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Vacuum-assisted Fluid Flow in Microchannels to Pattern Substrates and Cells

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Abstract

Substrate and cell patterning are widely used techniques in cell biology to study cell-to-cell and cell-to-substrate interactions. Conventional patterning techniques work well only with simple shapes, small areas and selected bio-materials. This paper describes a method to distribute cell suspensions as well as substrate solutions into complex, long, closed (dead-end) polydimethylsiloxane (PDMS) microchannels using negative pressure. Our method builds upon a previous vacuum-assisted method used for micromolding (Jeon, Choi et al. 1999) and successfully patterned collagen-I, fibronectin and Sal-1 substrates on glass and polystyrene surfaces, filling microchannels with lengths up to 120 mm and covering areas up to $13 \times 10 \text{ mm}^2$. Vacuum-patterned substrates were subsequently used to culture mammalian PC12 and fibroblast cells and amphibian neurons. Cells were also patterned directly by injecting cell suspensions into microchannels using vacuum. Fibroblast and neuronal cells patterned using vacuum showed normal growth and minimal cell death indicating no adverse effects of vacuum on cells. Our method fills reversibly sealed PDMS microchannels. This enables the user to remove the PDMS microchannel cast and access the patterned biomaterial or cells for further experimental purposes. Overall, this is a straightforward technique that has broad applicability for cell biology.

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Disclosures

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Keywords

Substrate patterning; cell patterning; soft lithography; microfluidic device; vacuum-assisted microchannel filling

Introduction

The use of substrate and cell patterning techniques to control the spatial organization of cultured cells, extracellular matrix proteins, and other biomolecules has increased over the last four decades in the fields of cell biology (Kane, Takayama et al. 1999), tissue engineering (Lin, Ho et al. 2006) and biosensing (Veisoh, Zareie et al. 2002). These techniques have proven useful to study the interaction between substrate and cells (Dickinson, Lutgebaucks et al. 2012) and between cells of the same or different types (Khademhosseini, Ferreira et al. 2006, Bogdanowicz and Lu 2013), to guide cell growth (Choi and Lee 2005), and to immobilize biomolecules in the fabrication of biosensors (Hwang, Kuk et al. 2011).

Two popular methods used to pattern substrate are photo-patterning and micro-contact printing (Thery, 2010). The photo-patterning method uses photosensitive material. Usually UV-sensitive material is cross-linked using a photo-mask which is transparent to UV in a patterned region. The patterned region is then used for subsequent attachment of cells or biomolecules (Clark, Britland et al. 1993). However, this technique is restricted to radiation-curable materials (Douvas, Argitis et al. 2002). Micro-contact printing (Alom and Chen 2007) is the process of transferring a pattern from a polymer (usually PDMS) stamp onto culture plates. In this process, the polymer stamp is first soaked in a solution and then placed onto a glass or Petri dish to transfer the pattern. While the micro-contact printing is an easy process, it only works with materials that can be adsorbed onto the surface of PDMS (Carola 2007). PDMS becomes hydrophobic upon exposure to the atmosphere for more than 30 minutes and thus must have corona or plasma treatments (Zhou, Ellis et al. 2010) to render its surface hydrophilic and wettable for patterning biochemical solutions.

Cells can be indirectly patterned by immobilizing them on a surface patterned with cell adhesion molecules (Bhatia, Toner et al. 1994) or by utilizing a substrate that can be switched to either repel or attach cells using electrical (Yeo, Yousaf et al. 2003), optical (Edahiro, Sumaru et al. 2005) or thermal (Yamato, Konno et al. 2002) excitation. Cells have been directly patterned using a stencil-based method (Folch, Jo et al. 2000) and microfluidic channels (Takayama, McDonald et al. 1999). However, all these techniques have several issues which limit their usefulness. Patterning using switchable substrate, for instance, is not compatible with all cells. This method also requires significant optimization in protocol to ensure reliable and reproducible patterning. Despite the versatility of stencil-based patterning, fabrication of thick stencils with holes at single cell resolution is difficult whereas working with thin stencil membranes without trapping air bubbles is cumbersome. Finally, the difficulty in injecting fluid into complex microchannels has limited the use of microfluidic devices to those with parallel stripes (Takayama, McDonald et al. 1999). The absence of a patterning method that can produce a complex pattern compatible with cells

and other biomaterials has severely limited patterning to small, simple geometric areas and selected substrate biomaterials.

This paper expands the vacuum-assisted micromolding in capillaries (MIMIC) technique (Jeon, Choi et al. 1999) and describes a method to pattern biologically-relevant substrates and cells using microfluidic devices and negative pressure (vacuum). The surface tension between the microchannel walls and solution is high due to the microscale dimensions and the hydrophobic surface of PDMS used to make the microchannels (Kim, Lee et al. 2002). As a result, injection of liquid into microchannels is challenging and limited to simple microchannels with both an inlet and an outlet. Using an inlet and an outlet, vacuum-assisted MIMIC has been used to fabricate polymer microstructures by filling polymer precursor in PDMS channels (Kim, Xia et al. 1995, Kim, Xia et al. 1996, Jeon, Choi et al. 1999). Unlike vacuum-assisted MIMIC, our method takes advantage of the gas permeability of PDMS (Merkel, Bondar et al. 2000) and uses vacuum to distribute biological solutions of substrates or cell suspensions inside closed (dead-end), complex microchannels, thus demonstrating the biological application of this technique. Our method, like the previous method, does not rely on the adsorption of solution onto the PDMS surface, making it possible to pattern cells and other biomolecules on a surface without the need to modify the surface of the PDMS microchannels and uses microfluidic channels constructed by reversible as opposed to irreversible sealing of the patterned PDMS with the culture plate surface. The ability to fill reversibly sealed microchannels without any leakage enables the user to peel off the PDMS and expose the patterned substrate or cells. Exposing patterned cells in turn allows the user to culture cells without problems such as limited accessibility of the culture medium and oxygen (Peng, Liao et al. 2013), which can occur with use of PDMS microfluidic culture devices. This study highlights the use of these techniques with a variety of cell adhesion molecules and cell types on two different culture surfaces, glass and polystyrene.

