

Hollow fiber integrated microfluidic platforms for *in vitro* Co-culture of multiple cell types

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Abstract This study demonstrates a rapid prototyping approach for fabricating and integrating porous hollow fibers (HFs) into microfluidic device. Integration of HF can enhance mass transfer and recapitulate tubular shapes for tissue-engineered environments. We demonstrate the integration of single or multiple HFs, which can give the users the flexibility to control the total surface area for tissue development. We also present three microfluidic designs to enable different co-culture conditions such as the ability to co-culture multiple cell types simultaneously on a flat and tubular surface, or inside the lumen of multiple HFs. Additionally, we introduce a pressurized cell seeding process that can allow the cells to uniformly adhere on the inner surface of HFs without losing their viabilities. Co-cultures of lung epithelial cells and microvascular endothelial cells were demonstrated on the different platforms for at least five days. Overall, these platforms provide new opportunities for co-culturing of multiple cell types in a single device to reconstruct

native tissue micro-environment for biomedical and tissue engineering research.

Keywords Co-culture · Microfluidic device · Hollow fiber · Tissue engineering · Cell seeding

1 Introduction

Microfluidics has shown great promise for cell and tissue culture applications (Mehling and Tay 2014; Velve-Casquillas et al. 2010). These platforms can dramatically overcome limitations of cell cultures on 2D plates for tissue regeneration (Taylor et al. 2005; Ghaemmaghami et al. 2012) and drug discovery (Wu et al. 2010). Today's cell/tissue culture platforms require the integration of very specific spatial and temporal features to aid developments. For example, microvascular cells require shear stress, alveolar epithelial cells requires cyclic stress (Huh et al. 2010), skin tissue require air-liquid interface (El-Ghalbzouri et al. 2004), and cartilage/bone tissue require cyclic loading for *in vitro* regeneration of functional tissue (McCarron et al. 2012). By integrating flow and planar structures, microfluidic platforms can create many of these features to control cell behavior. Additionally, microfluidics offer rapid response time, low cell requirements, co-culture in one system, high-throughput processing, and easy to integrate flow controls. In recent years, the ability of microfluidics to recreate physiologically relevant structures has led to the development of a large collection of devices known as organs-on-a-chip (Huh et al. 2011). The more complex the requirement is to reconstitute an *in vitro* system, the more flexibility is required in the ability to fabricate complex microfluidic devices. Conventional microfluidic devices need to be more adept to facilitate the complexity required by an *in-vitro* model. Not only should they be compatible with routine laboratory tasks such as sterilization, coating applications, cell seeding, imaging

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etc., but also they should be able to sustain co-culture of cells, tissue generation, and long term culture.

In this work, we focus on the development of microfluidic platforms that can integrate commercially-available hollow fibers (HF). Typically, the cell/tissue generation scaffolds inside microfluidic systems for cell culture are provided by integrating flat and porous membranes (Huh et al. 2010). While conventional microfabrication processes facilitate the integration of flat membranes, such planar structures can limit the ability to provide critical spatial features required to mimic physiological traits. Therefore, the integration of HFs into microfluidic devices can introduce additional features such as higher surface-to-volume ratio for increased mass transfer and tubular environments to mimic the shape of several *in vivo* organs.

HFs are tubular membranes containing defined pores on the tube wall. These pores can allow mass transfer between the lumen of the tube and the outside environment. Because of their tubular shape and mass transfer characteristics, HFs have been widely utilized in commercial bioreactors for large scale production of biomolecules (Diban and Stamatialis 2014; Wung et al. 2014; Bettahalli et al. 2011), dialysis (Oo et al. 2011), and artificial organs (Mueller et al. 2011). Depending on the type of application, HFs can provide unique advantages over planar membranes. In addition to providing large surface-to-volume ratio (Bettahalli et al. 2011), HFs offer selective mass transfer between two different fluids (gas or liquid) passing through the lumen and the outside of the HF, which can provide critical functions for cell growth such as gas-exchange (Bettahalli et al. 2011). Furthermore, the tubular shape can recapitulate the form factors of specific tissue components such as vasculatures (Unger et al. 2005; Janke et al. 2013) and bronchi (Grubb et al. 1997; Matsui et al. 1998). Therefore, integration of HFs into modern microfluidic devices could lead to useful applications. However, there have been limited examples of the integration of HFs into a microfluidic device, possibly due to the complexity of integration.

