

Nanoporous membrane-sealed microfluidic devices for improved cell viability

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Abstract Cell-laden microfluidic devices have broad potential in various biomedical applications, including tissue engineering and drug discovery. However, multiple difficulties encountered while culturing cells within devices affecting cell viability, proliferation, and behavior has complicated their use. While active perfusion systems have been used to overcome the diffusive limitations associated with nutrient delivery into microchannels to support longer culture times, these systems can result in non-uniform oxygen and nutrient delivery and subject cells to shear stresses, which can affect cell behavior. Additionally, histological analysis of cell cultures within devices is generally laborious and yields inconsistent results due to difficulties in delivering labeling agents in microchannels. Herein, we describe a simple, cost-effective approach to preserve cell viability and simplify labeling within microfluidic networks without the need for active perfusion. Instead of bonding a microfluidic network to glass, PDMS, or other solid substrate, the network is bonded to a semi-permeable nanoporous membrane. The membrane-sealed devices allow free exchange of proteins, nutrients, buffers, and labeling reagents between the microfluidic channels and culture media in static culture plates under sterile conditions. The use of the semi-permeable membrane dramatically simplifies microniche cell culturing while avoiding many of the complications which arise from perfusion systems.

Keywords Microfluidics · Membrane · Static culture · Microniche · Cell culture

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1 Introduction

Microfluidic based cell culture devices allow for well controlled manipulation of the local environment within microchannels, and thereby potentially enables recapitulation of the complexity of cellular microniches (El-Ali et al. 2006). While a broad range of biomedical applications including drug discovery and tissue engineering could benefit from cell-laden devices, limited cell viability has narrowed its utility. Thus, the design of reliable and approachable methods to improve culture conditions within microdevices will be integral to its broadened use.

The limited viability of cells within microfluidic devices, especially in long term cultures, has been attributed to poor transport of nutrients and gases to cells, especially in long microchannels. Compared to standard culture techniques, microscale culture systems have increased surface area-to-volume ratios, which greatly decreases the average amount of media available per cell (Korin et al. 2009a, b). As these systems are generally sealed with polydimethylsiloxane (PDMS), glass, or other surface with limited or no permeability, gas delivery and removal is further compromised (Mehta et al. 2007). Perfusion-based systems have been used to overcome these diffusive limitations by allowing for constant replenishment of soluble factors. However, active perfusion typically requires complex pumps and increases the likelihood for media contamination and the introduction of destructive bubbles. Active perfusion also introduces shear stress with flow which can induce a wide range of phenotypic effects in shear sensitive cells (Walker et al. 2004, 2005). Several approaches have been developed to limit the amount of shear introduced to cells, including periodic ‘flow-stop’ perfusion (Korin et al. 2009a, b), microgrooved glass substrates (Park et al. 2005), and orthogonal networks (Tourovskaja et al. 2005). However, the need for specialized control equipment, more complicated

fabrication approaches, and supplies with these methods limits its practical value to most laboratories. The issue of cell viability becomes even more challenging within three-dimensional (3D) cultures where cells are grown not on the walls of a microchannel but within a suspending matrix. While 3D cultures provide increased physiological relevance, increased transport limitations often lead to cell necrosis (Ling et al. 2007). Perfusion within these cell-encapsulated scaffolds is difficult because flow can disrupt the integrity of the material and bubbles can completely destroy the architecture. While systems have been designed to overcome some of these drawbacks, most techniques fail in the long-term.

In addition to shear effects, the convective delivery of nutrients has other drawbacks. With traditional static culture, diffusion is the primary modality for transport. Thus, locally secreted factors are available in the microenvironment in a manner similar to *in vivo* conditions. However, in both two-dimensional and three-dimensional perfusion based systems, convective flow removes these secreted factors which may be of importance to cell function and signaling. For example, Korin et al. noted significant differences in cell proliferation of human foreskin fibroblasts when comparing traditional culture techniques with perfusion and pulsed flow methods (Korin et al. 2009a, b).

Aside from issues with cell viability, a significant hurdle to overcome in cell-laden microfluidic devices is developing methods for efficient analysis. Sealed devices often limit the practicality of real-time analysis and complicate labeling of cells within the devices. While devices are attached to perfusion systems, the ability to image within the experimental duration is sacrificed or at best compromised. Further, histological stains can only reach cells within devices using the same perfusion modality, with associated resource limitations and burdensome time requirements, and can often yield inconsistent results.

