Investigation of Microfluidic Hepatocyte and Hepatic Progenitor Cell Cultures In Vitro

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Abstract

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(Under the guidance of Glenn M. Walker, Ph.D)

The in vitro culture of liver cells has applications in extracorporeal bioreactors, generation of grafts for transplantation, and drug screening. It was postulated that hepatic cells could be cultured within a microfluidic environment. A series of microchannels of varied width was tested with hepatic progenitor cells (HPCs) and showed maintained viability and a measurable level of albumin. Then HepG2 hepatocarcinoma cells were tested in a “Spheroid Chamber” design to attempt to encase the cells and maintain cell viability. Another device consisting of a channel layer and a well layer was then tested, resulting in successful seeding of the device but without maintained cell viability. Then viability and albumin production were tested in a series of channels, showing albumin fluorescence and maintained viability. In conclusion, we were able to show that HPC and HepG2 viability and hepatocellular behavior can be maintained in a microfluidic environment.
Dedication

To my parents.
Acknowledgments

I would first like to thank my advisor, Dr. Glenn Walker, for his guidance and support. I would like to thank Dr. David Gerber and Dr. Lola Reid for their guidance. I would like to thank the other members of my lab, Adrian O’Neill and Jeffrey SooHoo, and the members of the Transplant Lab: Lisa Rice, Natasha Wright, Prakash Chandrasekaran, and Maggie Walkup. I would like to thank Eliane Wauthier in the Liver Stem Cell Lab. I would also like to thank the Immunohistotechnology Core, and the funding source. I would finally like to thank my family for their support.
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# List of Abbreviations and Symbols

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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Antimycotic-Antifungal</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ECV</td>
<td>Effective Culture Volume</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>EthD-1</td>
<td>Ethidium homodimer-1</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-Transferrin-Selenium</td>
</tr>
<tr>
<td>HPCs</td>
<td>Hepatic Progenitor Cells</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethyl siloxane)</td>
</tr>
<tr>
<td>SAV</td>
<td>Surface Area to Volume</td>
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**Greek Symbols**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
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1 Introduction

1.1 Rationale and Goals for the Project

Current high-throughput hepatic culture systems used for screening drug candidates lack the complexity of the *in vivo* tissue environment, which limits their ability to accurately predict candidate efficacy. Recently, advances in the field of microscale tissue engineering have demonstrated the potential for culturing tissue for high-throughput assays in academic, pharmaceutical, and biotechnological applications. Hepatic tissue regeneration in vitro could address the need for improved drug candidate screening and provide a potential source of implantable liver tissue. Replacing the current two-dimensional hepatic culture systems with microscale three-dimensional systems could increase the precision of therapeutic readouts and enable early drug candidate validation at the tissue level in a cost-efficient setting.

We envision our microfluidic devices eventually being used to screen for the efficacy of drug candidates on arrays of microscale hepatic tissue cultures. In this project hepatic progenitor cells (HPCs) and hepatocarcinoma HepG2 cells were cultured in both microchannels and microwells to observe the effect of these cell culture environments on the cells. The primary goal was to develop a device that is optimized for hepatocyte cell culture.
The hypothesis tested is that hepatic progenitor cells and HepG2 cells can remain viable and maintain hepatocellular function in microfluidic devices.

The microfluidic devices provided useful information on HPC and HepG2 viability, differentiation, maturation, and proliferation in the microenvironment. The microfluidic devices developed allowed us to investigate how hepatic progenitor cells and HepG2 cells behave when cultured in a microenvironment that more faithfully mimics in vivo conditions than current high-throughput culture systems.
2 Background

2.1 Liver Culture Approaches

Liver cell culture in vitro has many potential applications. These include extracorporeal liver support, engineering of grafts for transplantation, drug screening in vitro, and research on hepatocellular behavior. A variety of liver cell culture methods have been developed to promote retention of hepatic function under in vitro conditions.[1] Various microscale cell culture bioreactor designs exist in addition to macroscale cell culture bioreactor designs.