In this work, we present a systematic approach to integrate HFs into microfluidic platforms for *in vitro* cell culture applications. First, we describe a rapid prototyping method based on laser-based microfabrication and lamination to integrate HFs. Second, we characterize the ability to seed cells on the inside of the HFs within the microfluidic device. Finally, we demonstrate the ability to perform co-culture of two different types of cells inside HF integrated microfluidic device.

2 Materials and methods

2.1 Design

Typical bioreactors that integrate HFs are composed of three primary components: (i) HFs; (ii) a chamber that houses the HFs, and (iii) two side compartments. The HFs are exposed to

the side compartments in such a way that fluids from these compartments are able to flow through the lumen of the HFs. Fluid in the housing side provides flow on the outer side of the HFs establishing a mass transfer path between the two fluids through the pores of the HFs. Miniaturization of HF bioreactors into a microfluidic platform can lead to channel dimensions that are in the same order of a single or a few HFs. We have designed miniaturized HF-integrated bioreactors that have different configurations to allow maximum flexibility of operation. The first design is composed of a single HF integrated into a planar chamber. In this design the side compartments of a typical HF bioreactor are eliminated since the single HF could be directly connected to a tube for flow (Fig. 1a). The second and third configurations were composed of multiple HFs to allow for a higher surface area. In the second design each of the HFs can be individually addressed by connecting them directly to separate feed tubes and does not have the side chambers (Fig. 1b). The third configuration is designed with side compartments such that multiple HFs are connected to a single fluid input simultaneously (Fig. 1c).

2.2 Fabrication

The key challenge of integrating HFs into microfluidic platforms is the requirement of placing tubular structures into planar systems. It is not trivial to develop tubular structures *in situ* using common the fabrication techniques available for microfluidics (Sia and Whitesides 2003). However, prefabricated HFs can be obtained and manually installed into microfluidic chambers. For fabrication, we used a rapid prototyping method based on laser-based micro-patterning and lamination techniques (Nath et al. 2010). In addition to rapid fabrication, the benefit of our chosen technique is the flexibility of the type of materials that can be used. Any materials that can be cut with a laser cutter are available for our fabrication method, which allows the use of multiple biocompatible materials to facilitate manual integration of the HFs.

Figure 2 shows the different layers and materials used to assemble the single HF integrated bioreactor. The top and the bottom chamber were made with 1/16" acrylic sheet (Plaskolite Optix, Columbus, Ohio). The middle layer was composed of the same acrylic sheet but with laminated adhesive transfer tapes (9122, 3 M Company) on both sides. The patterns for each layer were designed using Solid Edge 2D Drafting ST4 software (Siemens PLM Software). The resulting designs were cut using a CO₂ laser cutter (M-360, Universal Laser Systems). The components of the device were then cleaned by sonication in distilled water for 10 min and dried in a stream of compressed air to remove the burn residues generated by the laser cutting process.

Biocompatible mixed cellulose ester (ME) HFs (pore size 0.2 μm; lumen size 0.6 mm, Spectrum Labs, Rancho Dominguez, CA) were cut to the length of 1 cm. Both sides