In the present study, we have developed a simple technique to preserve cell viability and simplify labeling within microfluidic networks. Instead of bonding a PDMS microchannel network to an impermeable substrate, the network is bonded to a semi-permeable nanoporous membrane, which allows for free exchange of gases, proteins, nutrients, buffers, and labeling reagents between the microfluidic channels and culture media in static culture plates. While this system is amenable to perfusion for desired experimental needs (i.e. pulsed delivery of soluble factors), the porous membrane removes its constant requirement. We have validated the device for 2D and 3D cell culture under static and perfused conditions. We also demonstrate the ability to stain these cell-laden devices without the need for laborious perfusion. Finally, we show the ability to maintain pattern fidelity for 3D cultures within membrane-sealed devices. Collectively, the technique promises to introduce significant time and cost savings

without compromising the hallmarks of microculture systems.

2 Methods

2.1 Device fabrication

Microfluidic networks were formed using standard soft lithography techniques (Whitesides et al. 2001). Briefly, a PDMS solution was poured over a silicon wafer with the SU-8 relief of the network, and left to polymerize at 70°C overnight. The network comprised a single, straight channel 6 mm long, 500 µm wide and 100 µm deep. Inlet and outlet holes were punched through the PDMS with a 19-gauge blunt syringe. The microchannel-side of the devices was inked with a thin layer of a cell-tolerated silicone sealant (Dow Corning). The silicone coated devices were immediately sealed to either a polycarbonate membrane with a 0.4 µm pore size (Whatman, Piscataway, NJ) or a clean glass microslide. We note that the membrane was bonded to the PDMS with a silicone sealant, but any approach that reliably bonds the membrane to the substrate is feasible. In static culture and at low perfusion rates, the stress on the membrane-substrate interface is minimal, but in cases where perfusion pressures are elevated, an approach where an irreversible bond is formed may be warranted (Aran et al. 2010). Devices were allowed to cure for 24 h, after which Tygon tubing with a Luer hub adapter was inserted into the inlet holes. Open devices, placed channel-side up and which were not sealed with membrane or glass, were used as controls. Immediately before inoculation, devices were sterilized using ultraviolet light for 15 min followed by O₂ plasma treatment.

2.2 Cell culture

Primary rat dermal fibroblasts (RDFs) were isolated from transgenic rats engineered to express green fluorescent protein (GFP) via an actin promoter. When metabolically active, the cells emit fluorescence at 509 nm when excited at 395 nm, and lose their fluorescence rapidly upon dying. Cultures were maintained in a 37°C humidified environment with 5% CO₂. Media consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (L-glut), and 1% Penicillin/Streptomycin (P/S). Cells were harvested using 1× trypsin/EDTA.

2.3 Device inoculation

In separate Petri dishes, glass and membrane-sealed devices were positioned microchannel-side up on a PDMS block, which provided clearance for inlet tubing. For two-

dimensional cultures, RDFs were resuspended at 5×10^5 cells/ml in culture media and transferred to a 3 ml syringe. The suspension was flowed into the device and cells were allowed to attach for 1 h. Following this initial attachment time, inlet tubing was removed and devices were covered with culture medium. In separate Petri dishes, open device controls were filled with the RDF suspension using a micropipette. Devices were incubated for 1 h to allow for cell attachment and then covered with culture medium.

For three-dimensional cultures, RDFs were encapsulated within a type I collagen hydrogel using previously described methods (Shreiber et al. 2001). Briefly, lyophilized collagen was reconstituted to 3 mg/ml in 0.02 N acetic acid. The reconstituted solution was neutralized with the following materials: 20 μ l 1 M HEPES (Fluka), 140 μ l 0.1 N NaOH, 100 μ l 10 \times Minimum Essential Medium (Sigma), 1 μ l P/S, 10 μ l L-glut, 677 μ l type I collagen (Elastin Products), and 52 μ l of RDFs resuspended to 1×10^6 cells/ml in M199. This solution was transferred to a 3 ml syringe and flowed into the microchannel. Open device controls were filled using a micropipette tip. All three device configurations were incubated at 37°C for 1 h to allow for self assembly of the collagen network. Following this initial incubation, tubing was removed and devices were transferred into individual Petri dishes and covered with culture medium.