2.1.1 Cell Culture on Scaffolds

Although the classical static method for liver cell culture consists of the uncoated plastic tissue culture dish, synthetic and hybrid biodegradable scaffoldings are in development for the purpose of in vitro maintenance and expansion of cells as well as in vivo transplantation of the cells. Natural degradable materials, e.g. collagen and laminin, have also been used in tissue engineering to enhance cell attachment, with the advantage of close compatibility with the extracellular matrix (ECM) environment found in the liver. However they often lack mechanical integrity, may be immunogenic, and are often limited in supply. Therefore various biodegradable scaffoldings are in development, and these are used to coat surfaces including polystyrene, polyurethane, and cross-linked dextran. The biodegradable scaffoldings most used are made from the poly (\(\alpha\)-hydroxy acid) family of polymers,
including poly(glycolic acid), poly(lactic acid), poly(D,L-lactide-co-glycolide) copolymer, and their modified derivatives. Hybrid polymers are also in development and these are intended to solve the issues of the interaction between seeded cells and degradable scaffolds and the host response to the tissue engineered construct. These hybrid devices generally are designed to combine the biocompatibility of natural biopolymers with the desirable physical properties of synthetic polymers.[2]

2.1.2 In Vitro Three-Dimensional Bioreactors

The types of three-dimensional culture systems in development include methods for spheroid culture, cell culture on biodegradable microcarriers kept in suspension, cell encapsulation, and hollow-fiber bioreactors.

For the culture of spheroids, hepatocytes have been cultured primarily either on tissue culture dishes and placed on a rotary shaker at a constant speed or cultured on a nonadherent substratum in static culture. Spinner flasks or Erlenmeyer flasks have also been used when large-scale spheroid formation was necessary. In addition, novel methods including the combination of the static and shaking methods, such as the use of poly(2-hydroxyethylmethacrylate (PHEMA)-coated six well plates on a rotary shaker, have been described. This method, when done at 90 rpm, is advantageous as it generates spheroids of less than 250 µm diameter, thus avoiding the decreased oxygen and nutrient diffusion that occurs with necrotic centers that are generated with spheroids greater than 250-300 µm in diameter.

The method of culturing cells on biodegradable microcarriers kept in suspension is another method that has been developed for the three-dimensional
culture of liver cells. The use of porous PLGA beads as biodegradable microcarriers has been demonstrated. With this method, the beads are commonly first coated with collagen prior to addition of the cells for cell attachment.[2]

A method has also been described for the encapsulation of hepatic progenitor cells in alginate beads. This method sought to use electrostatic encapsulation to generate an ex vivo environment for murine hepatic progenitor cells (HPCs). In vitro analysis of the encapsulated beads demonstrated extended periods of viability and function based on albumin production, urea metabolism, and glycogen storage. This study demonstrated that HPC encapsulation fosters the differentiation of HPCs into functional cells while maintaining their viability in long-term culture.[3]

Hollow fiber bioreactors are another commonly used type of bioreactor for liver cell culture. Hollow fiber bioreactors are perfusion vessels comprising semi-permeable fibers and composed of various polymers. Hollow fiber bioreactors have been used in numerous bioartificial liver studies.[4-12]

2.1.3 Microfluidic Bioreactors

Miniaturization of bioreactors to the microscale offers many advantages. These include high throughput, cost effectiveness, portability, and lowered cell numbers and reagent volumes. In addition, the culture of cells at the microscale introduces the cells to a set of physical phenomena (discussed in Section 2.2.1) that may allow for a more in vivo-like environment. Following are examples of existing microfluidic bioreactors for liver cell culture.