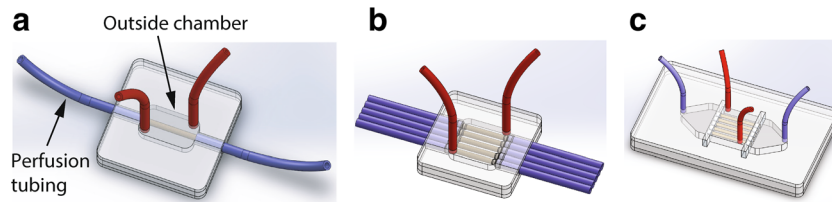


Fig. 1 a Schematic of single HF integrated microfluidic bioreactor where independent liquid stream can flow into the HF or the outside chamber. b Schematic of a multiple HF integrated bioreactor, where each HF can

have independent liquid stream c Schematic of a multiple HF integrated bioreactor where the all the HFs are permitted the same liquid stream

of the HFs were inserted in high-purity silicone rubber tubing (0.030 in. I.D., 0.065 in. O.D., 0.018 in. wall, McMaster-Carr). Medical grade silicone glue (A-4100 RTV, Factor II) was applied to seal the connection.

After preparing all of the components, they were manually assembled to obtain the final bioreactor. The middle layer was

first bonded to the bottom layer. The HF component was then placed into a slot that was patterned by the laser cutter on the middle layer. The top layer was then bonded to enclose the chamber to house the HF assembly. Medical grade silicone glue was used to seal the HF/silicon tube assembly from the sides. The top acrylic layer was designed to have access holes to establish inlet and outlet connections to the chamber that houses the HF. Silicone tubing was inserted into these access holes and glued to complete the fabrication process. A similar approach was utilized to fabricate the other two designs of the HF bioreactors that contain multiple HFs.

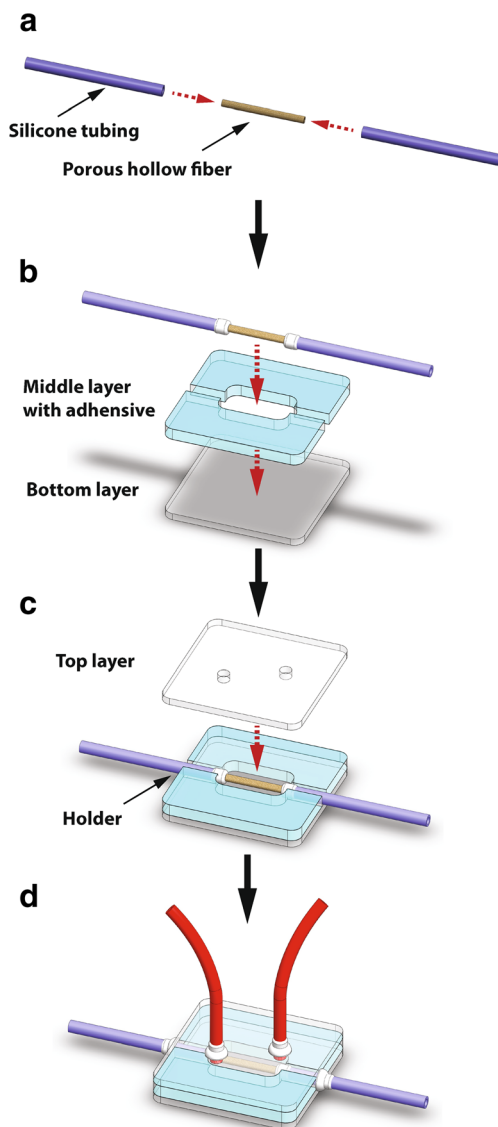


Fig. 2 Schematic showing the different steps to fabricate a single HF integrated microfluidic bioreactor