2.4 Perfusion

Inlet tubing used for perfusion studies was connected to three-way valves. Equivalent inoculation methods were used as described above. Following cell attachment and fibrillogenesis under static culture conditions, inlet valves were connected to 1 ml syringes driven by a syringe pump (Harvard Apparatus). Both 2D and 3D cultures were perfused at 0.05 μ l/min, a suitable flow rate based off of published results (Kim et al. 2006). Both 2D and 3D device configurations were submerged in excess media for the duration of perfusion.

2.5 Cell viability

Microchannels were inspected at various time points with epifluorescent microscopy using an Olympus IX81 inverted microscope. Images were acquired using a 10 \times objective and analyzed using ImageJ (NIH). Devices were visualized one hour post-seeding to ensure uniformity of cell number

and distribution. At 12 and 24 h, the number of GFP-expressing cells in 2D and 3D configurations was quantified for static conditions. For perfusion studies, cell viability was assessed quantitatively 12 h after initiation of flow. Long term cultures were monitored for up to 7 days and qualitatively assessed for cell proliferation and survival.

2.6 Cell labeling

2-D cultures were fixed by replacing the culture media with 4% paraformaldehyde for 1 h at room temperature followed by several rinses with an immunobuffer solution consisting of phosphate buffered saline (PBS) supplemented with 0.5% Triton-X and 1% bovine serum albumin (BSA). Devices were then prepared for either histological or immunohistochemical labeling of cytoskeletal elements. For histological staining, cells were incubated with 1 μ M TRITC-conjugated phalloidin and counterstained with 300 nM DAPI for 1 h at room temperature. For immunohistochemical labeling, devices were blocked with goat serum, incubated with mouse anti-tubulin (1:100) overnight at 4°C, fluorescently tagged using goat-anti mouse AlexaFluor 568 conjugated secondary antibody, and also counterstained with DAPI.

2.7 Maintenance of predicted flow

A Y-shaped device, intended to produce two parallel stripes of solution, was designed with two inlet channels, each 500 μ m in width, using standard soft lithography techniques. The device was sealed using a semi-permeable membrane as described above. One inlet was connected to a FITC-tagged collagen solution and the other to a native collagen solution; solutions were flowed into the device manually. Immediately following filling, devices were carefully moved into the incubator to allow for fibrillogenesis. Devices were then imaged using bright field and epifluorescent microscopy to assess stripe formation.

3 Results and discussion

3.1 Cell viability in static culture

Three device configurations, as shown in Fig. 1, were assessed for their effects on cell viability in both 2D and 3D

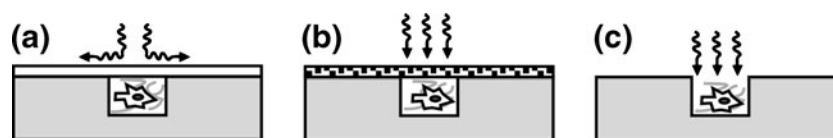


Fig. 1 Schematic of approach to improve cell viability by sealing channels with a semi-permeable membrane. (a) Culture sealed with a glass microslide or coverslip. Medium enters the culture through inlet

and outlet ports (not shown in schematic). (b) Sealing the culture with a semi-permeable membrane allows transport into the culture similar to an open channel (c)

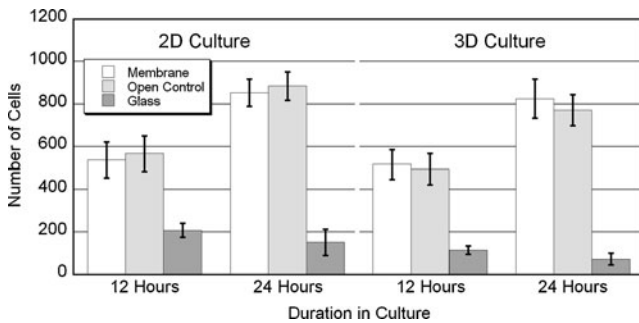


Fig. 2 Cell viability in static cultures (average \pm standard error of the mean). Equal numbers of fibroblasts were seeded in glass-sealed, membrane-sealed, and open microfluidic channels. At 12 h and 24 h, cell number was significantly greater in the membrane-sealed and open networks than those sealed with glass. Cell number significantly increased from 12 to 24 h in membrane- and glass sealed networks, but decreased in glass-sealed ones