1. Method and Materials

1.1. Microchannel fabrication

SU-8 master—Masters with complex patterns of micrometer features for soft-lithography fabrication were fabricated using photolithography. Briefly, a 50 μm thin layer of photoresist SU-8 50 (MicroChem, USA) was spin-coated onto a 3 inch silicon wafer (University wafers, USA) and exposed to 365 nm UV light through a transparency photo-mask after pre-exposure bake on hotplate. Unexposed photoresist was removed by developing the wafer in Propylene Glycol Monomethyl Ether Acetate (PGMEA, Sigma Aldrich, USA) after post-exposure bake on a hotplate. The wafer with patterned photoresist was rinsed with isopropanol, dried by blowing nitrogen gas and exposed to (Tridecafluoro-1, 1, 2, 2-Tetrahydrooctyl)-1-Trichlorosilane vapors (United Chemical Technology Inc., USA) under vacuum. This was necessary to prevent sticking of the Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning Inc., USA) to the mold during soft-lithographic replication.

Adhesive tape master—Adhesive tape masters (Shrirao, Hussain et al. 2012) were used to fabricate simple microchannels with dimensions larger than 300 μm . Briefly, a piece of adhesive tape was attached onto a clean glass slide and cut as per the layout of

microchannels using a scalpel. Unwanted tape was removed and the glass slide with patterned tape was rinsed with isopropanol. The mold was used for replication after heating at 65°C for 10 minutes.

PDMS device—The PDMS elastomer and its curing agent were mixed 10:1 (wt/wt) and degassed under vacuum to remove bubbles. The mixture was poured onto the mold to form a 1–2 mm thick layer and degassed again to remove any bubbles. The PDMS was cured in an oven at 65°C for 2 hours. Cured PDMS on top of the pattern was cut and peeled off the master. Inlet holes were made to access the microchannel engraved in patterned PDMS using coring tool with a diameter of 1–2 mm. The PDMS casts were sterilized prior to use by rinsing with 70% (v/v) solution of ethanol in DI water and then dried under sterile conditions. The microchannel device was constructed by affixing sterile PDMS cast with inlet on either bottom of Petri dish or glass slide as patterned PDMS casts exhibit conformal sealing with both surfaces.

1.2. Substrate patterning

Collagen-I—Collagen type 1, rat tail (Cat# 40236, BD Biosciences, Bedford, MA 01730, USA) with a final concentration of 3.2 mg/ml was prepared by dissolving collagen-I in DI water. After vacuum-assisted channel filling, cast removal and rinsing in DI water, a 1 ml solution of One Step Trichrome (Cat# NC9867252, Fisher Scientific, Pittsburg, PA 15275, USA) without any dilution was added to the Petri dish at room temperature for 1 hour. The Petri dish was rinsed with DI water and the pattern imaged under phase-contrast and brightfield microscopy.

Fibronectin—Human fibronectin (Cat# 40008, BD Biosciences, Bedford, MA 01730, USA) with a final concentration of 200 µg/ml was prepared from 1 mg/ml stock solution by diluting in DI water. A 5 µg/ml rabbit anti-fibronectin primary antibody (Cat# F3648, Sigma-Aldrich, St. Louis, MO 63103, USA) and 5 µg/ml Anti-rabbit IgG Alexa Fluor 594 (Cat # A-21207, Invitrogen, Eugene, OR 97402, USA) secondary antibody was added and mixed with the fibronectin solution. Premixing of anti-fibronectin, secondary antibody, and fibronectin was necessary to image fibronectin patterns. However it is possible that the primary and secondary antibodies bound not only to the fibronectin but to the surface of the Petri dish as well and may have contributed to some slight unevenness in the substrate coating. Nonetheless the technique allowed us to visualize the patterned fibronectin. After vacuum-assisted microchannel filling, PDMS cast removal and rinsing with PBS, the pattern was imaged with a fluorescence microscope.

1.3. Preparation of cell suspensions

PC12 cells—PC12 cells, a rat pheochromocytoma cell line (generously provided by Dr. Treena Arinzeh, New Jersey Institute of Technology, Newark, NJ), were detached from culture dishes by incubating with trypsin (Cat # BW17-161E, Fisher Scientific, Pittsburg, PA 15275) at 37 °C for 5–10 minutes. PC12 culture medium, high glucose Dulbecco's Modified Eagle Medium (DMEM, Cat # SH3008101, Fisher Scientific, Pittsburg, PA 15275) with 5% fetal bovine serum, 5% horse serum, 1% penicillin/streptomycin, was added to the Petri dish and the resulting cell suspension was centrifuged at 700 RPM for 5 minutes.

A cell pellet was obtained by aspirating the supernatant and re-suspending the pellet in 1 ml PC12 culture medium to count the total number of cells. The suspension was again centrifuged and then added with the necessary amount of serum-free PC12 culture medium (High Glucose DMEM, 1% penicillin/streptomycin) to obtain a cell suspension with a density of 5000 cells/ μ l (Figure 4) or 200 cells/ μ l (Figure 5).

Fibroblast cells—The same process was used to prepare the fibroblast cell suspension, Murine 3T3-J2 fibroblasts (Howard Green, Harvard University), with a cell density of 5000 cells/ μ l (Figure 6) or 6000 cells/ μ l (Figure 9). Fibroblast culture medium (High Glucose DMEM, 10% horse serum, 1% penicillin/streptomycin) was used instead of PC12 culture medium.