2.3 Cell seeding in HF bioreactors

Prior to the seeding process, HF embedded devices were sterilized using 70 % ethanol wash and overnight UV irradiation at 254 nm. The devices were then washed and rinsed with phosphate buffered saline (PBS, Life Technologies). After the washing process, cell culture medium was injected into the device to replace the remaining PBS. For the single HF bioreactor, human alveolar basal epithelial cells (A549, ATCC) were used in this study. A549 cells were first cultured in a 2-D cell culture plate with DMEM culture medium (Life Technologies) containing 10 % FBS (Life Technologies) and 100 U/mL of penicillin-streptomycin (Life Technologies). After 3–5 days, when the culture reached 60–80 % confluence, the cells were re-suspended in culture medium using trypsin-EDTA (0.25 %, Life Technologies). The cell stock was diluted with culture medium to 2×10^5 cells/mL. Diluted cell solution was then aspirated using 1 mL syringe (BD Biosciences). The inlet of the device (HF port) was connected to a syringe through a blunt needle, and the flow rate of the syringe was controlled with syringe pumps (SPL, World Precision Instruments). The cell seeding protocol was composed of two major steps. First, the HF was filled with cell seeding solution and the outlet tubing was closed using Luer lock plugs. Second, the cell seeding was initiated by starting the syringe pump at flow rate of 0.5 mL/min for 20 s. Under this condition, cells are seeded into the inside wall of the HFs as the solution is forced to pass through the HF walls. After the cells were seeded, the HF integrated device was placed at 37 °C in a 5 % CO₂ incubator for at least 16 h to ensure that the cells adhere to the HF.

The medium was continuously circulated into the system using the inlet tubing (chamber port) of the device which was connected to a medium reservoir (5 mL). To maintain the growth of cells, the flow rate in the outside chamber was adjusted to 10 $\mu\text{L}/\text{min}$. The cell culture medium was replaced every week.

2.4 Co-culture in HF bioreactors

Co-culture of different cell types into a single bioreactor has become an important technique in regenerative medicine (Kirkpatrick et al. 2011; Paschos et al. 2015). Co-culturing multiple cell types may require significantly different fluid management protocols including different seeding conditions and multiple media. We investigated the ability to perform co-culture of cells inside our HF-integrated microfluidic platforms using two configurations (Figs. 4a and 5a). For both the methods cells were co-cultured for 5 days. The devices were then sacrificed and live-dead staining was performed to measure the cell viability under the given co-culture configurations.

2.4.1 In single HF bioreactors

After sterilization of the single HF bioreactor, human lung microvascular endothelial cells (HLMVEC, Cell Applications) were seeded on the bottom surface of the outside chamber and human bronchial epithelial cells (BEAS-2B, ATCC) were seeded inside the HF. Before seeding, the seeding regions were coated with 30 $\mu\text{g}/\text{mL}$ of fibronectin (BD Biosciences) and 50 $\mu\text{g}/\text{mL}$ of collagen type I (rat tail, BD Biosciences) for the region growing the HLMVE and BEAS-2B, respectively. The coated bioreactor was incubated at 37 °C for 1 h and washed with PBS. The cell culture medium was injected into the device to replace the remaining PBS. On the chamber side, which was seeded with HLMVECs, the PBS was replaced with microvascular endothelial cell growth medium (Cell Applications) containing 100 U/mL of penicillin-streptomycin. For the luminal side of the HF, which was seeded with BEAS-2B cells, the medium was changed to bronchial epithelial cell growth medium (Lonza).

2.4.2 In multi-HF bioreactors

With the multi-HF integrated bioreactors, cells were seeded only in the luminal space of the HFs. For co-culture, the human lung microvascular endothelial cells (HLMVEC, Cell Applications) were seeded inside HF #2 and #4, where human bronchial epithelial cells (BEAS-2B, ATCC) were seeded inside HF #1, #3, and #5. To facilitate cell attachment, the HFs were coated with 30 $\mu\text{g}/\text{mL}$ of fibronectin (BD Biosciences) and 50 $\mu\text{g}/\text{mL}$ of collagen type I (rat tail, BD Biosciences) for the region growing HLMVE and BEAS-2B, respectively. The coated bioreactor was incubated at 37 °C for 1 h and washed with PBS. The cell culture medium was injected into the device to replace the remaining

PBS. The PBS was replaced with microvascular endothelial cell growth medium (Cell Applications) containing 100 U/mL of penicillin-streptomycin in HF #2 and #4 and the bronchial epithelial cell growth medium (Lonza) in HF #1, #3, and #5.