static cultures. An equal number of primary fibroblasts were seeded in glass-sealed, membrane-sealed, and open microchannels. Cell viability in the three device configurations was quantified at 12 and 24 h. Results show that viability was compromised significantly in traditionally sealed microfluidic channels exposed statically to culture medium only through inlet and outlet holes. However, viability when sealed with a semi-permeable membrane was statistically equivalent to fully open cultures (Fig. 2). Consistent with cell proliferation, there was a significant increase in total cell number in the membrane-bonded devices (ANOVA, followed by post hoc comparisons with Tukey's test, $P=0.007$) from 12 to 24 h. The number of cells in these devices was significantly greater than the

number of cells in the glass-bonded devices at both 12 and 24 h ($P=0.003$ and $P<0.001$, respectively). Cell number in the membrane-bonded devices was not significantly different from that in the open devices at 12 and 24 h ($P=0.867$ and $P=0.821$, respectively).

RDFs demonstrated typical fibroblast morphology and spreading kinetics in the 2D (Fig. 3(a–c)) and 3D (Fig. 3(d–f)) microculture experiments. Interestingly, at 24 h cells were markedly aligned in the 3D cultures, but not the 2D cultures, which may reflect the alignment of the collagen fibers induced by the channel geometry (Lee et al. 2006). In longer term cultures, cells in membrane-sealed devices continued to proliferate and reached confluence by 7 days (Fig. 4). By 3 days, however, no cells remained in the glass-bonded devices.

Different cells are variably sensitive to culture conditions, including nutrient and oxygen availability. Even in perfused systems, cell viability is often dependent on distance from the source of media replenishment. For example, Ling et al. noted a significant decrease in cell viability as the distance from the perfusion channel increased within their cell-embedded agarose channels at 3 days post-seeding (2007). Additionally, oxygen gradients can form in the direction of flow as a result of consumption by cells, and this phenomenon can be particularly strong in the case of cells with high oxygen requirements, like hepatocytes (Korin et al. 2009a, b; Mehta et al. 2007). In membrane-sealed devices, the pore size of the membrane was sufficiently small to preclude escape of cells and ECM components but large enough to allow the transport of crucial proteins, nutrients, and gases into the channel along