Retinal cells—Retinal cell suspensions were prepared as previously described (Nachman-Clewner and Townes-Anderson 1996, Clarke, Hognason et al. 2008). Briefly, adult aquatic-phase tiger salamanders (*Ambystoma tigrinum*), were decapitated, pithed, and enucleated. The anterior portions of the eye were removed and the retina scooped out of the eyecup using a pair of curved forceps. The retina was then washed twice with salamander Ringer solution (85 mM NaCl, 1.5 mM KCl, 25 mM NaHCO₃, 0.5 mM CaCl₂, 0.5 mM NaH₂PO₄, 24 mM glucose, 1.0 mM sodium pyruvate), and enzymatically digested in a papain solution (14 U Papain, Roche 10108014001, Worthington, Freehold, NJ), 85 mM NaCl, 1.5 mM KCl, 25 mM NaHCO₃, 1.8 mM CaCl₂, 0.5 mM NaH₂PO₄, 16 mM glucose, 1.0 mM Na pyruvate, 2.7 mM DL-Cysteine) for 40 minutes with slight rocking. The retina was then washed twice with salamander Ringer solution, and gently triturated using a smooth bored pipette yielding a mixture of retinal cells.

1.4. Cell patterning and culture

PC12 and fibroblast cells—Collagen-I solution with a final concentration of 50 μ g/ml was prepared from 3.2 mg/ml stock solution by diluting in DI water. 20 μ l of the collagen-I solution was placed into an inlet of a PDMS microchannel cast affixed on the bottom of a Petri dish. The collagen solution was then distributed inside the microchannels using the vacuum method and incubated at room temperature for 1 hour. The Petri dish was filled with DI water to submerge the PDMS cast and the cast was carefully peeled off using tweezers. The Petri dish was rinsed three times with DI water and filled with 1 ml of 1% BSA solution. The Petri dish was incubated overnight at 37°C and then the BSA solution was aspirated from the Petri dish. 0.5 ml of PC12 cell suspension in serum-free PC12 culture medium or fibroblast cell suspension in fibroblast culture medium with a cell density of 5000 cells/ μ l was added to the Petri dish and incubated at 37°C for 1 hour. The excess cell suspension was aspirated from the Petri dish. The patterned cells were washed three times with PBS by applying gentle shaking for 10 seconds during each wash. PC12 and fibroblast cultures were incubated at 37°C after adding their respective culture medium.

Retinal cells—Tissue culture dishes were modified to fit PDMS microchannel casts. A 2 cm diameter hole was drilled into the center of a 35 mm Petri dish. Uncured PDMS was then spread inside the Petri dish around the lip of the 2 cm hole and a cleaned 25 mm square #1 coverslip was placed inside the Petri dish. The dish was cured for 24 hours in an oven at

60°C. Prior to use, each Petri dish was cleaned overnight by soaking in a 1% Tergazyme solution, rinsed with DI water, and sterilized under UV for at least 1 hour. 4 µl of Sal-1 was placed into an inlet of the PDMS microchannel cast affixed on the bottom of the modified Petri dish. The Sal-1 supernatant, the supernatant of a mouse hybridoma cell line producing a monoclonal mouse antibody raised against retinal cell membranes (generously provided by Dr. Peter MacLeish, Morehouse School of Medicine, Atlanta, GA), was then distributed inside the microchannels using the vacuum method and incubated at 10°C overnight. The PDMS cast was then removed and the dish washed twice with salamander Ringer and then finally filled with salamander medium (108 mM NaCl, 2.5 mM KCl, 2 mM HEPES, 1 mM NaHCO₃, 1.8 mM CaCl₂, 0.5 mM NaH₂PO₄, 1 mM NaHCO₃, 24 mM glucose, 0.5 mM MgCl₂, 1 mM Na pyruvate, 7% medium 199, 1× minimum essential (MEM) vitamin mix, 0.1× MEM essential amino acids, 0.1× MEM nonessential amino acids, 2 mM glutamine, 2 µg/mL bovine insulin, 1 µg/mL transferrin, 5 mM taurine, 0.8 µg/mL thyroxine, 10 µg/mL gentamicin, and 1 mg/mL bovine serum albumin (pH 7.7)). Retinal cell suspension was then plated into the patterned Petri dish and cells cultured for 7 days at 10°C.

1.5. Immuno-fluorescent culture labeling

PC12 cells—Cell cultures were fixed with 4% paraformaldehyde in 0.1M PBS solution at room temperature for 20 minutes. Cells were then permeabilized in 0.2% Triton X-100 in PBS for 10 minutes and washed three times with PBS. Cultures were incubated in a 1 µg/ml solution of Phalloidin-Tetramethylrhodamine B isothiocyanate (TRITC, Cat # P1951, Sigma-Aldrich, St. Louis, MO 63103, USA) in PBS for 30 minutes at room temperature, washed three times with PBS and then incubated in 1 µg/ml solution of DAPI (Cat # 157574, MP Biomedicals Inc., Salon, OH 44139, USA) in PBS for 10 minutes at room temperature. After washing three times with PBS, the cultures were imaged with fluorescence microscopy on a Nikon microscope (Eclipse Ti) coupled to a fluorescent lamp (X-Cite Series 120) using 359 nm (blue, DAPI) and 547 nm (red, TRITC) excitation filters.

Fibroblast cells—The same process was used for the immuno-fluorescent labeling of the fibroblast cells.

Retinal cells—Retinal cells cultured on a monoclonal Sal-1 substrate pattern were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer solution at 4°C. Culture Petri dishes were rinsed 3 times with PBS and then incubated with goat serum dilution buffer (GSDB, 16% normal goat serum, 450 mM NaCl, 0.1 % Triton X-100, 20 mM Sodium phosphate, pH 7.4) for 1 hour at room temperature. Dishes were then incubated with goat-α-mouse IgG (Alexa Fluor 594 M8642, Sigma-Aldrich, Saint Louis, MO 63103, USA) at 1:100 dilution in GSDB without Triton for 1 hour at room temperature. Dishes were rinsed 3 times with PBS and imaged with fluorescent microscopy.