2.5 Image analysis

To observe the coverage and uniformity of seeded cells inside the HF, the HF was removed from the devices and cut longitudinally using surgical blades. The cells were then stained with NucBlue Live Cell Stain (Life Technologies) for 20 min. Distribution of the cells over the HF surface was observed and recorded using a fluorescent microscope. The cells were counted using ImageJ software (National Institutes of Health). Cell viability can be verified using a LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies) to ensure the success of the seeding process and the long term of cell culture. Cut HFs were incubated in diluted calcein AM (2 μM) and EthD-1 (4 μM) solution and incubated at 37 °C incubator for 30 min. The dyed HF was then observed using a fluorescent microscope (Z1 microscope, Zeiss). The shape of the cells and surface coverage on the HF was also observed with scanning electron microscopy (FEI, Inspect F SEM).

3 Results and discussions

3.1 Characteristics of the HF integrated microfluidic systems

The primary purpose of a HF integrated microfluidic platform is to create a two-fluid interface microenvironment for cell cultures. Our assembly process allowed for the simple fabrication of HF integrated microfluidic devices through the use of stacked planar components and commercially available HFs. Laser based microfabrication precisely develops the mini/ μ patterns in biocompatible polymeric substrates (e.g. acrylic, polycarbonate, and the adhesives). Figure 3 shows photographs of the HF integrated microfluidic platforms with three different configurations. Modulating the volume and location of the flow chamber allows the flow patterns to be precisely adjusted for a given application. The use of pressure-sensitive tapes to assemble the different layers eliminates the need for specific equipment for bonding. Unlike PDMS based platforms, this method does not require a mold, allowing rapid opportunity for design modification that is typically necessary at any early stage device development process. Since our process involved manual insertion of the HFs into the assembly, it was important to prevent leakage along the insertion points. It was simple to prevent such leakage for single HF or individually addressed multi-HF bioreactor the HF component by applying glue at the insertion points. However, for multi-HF systems with single fluid input, some

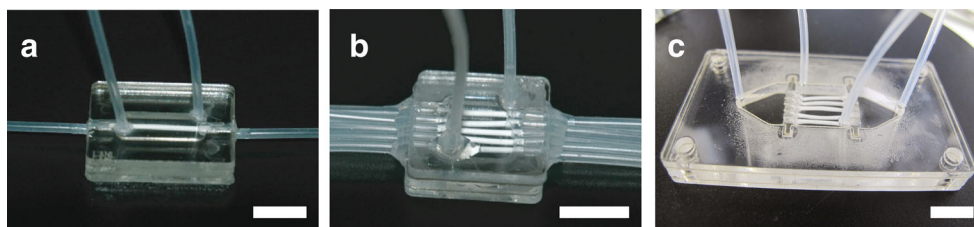


Fig. 3 Images of HF-integrated microfluidic bioreactors with different configurations (a) Single HF integrated device. (b) Individually addressable multi-HF device. (c) Multi-HF with single flow input. Scale bar = 1 cm

leakage occurred between top and middle layers, near the holder that was used to place multiple HF. It was not possible to access the holder to apply glue to stop the leakage. Therefore, by adding layers of laser patterned silicone membrane onto the top and the bottom plates, it was possible to prevent the leakage (Fig. S1).