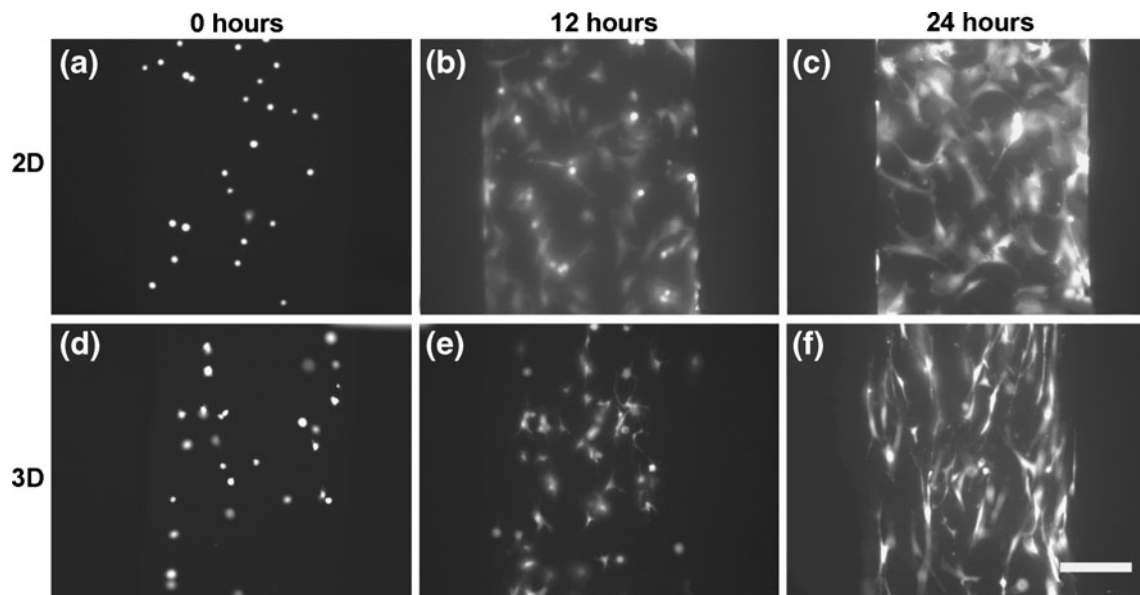


Fig. 3 Cell morphology in representative 2D (a–c) and 3D (d–f) microscale cultures in membrane-sealed networks. Initially rounded cells (a, d) began spreading by 12 h (b, e) and continued to spread and

multiply by 24 h (c, f). Cells in the 3D culture displayed noticeable alignment at 24 h (f). Scale bar=200 μ m

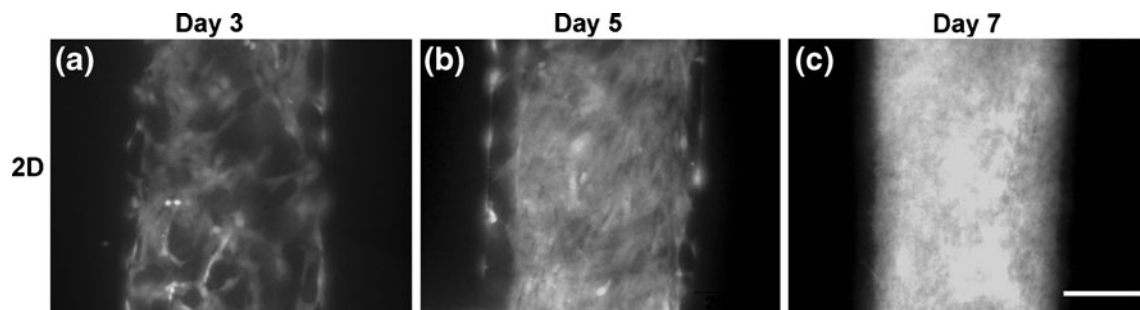


Fig. 4 Cell morphology in representative 2D microscale long-term cultures in membrane sealed networks. Fibroblasts continue to proliferate and spread from 3 days (G) to 5 days (H) and reach full confluence by 7 days in culture (I). Scale bar=200 μm

its entire length. Additionally, the growth to confluence of the cultures suggests no protein fouling of the membrane.

3.2 Cell viability in perfused culture

Glass and membrane sealed devices were subjected to pump-driven perfusion for both 2D and 3D microcultures to assess whether media replenishment could recover viability. Following an initial attachment period, cells were perfused at 0.05 $\mu\text{l}/\text{min}$ for 12 h and cell viability was quantified. As shown in Fig. 5(a), even with perfusion, membrane sealed devices retained viability better than glass sealed devices in both 2D ($P=0.0006$) and 3D cultures ($P=0.015$).

In glass-sealed 2D cultures, cell viability was highest at the inlet, decreased throughout the length of the microchannel, and recovered as a function of the distance from the outlet (Fig. 5(b)), whereas the viability of fibroblasts was uniform in membrane-sealed devices. As mentioned above, oxygen gradients can form within devices which may account for these results. Further, while membrane

sealed devices outperformed glass-sealed devices, the morphology of fibroblasts subjected to perfusion in 3D was rounded compared to the hallmark cellular extensions seen in static culture (Data not shown). These qualitative findings highlight the impact of flow-based systems on cellular phenotype, an important consideration in experimental design.

Generally, perfusion based systems require rigorous optimization in order to identify appropriate flow rates to minimize shear while maximizing cell viability. Further, these flow rates are dependent on a number of variables specific to the experimental setup, including cell type, seeding density, and device design. In our studies, we utilized a flow rate based on previously published reports and show that this was adequate for cell survival in membrane-sealed devices. Thus, membrane-sealed devices can be easily modified to include perfusion without sacrificing viability as this is not the sole modality by which cells receive their nutrients and oxygen.