1.6. Live-Dead Cell Assay

Live-Dead Cell Assay was performed using a Calcein-AM live-dead cell assay kit (Cat # L-3224, Invitrogen, Eugene, OR 97402, USA). Briefly, one ml of a solution of PBS containing 1 µl of 4 mM Ethidium homodimer-1 (EthD-1) and 1 µl of 2 mM Calcein AM was prepared and added to the culture dish. The culture was then incubated in a humidified

atmosphere with 10% CO₂ at 37°C for 10 minutes. The culture was washed with PBS and imaged with fluorescence microscopy with 485 nm (green, Calcein-AM) and 528 nm (red, EthD-1) excitation filters. The number of dead cells was directly counted from Figure 10(a). Live cells were calculated by multiplying the size of the patterned area with an average number of cells per mm² in the patterned area. The size of the patterned area was measured directly from the layout of microchannels used to pattern the cells. The average number of cells per unit area was calculated by counting the number of cells in three different regions, 3 mm x 0.5 mm, selected from the cell pattern shown in Figure 10(a).

2. Results

2.1. Vacuum-assisted microchannel filling

PDMS, which is biocompatible and widely used in biological research, was used to fabricate the microchannels. However, in order to avoid the creation of dead-spaces that would likely occur by injecting water-based solutions by vacuum into a complex system of hydrophobic PDMS channels with an inlet and outlet, we took advantage of the gas permeability of PDMS to inject fluid into closed-ended devices.

Microchannels were created using soft lithography. The master mold used for soft-lithography replications was fabricated using either photo-lithography or the adhesive tape method. An inlet hole was made using coring tools with a diameter of 1–2 mm to access the microchannels. The microfluidic device was assembled by placing the patterned side of the PDMS cast onto the bottom of a Petri dish. The PDMS formed a conformal seal with the planar surface of the Petri dish and provided reversible binding; it could be easily removed by peeling away from the culture surface. Such reversible but conformal sealing obviated the need of oxygen plasma or corona treatment to fabricate the device. However it was important to ensure the complete attachment of the PDMS microchannel cast to the culture surface. The presence of any particle or air bubble will destroy the seal between the cast and culture surface when the device is subjected to vacuum. Both surfaces were air dried and cleaned to remove any dust particles. Handling was done in a biosafety cabinet in order to reduce the probability of dust particles becoming trapped either on the PDMS or the bottom surface. Air bubbles trapped upon putting PDMS on a planar surface were removed by gentle squeezing with tweezers. The attachment between PDMS and hydrophobic/hydrophilic surfaces was tested by filling the channels with colored DI water (Figure 1B).

For vacuum filling, shown schematically in Figure 1A, a pipette filled with substrate or a cell suspension was inserted through the inlet hole until its tip touched bottom; then the solution was injected to form a droplet completely covering the inlet hole. The Petri dish was then placed under a laboratory vacuum with a magnitude of ~254 mmHgA for 10 minutes. The applied vacuum removed air from the microchannels as well as from the PDMS and created negative pressure inside the microchannels. Upon releasing the vacuum, the negative pressure inside the PDMS and microchannels, and the positive pressure outside the microchannels created a pressure gradient. As a result, the solution in the inlet hole flowed from a region of high pressure (outside) to a region of low pressure (inside microchannels) filling the microchannels. The method filled more than one microchannel simultaneously. The fluid flow started abruptly upon releasing the vacuum and eventually

slowed down as fluid neared the closed end of the channel. Although the microchannels were filled upon releasing the vacuum in less than 1 minute, the precise time taken to fill the microchannels depended on the length and dimensions of the channels and the viscosity of the solution used. We estimate the velocity of PBS in a channel that is 20 μm wide and 50 μm tall to be approximately 1 mm/sec.

2.2. Substrate Patterning

Collagen-I and fibronectin are glycoproteins of the extracellular matrix that bind to cell membrane-spanning receptors. Both molecules play important roles in cell adhesion, migration, growth and differentiation (Pankov and Yamada 2002). The vacuum-assisted PDMS microchannel filling method was used to pattern collagen-I and fibronectin substrates on standard Petri culture dishes. After removal of the PDMS cast, the presence of the substrate pattern was confirmed by suitable staining.

The PDMS device used to pattern collagen-I had 48 parallel microchannels each with a length of 13 mm, width of 20 μm , and separated by a distance of 200 μm covering an area of $13 \times 10 \text{ mm}^2$ (Figure 2a, b). Twenty μl of 3.2 mg/ml collagen-I in DI water was placed in the inlet and injected into the microchannels using the vacuum method. The device was incubated for 2 hours at 50°C on a hotplate to allow collagen-I to bind to the bottom of the dish. The PDMS cast was then peeled off the dish and the dish rinsed with DI water. The collagen-I pattern was stained with One Step Trichome and visualized with brightfield microscopy (Figure 2c). The stain revealed a blue pattern of collagen-I that had lines with widths of 20 μm and separations between the lines of 200 μm , similar to the dimensions of the PDMS microchannel device.

Similarly, fibronectin was also patterned onto Petri dishes using the vacuum method. The PDMS microchannel device shown in Figure 2(d, e) was used. This device had eight parallel microchannels each with a length of 120 mm, width of 25 μm and separated by a distance of 20 μm . These long microchannels had multiple turns covering an area of $11 \times 4 \text{ mm}^2$. Four μl of a solution containing fibronectin, anti-fibronectin and secondary antibody conjugated to Alexa-Fluor 594 (see methods) was placed in each inlet and injected into the microchannels using the vacuum method. The device was incubated for 1 hour at room temperature to allow fibronectin to bind to the bottom of the dish. The PDMS cast was then peeled off the dish and the dish rinsed with PBS. The complex fibronectin pattern was visualized due to the presence of primary and secondary antibodies in the fibronectin solution, with fluorescence microscopy (Figure 2f). The dimensions of the patterned fibronectin substrate and the microchannels were congruent.

In this way, the vacuum method was successfully used to obtain collagen-I and fibronectin substrate patterning with complex shapes over large areas. In all cases, patterns of substrate conformed precisely to the PDMS microchannels and the coating appeared homogeneous.