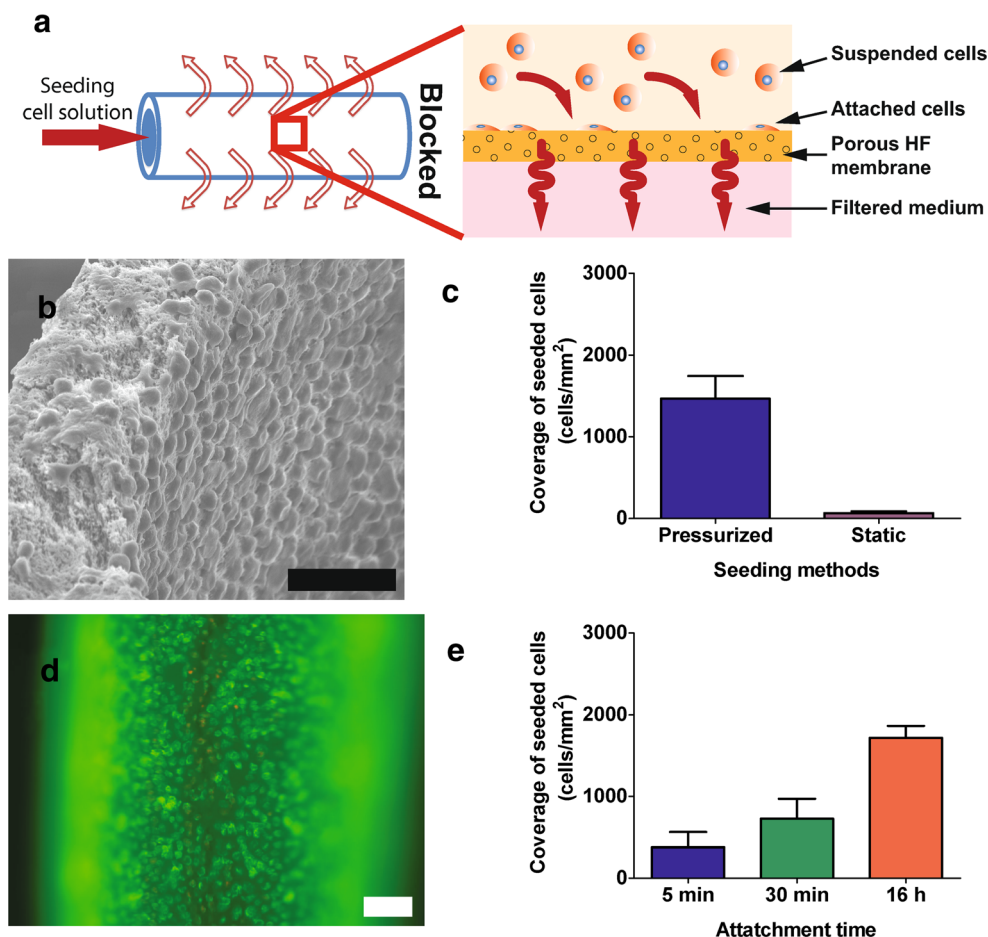
Individual fluid flow systems can be established through the different parts of the devices (e.g. inside the HFs and in the outside chambers) by connecting them to any commercial circulation and pumping systems to automatically control the flows for long-term culture. The platforms are suitable for sterilization with alcohol, washing, cell seeding, and long-term incubation. Furthermore, a device with multiple

channels connecting independent HFs allowed simultaneous testing of various co-culture conditions. Each HF can be connected to a different flow system to regulate the individual microenvironment relative to neighboring conditions. The flow rates inside and outside the HFs can also be easily tuned with a multi-channel pump using varying flow rates.

3.2 Cell seeding

Cell seeding is an important step in any cell culture experiment involving adherent cells. The surface coverage of the seeded cells and their viability can fluctuate with variations in the seeding process. The static seeding process is the

Fig. 4 Cell culture in HFs: **a** Schematic showing the cell seeding method using a pressurized flow. **b** SEM images showing the coverage of cells on the lumen of HF after seeding. Scale bar = 50 μm . **c** Graphs showing a comparison seeding techniques with and without (static) pressurized injection. **d** Microscope image (100X) showing live/dead (green/red) staining image of A549 cells after seeding in the lumen of HF. **e** Graphs showing the effect of surface coverage of seeded cells corresponding to different lengths of attachments times after seeding