An in vitro model of liver zonation has been developed. Allen et al.[13,14]
developed a polycarbonate-on-glass-slide parallel-plate perfused hepatocyte bioreactor that imposes physiologic oxygen gradients for an in vitro model of zonation and tested the bioreactor with a co-culture of primary rat hepatocytes and non-parenchymal cells. The location of cells in the liver determines their response to a toxic insult. Specifically, hepatocytes are distributed along the sinusoid in subpopulations into zones 1 to 3. As a result a toxic insult generates different responses in different zones. An in vitro hepatocyte bioreactor that represents liver zonation may provide a more accurate gauge of physiological hepatocytic responses to toxic insult by allowing for a measure of zonal hepatotoxicity. To control the oxygen gradients used to model liver zonation, Allen et al. used mathematical modeling and experimental validation. Experimental validation was observed in regional heterogeneity mimicking the distribution in the zonated liver, of CYP2B and CYP3A in co-cultures exposed to the oxygen gradients. Then, zonal hepatotoxicity was confirmed by perfusion of the co-cultures with acetaminophen (APAP) for 24 hours, which resulted in maximal cell death in the low oxygen outlet region similar to centrilobular necrotic patterns observed in vivo. The device could allow for the study of other zonation-dependent hepatocellular behavior.

Leclerc et al. have developed a multi-layer microfluidic poly-dimethylsiloxane (PDMS) bioreactor for the large-scale culture of hepatocytes, with larger cell numbers and cell densities. Large-scale hepatocyte culture might allow for more physiological hepatic function in vitro. The device consists of ten stacked PDMS layers comprising four cell culture chambers and an oxygen chamber in the middle of the ten stacked layers. To show that cellular activity was maintained in the
bioreactor, glucose consumption and albumin production were measured, and showed a gradual increase and saturation throughout the culture. The oxygen chamber was found to generate longer and healthier cultures. The cell density used in the bioreactor was $3-4 \times 10^7$ cells/cm$^3$, on the same order as typical macroscale bioreactors. Leclerc et al. conclude that the device could be a scalable method to realize the microfluidic bioreactor for large-scale cultures. Leclerc et al. have also constructed biodegradable tissue engineering scaffolds, with a photosensitive biodegradable polymer.[16,17]

Another feature added to microfluidic hepatocyte bioreactors is a membrane for cell attachment.[18] Two types of PDMS bioreactors were fabricated, one with a commercially available polyester membrane and one with a PDMS membrane with 5 µm x 5 µm hole sizes. The membrane was used to mimic the relative surface area between blood perfusion and hepatocytes seen in vivo. The microbioreactors with the PDMS membranes were shown to express greater albumin secretion than the polyester membrane as well as static cultures in tissue-culture-treated dishes and in cultures in inserts with the same polyester membranes. Ammonia removal was also shown to be seven times greater in perfused cultures compared to static cultures.

Because co-cultivation of parenchymal and mesenchymal cells has been widely utilized as a paradigm for the study of cell-cell interactions in vitro and because co-cultures of two cell types provide more functional tissue constructs for use in therapeutic or investigational applications, Bhatia et al. have developed bioreactors in which co-cultures of primary rat hepatocytes and murine 3T3-J2 fibroblasts were cultured in order to investigate the role of increasing fibroblast
density on hepatic function.[19] Randomly distributed co-cultures were performed, and microfabrication was also used to pattern the cell populations by patterning collagen onto the substrate, allowing for the isolation of fibroblast number as an independent variable in hepatic function. Collagen-modified borosilicate wafers were used for all the co-cultures. To hold constant the homotypic interactions, the hepatocyte patterns used were similar in all conditions, and the region of contact between the cell populations was also held constant. Synthetic and metabolic markers of liver-specific function were measured, and the data suggested that fibroblast number is a factor in modulation of hepatocellular response through homotypic fibroblast interactions. Information on the interplay between the two cell types could allow for the formation of more functional model tissues in vitro or functional tissue for implantation.