2.3. Cell Patterning

The vacuum method was used to pattern cells using two approaches: 1) Cell seeding on a substrate patterned onto a Petri dish and referred to hereafter as indirect cell patterning, and 2) injection of a cell suspension into microchannels and referred to hereafter as direct cell

patterning. Indirect cell patterning is shown schematically in Figure 3. The process of direct cell patterning is shown in Figure 1A; however in this case instead of substrate solution, a cell suspension is placed in the inlet hole.

2.3.1. Indirect Cell Patterning—PC12 and fibroblast cells were patterned using the indirect approach on patterned collagen-I substrate. In this approach, first collagen-I substrate was patterned on a polystyrene Petri dish by filling PDMS microchannels with 50 µg/ml solution of collagen-I using the vacuum method. The PDMS cast was peeled off the Petri dish after incubating collagen-I filled microchannels for 1 hour at room temperature. The Petri dish was rinsed with DI water and coated with 1% bovine serum albumin (BSA). The surface of the Petri dish without collagen-I, referred to hereafter as the non-patterned region, was therefore coated with 1% BSA; BSA was used to prevent cell adhesion to the non-patterned region. PC12 cells suspended in serum-free medium at a density of 5000 cells/µl were then seeded into the dish and incubated for 1 hour. Figure 4 (a) shows that before washing, cells were omnipresent. However, cells in the non-patterned regions were removed after washing with PBS leaving only cells that were attached to patterned substrate (Figure 4b). The lack of attachment in the non-patterned region can be attributed primarily to the BSA coating. The BSA was able to prevent most cell binding to the non-pattern region without any effect on the collagen-I substrate which is consistent with other studies of BSA coating after substrate patterning (Anderson and Hinds 2011). Despite the use of BSA coating, however, very small numbers of cells were found attached to regions without collagen; those that remained after washing showed only limited growth in all directions. Although BSA was used in this study, it is possible to use other commonly used coatings to prevent cell attachment such as PLL or PEG (Yang, Co et al. 2011, Poudel, Lee et al. 2013). This procedure allowed the PC12 cells to be patterned on large surface areas, up to 13 × 10 mm² in our case (Figure 4c).

After patterning, PC12 culture medium was replaced by a medium with NGF and cells were cultured for 7 days (Figure 5) to allow cells to differentiate and grow. Live-cell staining using Calcein AM (Figure 5a, b) showed little evidence of cell death after 7 days. PC12 cells plated at a density of 200 cells/µl and attached onto the patterned collagen-I, maintained a binary spindle morphology of their neurites along the substrate pattern. Neurite outgrowth increased noticeably on a daily basis (Figure 5c, d).

Similarly, fibroblast cells were successfully patterned on a large area of patterned collagen-I substrate. Figure 6 shows the phenotypical growth of fibroblasts cultured for 3 days on patterned collagen-I substrate. The fibroblast cells spread only on the collagen-I pattern, thus they were effectively isolated to the substrate pattern. The adherence of fibroblast cells to the substrate is critical for survival as pro-life (trophic) signaling pathways are relayed through contact between the cell and its surface (Ingber 1997).

The indirect cell patterning approach using the vacuum method was also used to pattern amphibian retinal cells. In this case, the Sal-1 supernatant containing a mouse monoclonal anti-salamander antibody raised against retinal cell membranes was patterned onto glass cover slips using the vacuum method. In this case BSA was not used to prevent cell attachment to other areas of the Petri dish. The retinal cell suspension was then plated onto

the patterned Sal-1 substrate (Figure 7a). It was unnecessary to subsequently wash the culture dish with Ringer or PBS to remove nonspecific binding of retinal cells to the glass since retinal cells bind specifically to Sal-1. All retinal cell types were able to attach to the stripes of Sal-1 including Mueller, bipolar, ganglion, amacrine, and photoreceptor cells (data not shown). The subsequent neuronal outgrowth of rod and cone photoreceptors as well as other nerve cell types occurred almost exclusively within the area of the patterned Sal-1 substrate (Figure 7b, c). After 7 days in vitro, thin filopodia were able to extend past the borders of Sal-1 but thicker, neuritic processes continued to grow only where Sal-1 was present. Various geometries of Sal-1 patterning were tested (data not shown) with the amphibian retinal neurons and all achieved similar adherence to the patterned substrate. Occasional cells which attached to uncoated areas generally demonstrated stunted growth and differentiation. The Sal-1 and cell patterns were maintained for at least 7 days in vitro. Thus, cell patterning of a variety of cells with micrometer resolution on a large surface area was achieved using the vacuum-assisted microchannel filling method.

2.3.2. Direct Cell Patterning—Amphibian retinal neurons and fibroblast cells were also patterned using the direct cell patterning approach. A PDMS device having parallel microchannels with a width of 50 μm and separated by a distance of 75 μm was used to pattern retinal cells. The PDMS cast was soaked in 1% BSA solution overnight to prevent cell adhesion to the PDMS and then attached to a glass coverslip. The cell suspension was distributed into the microchannels using the vacuum method shown in Figure 1A. Cells of all different sizes and shapes, from rod cells to retinal ganglion cells were able to freely move through the channels shown in video (Online Resource 1). Cells were then incubated for one day to allow cells to attach to the substrate. The cells were washed with PBS after peeling off the PDMS cast and cultured for 7 days to observe their growth (Figure 8). Although, no attempt was made to determine the amount of cell death in retinal neurons, qualitatively cell survival was similar to cell survival without exposure to vacuum. Importantly, the patterned photoreceptor cells exhibited normal growth (Figure 8c), even though subjected to vacuum during the patterning process.