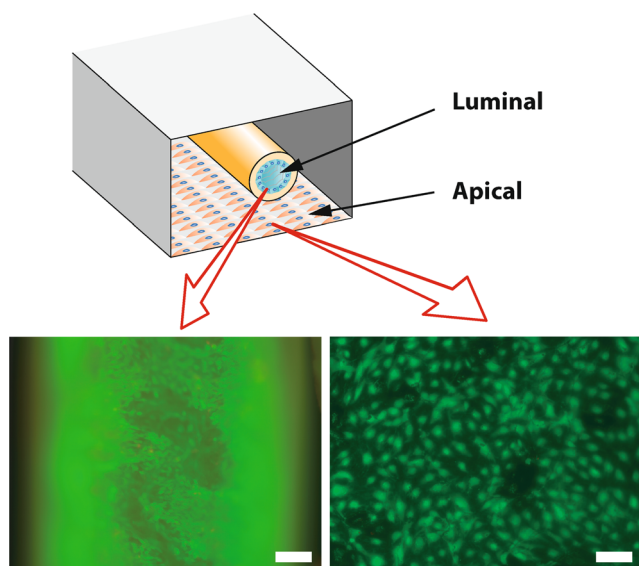


Fig. 5 Co-culture of cells in a single HF integrated bioreactor: BEAS-2B cells were cultured inside the lumen of HF, while HLMVE cells were cultured. Microscope images were acquired at 100X magnification. Scale bar =100 μ m

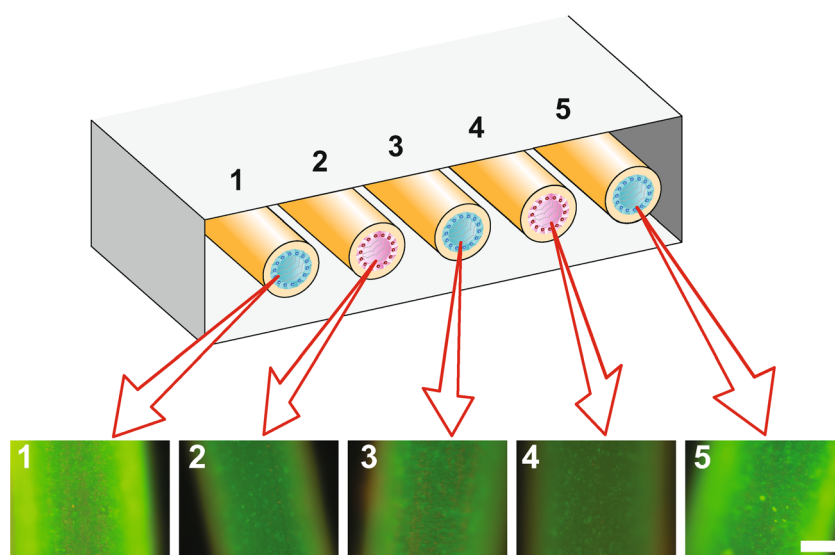
simplest and most widely used in adherent cell culture, where the technique relies on gravity and time to allow the cells to adhere to the surface. With 3D structures like the HF, application of static seeding cannot ensure proper surface coverage throughout the entire luminal surface of a fiber. Since the HF has walls that are permeable to fluid, we elected to use a dynamic cell seeding process to ensure higher surface coverage and reproducibility. By using a pressurized flow of the seeding solution through the luminal part of the HFs (Fig. 4a), it was possible to force the liquid pass through the membranes while leaving cells deposited onto the luminal surface. Using this technique we found that it was possible

to get cells deposited across the entire surface of the HF. Figure 4b shows an SEM image of the inside of the HF after the cells were deposited into the luminal surface using the pressurized method. When we compared the pressurized deposition method to the static method, we found that the pressurized method resulted in 22 times more cell deposition on the HF walls (Fig. 4c). However, the pressurized process can impart stress on the cells during the deposition process, which may lead to cell death due to high shear. The ability of the cells to withstand the shear depends on the cell types. In our case, we demonstrated the deposition using A549 cells, which did not show significant cell death (quantified by the live/dead staining, Fig. 4d) after the deposition process. Nevertheless, the deposition did not lead to immediate cell adherence onto the surface. Figure 4e shows the coverage of seeded cells that adheres to the surface over time. It took up to 16 h for the cells to adhere to the surface.