A silicon microbioreactor has also been developed for hepatocyte cell culture.[1, 20] Deep reactive ion etching (DRIE) of silicon wafers was used to pattern an array of through-holes with cell-adhesive walls. The sidewalls of the through-holes were made cell-adhesive due to the fluoropolymer remaining on the sidewalls because of the SF$_6$ etch followed by passivation in C$_4$F$_8$ involved in the DRIE process. The silicon scaffolds were placed in a reactor housing with a filter and support for the silicon wafer with the housing designed to deliver a continuous perfusate across the top of the array and through the three-dimensional tissue mass in each channel. Reactor dimensions were constructed to allow for estimated values of cellular oxygen uptake rates and also to allow for fluid shear stress at or below a physiological range (<2 dyne/cm$^2$). To test the reactor primary rat hepatocytes were
seeded and cultured for up to 2 weeks, and it was found that cells seeded into the through-holes rearranged extensively to form tissue like structures and remained viable throughout the culture period. It was also observed that preaggregation of the cells into spheroidal structures prior to seeding improved the morphogenesis of tissue structure and viability. In situ imaging of tissue structure and function using two-photon microscopy was also demonstrated.

A microchannel flat-plate bioreactor has also been developed to study the effects of oxygenation and flow on the viability and function of rat hepatocytes and 3T3-J2 fibroblasts in co-culture.[21, 22] The bioreactor, made of polycarbonate sheets, was made with and without an internal membrane oxygenator, to study the effect of oxygenation on cell viability and function. In addition a range of channel heights and flow rates was used to study the effect of varied lower wall shear stresses on hepatocyte function as measured by albumin and urea synthesis rates. The oxygenated bioreactor was found to perform better than the one without the oxygenator, showing stable albumin and urea synthesis rates for 10 days of perfusion. In addition, lower wall shear stresses (0.01 to 0.33 dyne/cm$^2$) were found to generate 2.6- and 1.9-fold greater albumin and urea synthesis rates than higher wall shear stresses (5 to 21 dyne/cm$^2$).

An array of microfluidic wells has also been developed for the purpose of culturing primary mammalian hepatocytes.[23] Each well supported a micropatterned coculture of primary rat hepatocytes in coculture with 3T3-J2 fibroblasts. Each of the wells was continuously perfused with medium and oxygen via separate channels. The hepatocytes in the array were shown to be capable of
continuous, steady-state albumin synthesis.

Biodegradable polymers have also been used for creating microfluidic tissue engineering scaffolds.[24] Poly(glycerol sebacate) scaffolds were used to create two types of tissue engineering scaffolds, termed “vascular” and “hepatocyte” constructs. The first exhibited constant maximum shear stress within each channel of the device with the purpose of imitating vasculature. The second allowed for high perfusion rates while also allowing for high levels of cell attachment, by adding staggered posts intended to protect the cells from the shear forces associated with the high perfusion rates.

An artificial liver sinusoid with a microfluidic endothelial-like barrier has also been developed for primary hepatocyte culture to imitate the mass transport properties of the hepatic microcirculation.[25] The unit consisted of a cord of hepatocytes fed by diffusion of nutrients across an endothelial-like barrier by a convective transport vessel. Rat and human hepatocytes were sustained for several days with this configuration with no extracellular matrix (ECM) coating. The channels were designed to allow for a ratio of convective to diffusive flow in the flow channel and a ratio orders of magnitude lower in the cell culture channel, to allow for lower shear stresses while still allowing for nutrient exchange through diffusion across the endothelial-like barrier.

Hepatocyte spheroids have been cultured in wells on a microfabricated chip by Fukuda et al.[26] Another microbioreactor, developed by Kim et al., consists of a device in which a biocompatible self-assembling peptide hydrogel, Puramatrix, is hydrodynamically focused in the middle of main channel.[27] Toh et al. have
developed a microchannel in which a collagen solution containing hepatocytes is immobilized in a microchannel with perfusion. Hepatocytes have also been cultured on a microfabricated poly(ethylene glycol) (PEG) brush surface.

2.2 Microfluidics

2.2.1 Advantages

Microfluidic cell culture offers a number of advantages over conventional cell culture methods. These include higher throughput, increased surface-area-to-volume ratios, smaller effective culture volumes, and laminar flow.