Attempts were also made to inject cell suspensions using the vacuum method into microchannels shown in Figure 2(a) and Figure 3(a) with widths as low as 20 μm (Online Resource 2). However, despite the ability of the method to fill microchannels with a width of 20 μm , it was difficult to obtain a pattern with a high cell density of cells as most of the cells injected into microchannels were detached while peeling off the PDMS cast after incubation. The cell detachment during peeling off the cast can be attributed to shear force exerted during peeling and the possibility of cells adhering to PDMS microchannel walls. The microchannels shown in Figure 8(a) with a width of 50 μm , a diameter greater than the size of the cells, was selected to minimize the shear force exerted on attached cells. However, even after injecting retinal cells in microchannels with a width of 50 μm , it was found that most cells remained in the inlet. This can be attributed to the ability of liquid to flow faster than cells filling most of the space of the microchannels with liquid.

Similarly, fibroblast cells were patterned onto polystyrene tissue culture dishes using the direct cell patterning approach. The PDMS device used for patterning was fabricated using an adhesive tape master and had four microchannels with a width and separation between

microchannels of $\sim 500\ \mu\text{m}$ (Figure 9a). A fibroblast cell suspension with a cell density of 6000 cells/ μl filled the microchannels during the vacuum method (Figure 9b). The cells were allowed to attach to the bottom of the tissue culture dish for 1 hour in an incubator. The PDMS cast was then peeled off and revealed patterned fibroblast cells which were cultured for one day (Figure 9c).

The effects of the applied vacuum on directly patterned mammalian fibroblast cells were analyzed by performing the Live-Dead cell assay immediately after the patterning the cells (Figure 10a). Only 3.8 % of the total numbers of cells were labeled as dead, suggesting that the applied vacuum does not have an adverse effect on cell survival. In addition, the growth of fibroblast cells after 5 hours in-vitro (Figure 10b, c, d) exhibited spreading on the pattern which is common to this particular cell type.

Although assessment of growth and cell survival are sensitive indicators of cell health, examination was also made of the effects of vacuum on the culture medium (Petrucchi, Ralph et al. 2007). Evaporation of liquid and formation of bubbles were observed during the vacuum procedure on the lid used to cover the microchannel device. The dynamics of reduction in the concentration of gases under negative pressure is complex and depends on several factors such as time, partial pressure of gas, temperature, and equilibrium solubility of solutes present in solution (Käppeli and Fiechter 1981, Vendruscolo, Rossi et al. 2012). The pH value of the solution is affected by the concentration of oxygen and carbon dioxide and is important for cell metabolism (Watanabe, Yamamoto et al. 1989). Thus we chose to measure this parameter directly. The pH of the cell culture medium subjected to vacuum with a magnitude of $\sim 254\ \text{mmHg}$ at room temperature was monitored for up to 30 minutes. The pH showed an increase from 7.64 without vacuum to 7.81 after 10 minutes of vacuum and to 7.85 after 30 minutes of vacuum. These changes are small and, as noted, did not appear to affect the viability of the mammalian cells, the fibroblast and PC12 cells, but also the primary amphibian neuronal cells, which are considered to be more sensitive and fragile than the connective tissue cells and the neuronal cell line. However vacuum-assisted microchannel filling may not be suitable for those processes which are very sensitive to small variations in pH.

3. Discussion

This study has described a method to inject solutions into long, complex shapes and closed (dead-end) PDMS microchannels using vacuum. The method successfully patterned substrates and cells over large areas. Moreover, the microchannels could be reversibly sealed with glass or polystyrene surfaces and thus removed for cell plating or for continued long-term cell culture when cell suspensions had been injected. The advantages of this vacuum method are simplicity, biocompatibility, broad applicability and inexpensiveness.

Unlike a stencil-based method, the fabrication of microchannels is done using soft lithography which is simple, inexpensive, requires no prior knowledge of microfabrication and is feasible in a conventional cell biological research laboratory. Compatibility with long-term cell culture is insured because the vacuum method, which requires only a desiccator and laboratory vacuum, can be performed sterily inside a culture hood. Additionally, the

vacuum method injects solution into PDMS microchannels in spite of the hydrophobicity of the microchannel walls, and regardless of the bottom surface of the device, or the composition of the solutions. Thus, the vacuum method should be suitable to pattern a large variety of substrates and cells; we have demonstrated its use with several cell substrates (collagen-I, fibronectin, and Sal-1) and cell types (fibroblasts, PC12 cells, and primary retinal neurons).

The equipment necessary for the vacuum method is also simple and widely available in most laboratories. The vacuum used in all of our experiments is rated at ~254 mmHgA which is easily obtainable with inexpensive single stage mechanical vacuum pumps or is provided as a central service in many laboratories. Additionally, any vessel which can hold a vacuum can be used to perform this technique. As with any technique utilizing a vacuum, the vessel should be rated for the vacuum being applied to mitigate the risk of implosion.

It is important to note that the vacuum method fills substrate solutions or cell suspensions simultaneously into more than one closed (dead-end) microchannel regardless of their width, length, and complexity (Figures 2a, d, and 9a). The method thus enables the user to efficiently perform patterning with complex shapes. Moreover, it is possible to inject distinct substrate solutions or cell suspensions in each microchannel. This would make it possible to obtain patterning with more than one substrate or cell type for co-culture without need for alignment and issues related to registration of more than one pattern. In addition, very small amounts of experimental solutions are required. Less than 4 μ l of solution filled a microchannel with a width of 25 μ m and a length of 120 mm. The method has patterned an area of 13×10 mm² using less than 40 μ l of substrate solution. The use of small amounts of substrate solutions and cell suspensions avoids waste of expensive reagents and difficult to produce cell suspensions. The ability of the vacuum method to consistently pattern large areas with micrometer resolution using small volumes of reagents should prove very useful for studies in cell biology especially in neuronal growth studies, due to the long length of neurites.