3.3 Co-culture

Different cells have different growth rates and may require different media for growth. Therefore, it is important for a platform to accommodate the ability to manage multiple fluids independently. The designs of the HF-integrated platforms were suitable to flow different fluids in the luminal and apical sides independently. For the single HF integrated system, we demonstrate that it was possible to seed and grow BEAS-2B cells inside the HFs, while growing HLMVE cells at the bottom surface of the HF housing chamber (Fig. 5a). Both types of cells require different media for optimal growth. Figure 5b and c show the live/dead staining of the two regions after five days of co-culture. The co-culture approach in this configuration is similar to co-cultures carried out in commercially available Transwell plates (Miki et al. 2012), which have been used

Fig. 6 Co-culture of cells in a multiple HF integrated bioreactor. In this bioreactor the luminal side each HF was individually cultured with BEAS-2B and HLMVE cells. BEAS-2B cells were cultured inside HF # 1, 3 and 5, while HLMVE cells were cultured inside HF # 2 & 4. Microscope images were acquired at 100X magnification. Scale bar =100 μ m



for tumor microenvironment applications. However, in this case, the HF offers 3D scaffolds for the cells to grow. Unlike Transwells, which is a static culture system, our platform offers the ability to integrate flow and thereby, enabling long term cell culture. Additional flexibility of the integrated system is the ability to create flow conditions required by the overall co-culture process. In this case, we were able to use a combination of flow based (luminal side) and static (chamber side) cell seeding method, to seed the two types of cells. After the cells adhere to their respective surfaces, it was possible to switch to flow conditions (e.g. 10 $\mu\text{L}/\text{min}$ on the luminal side and 10 $\mu\text{L}/\text{min}$ on the chamber side).

Similarly, on the multiple HF system, different types of cells could be cultured simultaneously inside the different HFs. Figure 6 demonstrates a co-culture experiment that was performed on a multi-HF integrated microfluidic system. Here, both the cell types were seeded inside the HF using the dynamic seeding method. Since the cells are deposited on the luminal surface of the HFs, each HF can flow different media to aid the respective cell adherence and growth. After 5 days of culture, live/dead staining results showed both cell types maintained high viability (Fig. 6c). The ability to manipulate flow and the media type depending on the type of application with our HF integrated microfluidic platform will be important to generate diverse co-culture models critical for wide applications including drug development and regenerative medicine.

4 Conclusions

The ability to integrate single and multiple HFs into polymeric microfluidic devices using laser patterned plastic sheets/films and lamination have been demonstrated. Microfluidic devices integrated with HFs provide new challenges in terms of cell seeding, which was addressed by developing a cell seeding method based on pressurized flow. The pressurized cell seeding process showed dramatic enhancement of the cell attachment into the lumen of the HFs. Upon the optimization of the cell seeding technique, co-culture of different cell types was demonstrated. Two different formats of co-cultures were possible. In the first configuration one type of cells were cultured inside the lumen and the second type of cells were cultured on the bottom surface of the chamber that holds the HF. Using this configuration simple micro-physiological models can be established to study cell-cell interactions in a microfluidic platform. The second format of co-culture was demonstrated on a more complex HF-integrated system comprising of multiple HFs. Different cells were cultured inside different HFs. Such a system may be utilized to create more complex micro-physiological models that can integrate more than two cell types requiring different specialized media. This system may be applied to study complex interaction between

cells to closely mimic *in vitro* conditions. In this paper, our primary objective was to present the rapid method of integrating HFs into microfluidic platforms such that they can be adapted to practical applications. Since the rapid prototyping method relies on commercially available manufacturing techniques, the prototyping protocol is not limited to laboratory developments only. Rather, the demonstrated method can be readily adapted to commercial production schemes with little or no modifications.

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