Microfluidic cell culture allows for higher throughput. It allows for the use of lower cell numbers and lower reagent volumes. In the case of drug screening, for example, microfluidic cell cultures could allow for greater efficacy.

Another advantage of microfluidic cell culture is the higher surface area-to-volume ratios at the microscale. For example, whereas a 35 mm dish with 1 mL of medium in it has a SAV ratio of 11 cm⁻¹, cells cultured in a microchannel of dimension 100 μm x 100 μm x 3 cm (H x W x L), a volume of 300 nL, are in an environment with a SAV of 400 cm⁻¹. Larger SAV ratios allow for more efficient mass transport of gases by diffusion to and from cells, assuming the microchannel is constructed of a gas-permeable polymer. Figure 1 illustrates qualitatively the difference in SAV ratio between a dish and microchannels.
The concept of effective culture volume (ECV), introduced by Walker et al. (2004) [31], indicates the ability of the cell to control its microenvironment during culture. Factors affecting the ECV include the magnitude of mass transport along each axis and the effects of diffusion and convection. For a cell in a microchannel, mass transport is limited to the $x$-axis along the length of the microchannel due to the short diffusion distances along the $y$- and $z$-axes, across the span of and from the top to the bottom of the microchannel, respectively. In contrast, the ECV of a cell in a 35 mm dish is less controlled, as mass transport is not limited to one dimension. Convective and diffusive transport may occur equally along all three axes as opposed to one axis at the microscale. Because in vivo environments are defined by small ECVs, cell culture in a microfluidic environment may allow for a more in vivo-like environment than cell culture at the macroscale.

Laminar flow is a condition in which the velocity of a particle in a fluid stream is not a random function of time. The flow regime of a fluid flow, laminar or turbulent, is determined by its Reynolds number. The Reynolds number can be calculated by

$$R_C = \frac{\rho V D}{\mu}$$

Equation 1
where $\rho$ is the fluid density, $\nu$ is the characteristic velocity of the fluid, $\mu$ is the fluid viscosity, and $D_h$ is the hydraulic diameter. The hydraulic diameter is a computed value that depends on the channel’s cross-sectional geometry. $Re < 2300$, as calculated by the above formula, generally indicates a laminar flow. Due to the small size of microchannels, flow is almost always laminar. For the purpose of liver cell culture, laminar flow may be advantageous as the fluid flow over the cells is more steady and predictable and this may be advantageous for autocrine as well as paracrine cell signaling.[30]
3 Materials and Methods

3.1 Microfabrication

3.1.1 Microfluidic Device Materials

The methods that exist for fabricating microfluidic devices include soft lithography, micromachining, embossing, in situ construction, injection molding, and laser ablation.[30] We used PDMS devices generated from a soft lithography process of molding PDMS from SU-8 photoresist patterns on silicon wafers in our experiments. PDMS molding is faster and less expensive than the other methods mentioned, but this method was primarily chosen due to the high gas permeability and biocompatibility of PDMS. The following equations for oxygenation[32], when applied to our PDMS devices along with the oxygen consumption and oxygen gradient values given with the equations, demonstrate that our devices would be adequately oxygenated.

\[ X_{O_2 consumption} = N_{cells/cm^2} X_{onecell} S_{device}, \]

Equation 2

where oxygen consumption in mols/device/day \( X_{O_2 consumption} \) is calculated from the cell density in the microdevice \( N_{cells/cm^2} \), the oxygen consumption of a single hepatocyte per second \( X_{onecell}=1 \times 10^{-16} \) mols/cell/sec, and the area for the cell culture \( S_{device} \).
where \( F_{\text{max}} \) is the amount of oxygen that could be supplied into a device in mols/device/day and is estimated from the permeability of oxygen in PDMS \( D_{\text{PDMS}} \), the thickness of the PDMS wall of the device \( \Delta z \), and the oxygen concentration gradient across the wall of the device \( \Delta C \).