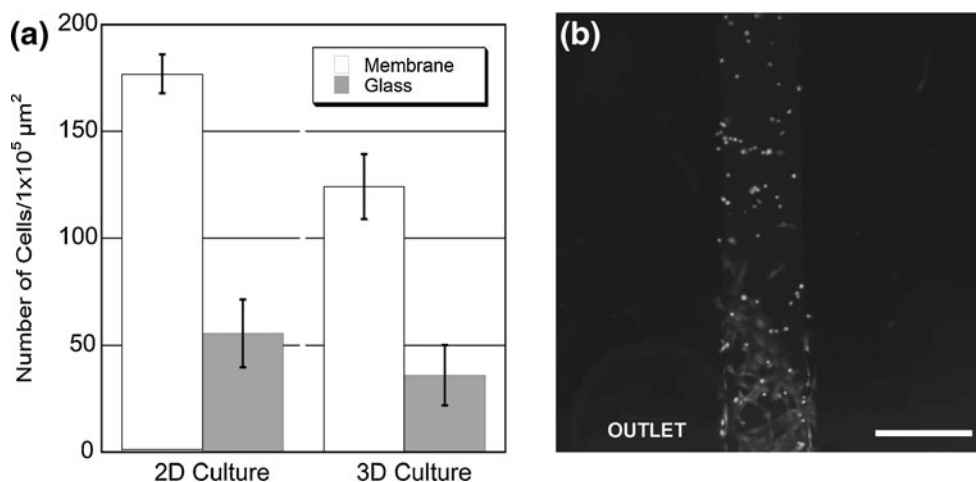
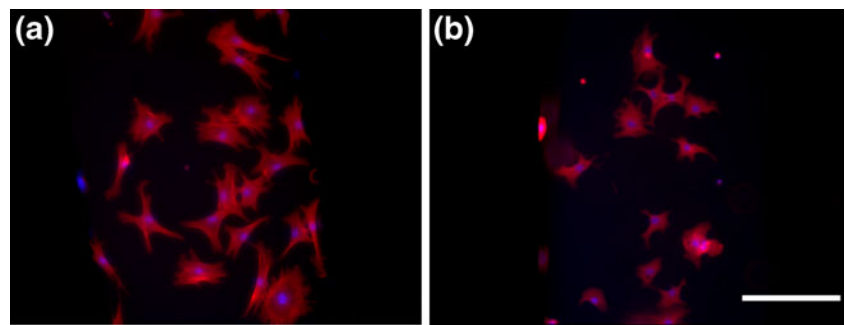


Fig. 5 Cell viability in membrane and glass-sealed perfused cultures. (a) Cell viability in 2D and 3D perfused cultures (average \pm standard error of the mean). Equal numbers of fibroblasts were seeded in glass-sealed and membrane-sealed microfluidic channels. After 12 h of pump-driven perfusion, cell number was significantly greater

in the membrane-sealed networks than those sealed with glass for both 2D and 3D cultures. (b) Cell morphology in representative 2D glass-sealed perfused microchannel. There is a noticeable decrease in cell number and a more rounded morphology as the distances from the outlet increases. Scale bar=500 μm

Fig. 6 Cell labeling within membrane-sealed networks. **(a)** DAPI-labeled nuclei and TRITC-phalloidin labeled F-actin at 24 h post seeding. **(b)** DAPI-labeled nuclei and immunohistologically labeled tubulin at 12 h post seeding. Scale bar=200 μm



3.3 Staining in devices

Devices that are sealed using an impermeable surface require perfusion for histological processing, which uses larger volumes of expensive labeling agents, and can often be a long, laborious process with inconsistent results. In addition to improving cell viability, the enhanced mass transport using membrane-sealed devices simplifies labeling of cells within the microculture. At discrete time points, separate membrane-bonded cultures were fixed and labeled with DAPI, a nuclear probe, and TRITC-conjugated phalloidin, a cytoskeletal probe. As shown in Fig. 6(a), the semi-permeable membrane allowed simplified and uniform fluorescent labeling in an hour.