3.1. Effect of applied vacuum on cells

Our results show that vacuum has little or no effect on a variety of cells which is consistent with previous research (Solchaga, Tognana et al. 2006, Ferrell, Gallego-Perez et al. 2010). The loading of cells after degassing a microfluidic device (Wang, Ni et al. 2009) has been used to culture mammalian cells in various sized chambers. Cells did not experience vacuum directly in this approach. In addition, unlike the vacuum method, this report used irreversibly sealed microchannels which cannot be removed to access cells after injection into the microchannels. However, vacuum-assisted cell seeding has been applied to mesenchymal stem cells using a 3D unpatterned HYAFF-11 sponge scaffold. The cell viability and chondrogenic potential of the human stem cells were unaffected by applied vacuum of 85 mmHgA (Solchaga, Tognana et al. 2006), a vacuum greater than that used in our study. In another, similar approach, vacuum of 128–638 mmHgA was used to load adherent (mammalian fibroblast and epithelial cell lines) and non-adherent (human leukemia) cells into micro-wells without any adverse effects (Ferrell, Gallego-Perez et al. 2010). An earlier study also showed that A_L cells (human-hamster hybrid A_L cell line)

suspended in culture medium and glycerol remained viable when subjected to vacuum with a magnitude of 75 mmHgA (Feng, Wu et al. 2004) which is several fold greater than the vacuum used in our study. The results of cell growth and viability of cells patterned using vacuum in this study and others clearly indicates that cells can withstand vacuum with a magnitude of ~254 mmHgA.

In our report cell growth was used as evidence of the viability of the cells. However, patterned substrates may also be used to aid in cell growth measurements. PC12 cells are often used as a model of neural differentiation. Unlike their primary neural cell counterparts, PC12 cells are easily cultivated and upon addition of NGF exhibit nerve outgrowths similar to that of sympathetic neurons. However, these neurite outgrowths can form in random directions, making it difficult to measure the lengths of the outgrowths. Furthermore, isolating PC12 cells to a specific geometric formation can have a profound impact on PC12 cell proliferation and differentiation (Mahoney, Chen et al. 2005, Yang, Co et al. 2011, Cai, Zhang et al. 2012, Poudel, Lee et al. 2013). In this study PC12 cells and their neurite outgrowths were confined to collagen-I, patterned as lines, resulting in cells with a bipolar morphology in which only two neurites extended along the collagen-I pattern. Similar studies (Yang, Co et al. 2011, Cai, Zhang et al. 2012) in which PC12 cells were confined to a line pattern showed that decreasing widths, of similar dimensions to our 20 μm wide collagen-I patterns, led to an increase in cell alignment. Furthermore, widths of similar dimensions exhibited the formation of one or two neurites, as opposed to larger widths which exhibited formation of more than two neurites (Mahoney, Chen et al. 2005). This confinement to the pattern facilitated measurement of neurite outgrowths, which showed progressive growth over the course of 7 days (Figure 5d). Although cell viability was maintained, proliferation was not enhanced as images from initial seeding and seven days after showed no significant difference in the number of cells. This would be expected as the addition of NGF is known to terminate mitosis in PC12 cells (Greene 1978).

3.2. Technical Issues

Some precautions need to be taken for the optimal use of our technique. The width of microchannels used for direct cell patterning should be selected based on the size of cells to promote cell flow and to avoid detachment of cells while peeling off the PDMS cast. Prior to use, coating PDMS microchannels with 1% BSA helped to reduce cell adhesion to PDMS and hence reduce the cell detachment, however, use of wider microchannels provided better control in preventing cell detachments. This study has used microchannels with a width of 50 μm , for instance, to pattern the retinal neurons onto coated glass surfaces using the vacuum method.

In addition the height of the channels should be designed to both provide enough substrate solution to coat the glass or polystyrene surface and to reduce fluidic resistance. The fluidic resistance can be calculated using Poiseuille's law which depends on viscosity of fluid (η), width (w), height (h) and length (L) of the channel (Figure 11). The threshold pressure difference between two ends of the channel is necessary to pull the liquid front inside the channel against the surface tension of fluid and flow resistance (R) inside the channel. However, channels which have a very large aspect ratio can be difficult to produce using

conventional photolithography and soft lithography techniques. Therefore, some experimentation with the channel height may be necessary for other applications.

The thickness of the PDMS cast also requires some consideration. We used moderately thin PDMS cast with a thickness of 1 to 2 mm. While thicker PDMS will have a greater volume for air to diffuse back into the PDMS, it will also slow down the degassing process when vacuum is applied and hence require a longer degassing time as well as being less flexible which makes conformal sealing and detachment more difficult.

When utilizing different substrates with this technique, some experimentation by the user is required. If the substrate solidifies during patterning or if it binds significantly to the PDMS microchannels, the patterning may peel off after removal of the microchannel. While we never observed this phenomenon during patterning, it could potentially occur in other substrates used for cell culture.

The concentration of cells in cell suspensions and the width of microchannels are important parameters that need to be selected carefully to use the vacuum method for cell patterning and to accomplish experimental goals. Low cell density results in large distances between adjacent cells; this was useful to study neurite outgrowth which can extend over long distances. On the other hand, high cell density could result in placement of adjacent cells close to each other; this could be useful to study cell-cell interaction.

3.3. Limitations

Finally, the method has some limitations. The vacuum method can inject solutions into interconnected microchannels through a single inlet but requires an inlet to each isolated microchannel. This does not allow the vacuum method to pattern shapes which are not interconnected such as two dimensional arrays of dots. Other conventional methods such as micro-contact printing would be efficient to perform such dot patterning.

This technique cannot be used to fill microfluidic channels made of non-gas permeable material. In the case of PDMS channels, the applied vacuum creates negative pressure not only inside the channel but also inside the PDMS channel sidewalls due to the gas permeability of PDMS. Unlike in non-gas permeable materials, the pressure inside the sidewalls of the PDMS channels is unaffected by channel filling and is only affected by the atmosphere gradually diffusing into PDMS over time. As a result, fluid flow inside the PDMS channel continues until fluid reaches the end of the microchannel. Although not tested, our method can be potentially used to fill microchannels made from other gas permeable materials.

We determined that the pH of the culture medium changes when it is subjected to vacuum. Although this change in pH is insignificant and had no adverse effect on cells, the vacuum method may not be suitable to applications which are more sensitive to variations in pH.

