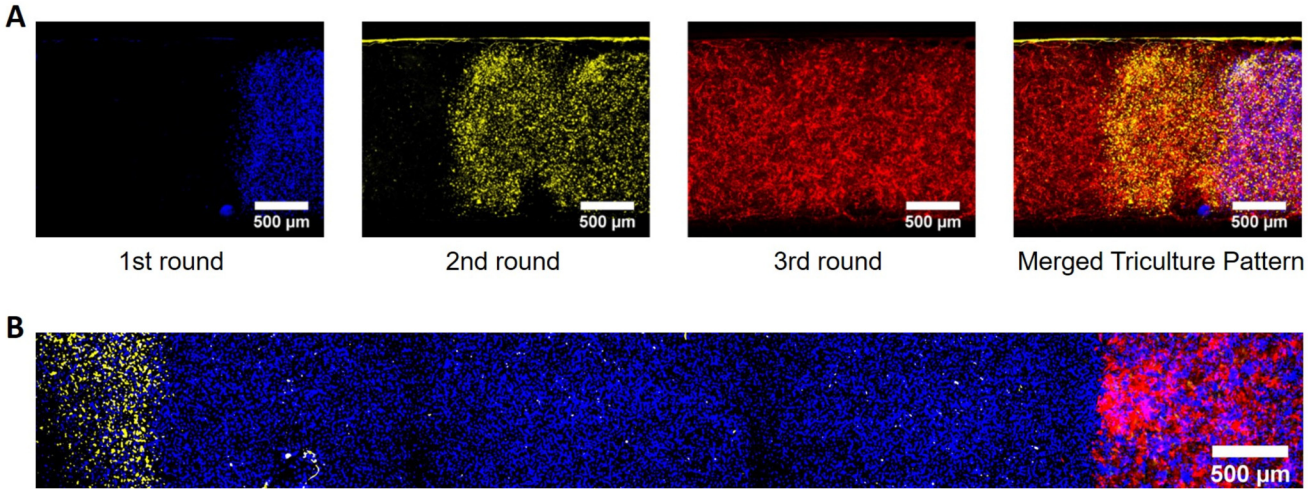

FIG. 6. Triculture patterning. (a) Single inlet patterning. Cell seeding is accomplished by partial filling of the microchannel in both the first and second rounds, but the cell suspension is injected further in the second round. In the third and final round of cell seeding, the channel is completely filled by the cell suspension. (b) Dual inlet patterning. The first two cell types (red and yellow) are seeded by partial filling from both the inlet and outlet of the microchannel. In the third round of cell seeding, the channel is completely filled with the cell suspension (blue). Here, NIH 3T3 cells with blue (H2B-mTurquoise2), red (mCherry cytoplasmic reporter), and yellow (H2B-Citrine) labels are patterned in each round.

which expresses a fluorescent signal upon uptake and hydrolysis by intracellular esterases in viable cells.[22] Fluorescent staining was prominent after 72 h of culture in microchannels [Fig. 3(b)], indicating good health. Additionally, we patterned cocultures within microchannels (0.5 mm height) and established strongly convection-dominated laminar flow (Peclet number of 2917 and Reynolds number of 0.56, as calculated from the channel geometry and flow rate[25]) by perfusing at 2.1 ml/h with a peristaltic pump. Cell adhesion and morphology under perfused and static conditions were similar in patterned NMuMG cells at 24 h (Fig. 5). Shear stress was maintained at 0.006 dyn/cm^2 at the cell surface, which is well below the critical value of 2 dyn/cm^2 that has been reported to affect cell adhesion.[23] This demonstrates that our patterned coculture method is compatible with convection-dominated flows capable of attenuating autocrine cell signaling.[13]

Finally, our patterning method can be extended to pattern three or more cell populations. The key change is that after the initial cell seeding, subsequent seedings must push the cell suspension beyond the border established by the previous seeding, while not filling the channel completely until the final seeding round. This capability is demonstrated by creating a patterned triculture in Fig. 6(a), but there is no firm limit on the number of seeding rounds that can be performed. In the case of the triculture demonstrated here, 15 μl was injected in the first seeding round, 25 μl was injected in the second round, and 40 μl was injected in the third and final round.

We were also able to minimize the cross-contamination by creating triculture patterns through cell seeding from opposite sides of the channel. This involved first seeding a 12 μl suspension of NIH 3T3 cells (mCherry cytoplasmic reporter) and incubating for 4 h. An additional 23 μl of media was then pushed into the channel from the same inlet before pushing 12 μl of a second cell suspension (NIH 3T3 H2B-Citrine) into the channel from the outlet. This created an air gap between the two different cell types. Once adhered, a 40 μl suspension of the third cell type filled the entire channel to establish the triculture. This allowed us to create a triculture pattern with sharp interfaces and minimal cross-contamination between the three cell populations as demonstrated in Fig. 6(b).

CONCLUSION

We have demonstrated a simple and robust method to pattern multiple cell populations inside a microfluidic channel. The technique is very simple, requiring only an unmodified microfluidic channel and a manual syringe. It produces very low non-specific cell attachment outside of the targeted region and allows more than two cell populations to be patterned. The method creates cell interfaces that are perpendicular to the microchannel, allowing flexibility in choosing the size of each region and ensuring that perfusion through the channel will wash soluble factors across the interfaces rather than along them, which may be useful for certain types of paracrine signaling experiments. We anticipate that this patterning technique will be useful for studies on cell–cell interaction that can benefit from the continuous perfusion or reduced media volume that is available in the microchannel culture.

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