For a Design 1 (see 3.2.1 4-Channel Design) microchannel (5 mm \( L \) x 200 \( \mu m \) \( W \) x 100 \( \mu m \) \( H \)) with a PDMS ceiling 3 mm thick, \( S_{\text{device}} = 5 \, \text{cm} \times 0.02 \, \text{cm} = 0.1 \, \text{cm}^2 \) and if the channel were to contain 500 cells (a number greater than that observed in our experiments) and \( N_{\text{cells/cm}^2} = 5,000 \, \text{cells/cm}^2 \), the oxygen consumption of the cells in mols/device/day would equal

\[
X_{\text{O}_2\text{consumption}} = 4.32 \times 10^{-9} \, \text{mols/device/day}
\]

where maximum oxygen available would be, assuming an oxygen permeability in PDMS of \( 4.1 \times 10^{-5} \, \text{cm}^2/\text{sec} \), an oxygen concentration gradient of \( 2 \times 10^{-7} \, \text{mol O}_2/\text{cm}^3 \) [32], and a PDMS ceiling thickness of 3 mm,

\[
F_{\text{max}} = 2.362 \times 10^{-7} \, \text{mols/device/day}
\]

for a cell culture area of 0.1 \( \text{cm}^2 \).

Therefore the amount of oxygen available is two orders of magnitude higher than that necessary to maintain the cell culture. The level of oxygen consumption is on the same or lower order of magnitude for the other device designs tested, as the
surface area $S_{device}$ was the same or greater with a similar or lower cell number for a cell density near or less than the value calculated above.

3.1.2 Masters and PDMS

To generate a PDMS device, a "master" was first fabricated from using photolithography. 75-mm diameter silicon wafers were spin-coated with SU-8 negative photoresist at a spin speed determined by the desired thickness of the pattern, i.e. the height of the microchannel or well. For example, a spin speed of 1650 rpm would be used to coat silicon wafers with SU-8 at a thickness of 100 µm. The coated wafers were then soft-baked on an aluminum hotplate or in an oven at 95°C after ramping from 65°C, with differing times depending on the thickness of the photoresist.

To pattern the photoresist, the pattern was first printed on transparencies with a resolution of 3556 DPI to create a photolithographic mask. The coated and soft-baked wafers, covered with the mask, were then exposed to UV to crosslink the areas exposed by the transparency. After a post-exposure bake at 95°C on an Al hotplate or in an oven, ramped from 65°C, for varying times depending on the photoresist thickness, the wafers were then developed to remove the unexposed photoresist. Then the patterned wafer was hard-baked on an Al hotplate or in an oven at 150°C to anneal the remaining patterned photoresist.

To create the final PDMS device, PDMS elastomer and curing agent were first combined in a 10:1 ratio, mixed, and desiccated to remove bubbles. Then after
placing the master in a 100 mm polystyrene petri dish, or in later experiments, in an aluminum “cup” shaped to hold the master, the PDMS elastomer and curing agent mixture was poured over the masters and cured at 80°C, or in later experiments at 150°C for a shorter period of time. The patterned PDMS was then peeled off of the master and sterilized and placed on a cell culture surface (polystyrene or collagen I) or plasma cleaned, bonded to another surface (another PDMS layer, or glass), and then sterilized and placed in a petri dish for cell culture. The process flow for making the masters and PDMS devices is shown in Figure 2.

Figure 2. Microfabrication process flow for PDMS devices used in cell cultures.

3.2 Device Designs

The device designs tested differed in the shape of the cell culture area but were all fabricated in PDMS. Initial experiments were done in a device design consisting of a microchannel. The next design consisted of a microchannel in which the cells were inserted into a chamber in the center that was encased by posts so
that the cells would potentially stay within the chamber. In the third design, we placed wells in a layer below a layer consisting of channels to see whether the cells would settle and stay viable and express hepatocellular functions while cultured in the wells. In our fourth design, we tested the cells again in channels with no wells but with a collagen I – coated glass surface instead of a collagen I – coated polystyrene surface as with the initial microchannel experiments, and with a different type of cell.