While DAPI and phalloidin are useful histological markers, immunohistochemical analysis is often used to label for specific ligands. However, IgG antibodies generally have a molecular weight in the range of 1.5×10^5 Da, which is two orders of magnitude larger than TRITC labeled phalloidin. As shown in Fig. 6(b), we successfully stained fibroblasts with an antibody against tubulin, a cytoskeletal protein, followed by a fluorescently tagged secondary antibody. Additionally, there was no significant background due to the presence of the membrane. Thus, these larger molecules diffuse through the membrane without significant obstruction. From our own experience, perfusion techniques used for labeling within cell-laden microfluidic devices can take several days, where the membrane-sealed devices can be

stained in several hours. Further, the number of syringe pumps required creates a resource limitation on devices that can be histologically processed at one time. Conversely, multiple membrane-sealed devices can be treated as separate slides or submerged simultaneously in a bath containing the appropriate probes providing additional time savings with batch processing.

3.4 Maintenance of predicted flow pattern

Spatiotemporally regulated signals are important to a diverse range of physiological processes, including embryogenesis, wound healing, and nerve regeneration. However, it is difficult to mimic these cues using standard tissue culture technique. Microfluidic systems allow for precise control of the presentation of stimuli and may aid in our understanding and manipulation of various biological phenomena (Khademhosseini et al. 2006). While many microfluidic device designs have focused on the use of surface-immobilized or soluble gradients, tissue engineering and biomaterial applications could benefit from the use of 3D microcultures. One methodology used to generate spatially reproducible gradients is patterning scaffolds with immobilized biomolecules, a technique used successfully with collagen (Sundararaghavan et al. 2011) and agarose (Luo and Shoichet 2004).

A representative 3D configuration was used to ensure bonding of a semi-permeable membrane does not interfere with the ability to pattern within microchannels. A

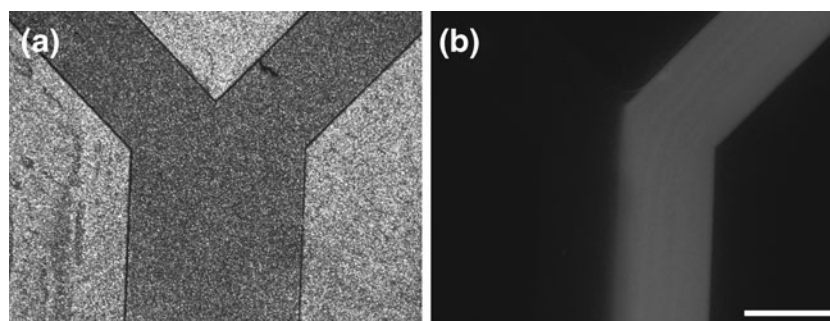


Fig. 7 Pattern fidelity in three-dimensional microcultures. A Y-shaped, stripe-generating microfluidic device was sealed with a nanoporous membrane **(a)**. Two solutions, native and fluorescently tagged type I

collagen, were pumped through individual inlets. The predicted flow was not significantly affected by sealing with the nanoporous membrane as shown by the boundary of fluorescence **(b)**. Scale bar=500 μm

“Y”-shaped, stripe generating device was used as a representative model (Takayama et al. 1999). By using fluorescently tagged type I collagen in one inlet and native collagen in the other, we were able to maintain predicted flow patterns and form a linear stripe with flow (Fig. 7). We speculate that this is expandable to other liquid-swelled polymers, including synthetic (PEG) and natural (alginate, chitosan) oligomers, as well as with 2D cultures exposed to laminar flow patterns.

4 Conclusion

Microfluidic systems offer great potential as customized microenvironments for cell culture for assays of genotypic and phenotypic behavior as well as regenerative medicine (El-Ali et al. 2006). However, extended culture in these systems has been difficult because of diffusive limitations for static culture and the complexity and unwanted, inconsistent, and perhaps destructive effects of active perfusion systems. Herein, we have presented a simple yet effective means of dramatically improving cell viability by sealing the microfluidic network with a semi-permeable membrane. The improved transport extends the period of time for cell culture, which broadly increases the potential for micro-scale assays of cell behavior as well as micro-tissue engineering. The simplicity allows for more cultures to be maintained at once. The approach also simplifies methods associated with evaluating cellular responses via soluble probes, and eases handling and use of disposable supplies, and is also cost-effective, as membranes can be purchased for less than 10 cents apiece. As such, the technique promises to introduce significant time and cost savings.

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