For us cell patterning using the vacuum method required a cell suspension with a relatively high cell density as most of the cells remained in the inlet after injecting cells into microchannels. In the case of narrow microchannels, fluid fills the microchannels before cells start to flow along with the fluid and flow stops once microchannels are filled. This

results in a large number of cells in the inlet and few cells in microchannels. However, we believe that this problem can be addressed by designing the end of microchannels in such a way that it helps to maintain fluid flow until enough cells flow into the narrow microchannels.

3.4. Future Directions

In the future, this technique may be expanded upon by utilizing two microfluidic devices on the same glass or plastic substrate. For example, a researcher could use the 25 μm microfluidic device to pattern stripes of collagen in one direction. They could subsequently remove the device, and utilize a second microfluidic device to pattern fibronectin in a direction perpendicular to the first device. This would create areas on the substrate that would simultaneously contain collagen and fibronectin. Other researchers have utilized a similar concept to create isolated areas of a fluorescently tagged antibody with an antigen (Bernard, Michel et al. 2000). In addition, this method may be able to create isolated patterns if combined with the use of a method of removing coating from the substrate such as oxygen plasma treatment (Rhee, Taylor et al. 2005). For example, if one microfluidic device was used to pattern collagen, and another device was placed perpendicularly to protect areas of collagen from oxygen plasma treatment. However, at this time we have not explored this possibility.

The vacuum-assisted technique may also be useful for three dimensional (3D) cell cultures. Hydrogels such as poly(ethylene glycol) diacrylate (PEGDA) are widely used in 3D cell culture to encapsulate cells and mimic the tissue-like microenvironment around the cell during in-vitro culture (Tibbitt and Anseth 2009, Durst, Cuchiara et al. 2011). So far, the patterning of such 3D cultures are limited to UV curable hydrogels due to the ease and accessibility of photolithography (Occhetta, Sadr et al. 2013). The ability of the vacuum method to inject solutions in complex, long, and dead-end reversibly sealed microchannels can be used to obtain 3D patterned cell cultures by injecting a hydrogel precursor solution mixed with a cell suspension into the PDMS microchannels. This will enable users to obtain complex 3D cell patterns critical for studies of tissue engineering, using a variety of hydrogels, such as alginate, chitosan etc.

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Fig. 1.

A) Schematic of the vacuum-assisted microchannel filling method for biological solutions.

B) Leak testing of microchannels using green food dye in DI water. Scale bar = 200 μm .

Fig. 2.

Collagen-I substrate pattern. (a) PDMS microchannel device (b) magnified region of the microchannel device shows microchannels of a width of 20 μm with separation of 200 μm between them. (c) Collagen-I substrate pattern on polystyrene Petri dish labeled with Trichrome after removal of the PDMS microchannel cast. Fibronectin substrate pattern. (d) PDMS microchannel device (e) magnified region of microchannel device showing eight microchannels running parallel to each other (f) Fibronectin substrate pattern on polystyrene Petri dish after removal of the microchannel device. Fibronectin was labeled prior to injection with anti-fibronectin and a secondary antibody conjugated to Alexa Fluor 594. Note that greater than 90% of the channel area is homogeneously covered by substrate.

Fig. 3.
Indirect cell patterning by cell plating on patterned substrate.

Fig. 4. Patterned PC12 cells cultured for 1 day on patterned collagen-I substrate. (a) PC12 cells incubated on patterned collagen-I substrate for 1 hour before washing; (b) after washing with PBS to remove non-attached cells; (c) PC12 cells in-vitro for 1 day demonstrating consistent patterning with micrometer resolution on a large area of Petri dish.

Fig. 5. Guided neuronal differentiation of PC12 cells (200 cells/μl) on the micropatterned collagen-I substrate cultured for 7 days. The cultures were double-stained with Calcein-AM and DAPI for cells and nuclei. (a) Calcein-AM, (b) DAPI, (c) Phase, (d) Measurement of neurite outgrowth of differentiating PC12 cells on the patterned collagen-I substrate over 7 days. Values represent mean $\pm$ standard error of mean.

Fig. 6.
Patterned fibroblast cells in-vitro for 3 days on patterned collagen-I substrate.

Fig. 7. Amphibian retinal neurons cultured on patterned Sal-1 substrate. (a) Neurons attached to Sal-1 substrate patterned on a glass coverslip in-vitro for 24 hours. Sal-1 substrate labeled in green was merged with a phase contrast image of cells. (b) Rod and (c) cone photoreceptors cultured for 7 days on patterned Sal-1 substrate. Thick neurites hug the edges of the Sal-1 substrate.

Fig. 8. Patterning of amphibian retinal neurons using vacuum method. (a) Cells after injection of cell suspension into the microchannels. (b) Cell pattern after peeling off PDMS cast after 1 day of culture in the microchannels. (c) Patterned cells cultured for 7 days showing neuronal outgrowth. Please note that although the cells were patterned, the substrate in these experiments was not, allowing cells to grow neurites in multiple directions

Fig. 9. Patterning of mammalian fibroblast cells using vacuum method. (a) PDMS microchannel device used to pattern the cells. (b) Fibroblast cells filled microchannels from a single inlet. (c) Fibroblast cell pattern on tissue culture dish after peeling off PDMS cast and culturing for 1 day.

Fig. 10.

Live-Dead cell assay of fibroblast cells patterned using vacuum method. (a) Fibroblast cell pattern labeled immediately after peeling off PDMS cast. Live cells are labeled with Calcein AM (green) and dead cells are labeled with Ethidium homodimer (red). (b) Fibroblast cells patterned using vacuum after 5 hours in-vitro showing typical growth and spreading. (c, d) Patterned fibroblast cells labeled after 5h in culture with DAPI (blue) for nuclei and phalloidin-TRITC (red) for actin filaments.

$$\text{Flow Rate} = \frac{P1 - P2}{R} \quad P1 > P2$$

$$R = \frac{12\eta L}{wh^3}$$

Fig. 11.
Schematic of fluid flow inside the microfluidic channel