Experiments with Design 1 used murine hepatic progenitor cells (HPCs). Experiments with Designs 2, 3, and 4 were done with cells from the human HepG2 hepatocarcinoma cell line instead of with HPCs. As shortcomings of each design were discovered the design evolved, thus the experiments consisted of testing each design in series.

3.2.1 Design 1 – 4-Channel Design with Varied Channel Width

Design

The first microfluidic device consisted of four microchannels with varied width and the same length and height. This variation allowed for the testing of the hypothesis that the channel width would affect the viability or the hepatocellular function of the cells.

Channel Dimensions: 50 mm x 100 µm x (100 µm to 400 µm) (L x H x W)

Port diameter: 4 mm (1.2 mm in design below but cored with larger cork borer)
Figure 3. Photolithographic mask

Figure 4. Si and SU-8 master for fabrication of the PDMS microchannel device. The substrate is a 75 mm-diameter silicon wafer with the SU-8 photoresist pattern raised above the wafer surface. Shown are the microchannels with numbers and lines marking each millimeter along the 50 mm microchannel, with a 1.2 mm diameter port at each end of each channel.
Device Operation

To load cells into the PDMS device made from the photolithographic mask shown in Figure 3 and the master shown in Figure 4 (device side view shown in Figure 5), the device ports first had to be cored with a cork borer to allow access to the microchannels. The devices were then dipped in 70% ethanol (EtOH) for a few seconds and dried under UV in a laminar flow hood for a minimum of 30 minutes. Then the PDMS molds were placed inside a 35 mm polystyrene petri dish. The bottom surface of the channels was coated with collagen I by flowing the collagen I through the channels, allowing it to sit at room temperature under UV for a minimum of one hour, rinsing with phosphate buffered saline (PBS) and sterile H₂O, and then rinsing with the medium used to culture the murine HPCs. The collagen solution used in all experiments was rat tail collagen type I at a concentration of 50 µg/mL of collagen in 0.02 N acetic acid. Control 35 mm dishes were coated with collagen I with the same set of steps as described for the devices.

Cell loading was done using various methods. Prior to seeding, the channel was already wetted with medium. The first method was to simply pipet the cells into one port and allow the fluid flow to drive cells into the channel. As this failed to drive
enough cells into the channel, another method that was tested was tilting the devices at an approximately $15^\circ$ to $30^\circ$ tilt immediately after loading a port with cells and allowing the cells to enter the channel for a couple of minutes with intermittent observation under a microscope. This method had better results than the first method. In later experiments with this device, the port size was increased to 4 mm by coring larger ports. This increased the ease of cell loading by allowing for larger volumes of the cell mixture to be added and facilitating medium changes and sample collection by increasing ease of access with the pipette.

In all experiments, devices were placed in Falcon™ (Becton Dickinson, Franklin Lakes, NJ) petri dishes for the cell culture. A couple of milliliters (0.5 mL to 5 mL depending on the dish volume) of the cell culture medium used for the culture were then pipetted into the area outside the device, to keep the area around the device humidified once the devices were incubated (at 5% CO$_2$ and 37$^\circ$C in all experiments). A lid was placed on the petri dish during the cell culture.

### 3.2.2 Design 2 – Spheroid Chamber Design

**Design**

The purpose of the second design was to attempt to create and maintain three-dimensional hepatocellular aggregates, or spheroids, within a microscale culture chamber. Spheroids are advantageous for mimicking hepatocellular functions because hepatocytes cultured to self-assemble into multicellular spheroids possess tight junctions and microvilli-lined channels that contain bile[33] and maintain liver-specific functions, such as high albumin secretion, urea excretion, and cytochrome
p450 activity over a period of several weeks, whereas non-aggregated hepatocytes do not. By allowing for liver tissue to be cultured in three dimensions in a potentially more *in vivo*-like environment at the microscale, the design would allow for greater accuracy and relevance of studies on the cultured tissue.

The second design (Figures 6-7) consisted of a rectangular cell culture chamber with a linear array of posts on two sides to prevent cell clusters from flowing out of the chamber and inlet and outlet microchannels to allow for fluid flow. This design allowed for direct access with a pipette to the rectangular chamber. By coring a cylindrical hole in the PDMS directly above the rectangular chamber the cells could be directly added with a pipette. The length and width dimensions of the rectangular area were large enough to allow for the formation of large clusters of hepatocytes.

**Dimensions**

Rectangular Cell Culture Chamber:
1.8 mm x 1.2 mm x 100 µm (L x W x H) (layer above is cored with a 1.2 mm cork borer)

Posts: 200 µm x 50 µm x 100 µm (L x W x H)

Gaps between Posts around Cell Culture Chamber: 100 µm wide

Inlet and outlet microchannels: 6 mm x 400 µm x 100 µm (L x W x H)

Port diameter: 4 mm (1.2 mm in design below but cored with larger cork borer)
Figure 6. *Top view of the spheroid chamber design.* Cells were added with a pipette to a port cored above the central rectangular cell culture chamber. The linear arrays of posts on each side of the rectangular area were added to prevent cell clusters from flowing out of the central area.

Figure 7. *Side view of the spheroid chamber design.*

Device Operation

The PDMS above the cell culture chamber in each device was first cored with a 1.2 mm cork borer. The ports were cored with a 4 mm cork borer. The devices were then sterilized by soaking in 70% ethanol for 30 minutes and drying under UV overnight. Each device was then placed in a separate uncoated tissue-culture-treated 35 mm polystyrene dish. In experiments with Design 2, the HepG2 cells
were cultured on uncoated tissue-culture-treated polystyrene as this was the standard method of culturing HepG2 cells in the lab.

Cells were seeded into the device using different methods. In all experiments the devices were wetted with medium prior to adding cells. The first method was to inject cells into the cored hole above the cell culture chamber of the device with a pipette. This was done by suctioning a few µL of the cell mixture into a pipet tip with a pipette, placing the pipet tip in the hole cored above the culture chamber, and pipetting the cells into the culture chamber. The pipette and pipet tip were then removed from the cored hole. The second method of adding cells was to, having suctioned cells into the pipet tip with a pipette, place and then remove the pipette from the hole cored above the cell culture chamber while keeping the pipet tip in place in the cored hole, thus allowing the cells in the cell mixture in the pipet tip to settle into the cell culture chamber via gravity. The first method of injecting the cells proved to be a more effective method as more cells were seeded using this method.

3.2.3 Design 3 – Connected 4-Channel and Well Design

Design

To see how the cells would react to being cultured in a layer separate from the perfusion channel, Design 3 incorporated a separate well layer for the cell culture. This method also allowed for the investigation of how cells react to being placed in a cylindrical structure; this method was tested as a potential method of inducing aggregation into a spheroidal aggregate.
In this design, a series of connected channels was placed over a linear array of wells so that each channel was placed over one well (see Figures 8-9).

Devices made from Design 3 were two layers whereas all other devices were comprised of one layer. The device fabrication required two masters; one consisting of a series of wells and one consisting of the pattern of channels placed above the wells. Separate molds were made from each master and these were then bonded after plasma cleaning.

**Dimensions**

Port diameter: 4 mm (1.2 mm in design below but cored with larger cork borer)

Channels: 500 µm x 100 µm (W x H)  Wells: 300 µm diameter x 100 µm H

Figure 8. Top view of both layers (channels and wells) of Design 3. The pattern above is an overlay of the masks used to generate both layers of the PDMS devices.

Figure 9. Side view of Design 3.
Device Operation

First the ports in the top layer were cored with a 4 mm cork borer. Then after cleaning the surfaces of the devices with Scotch tape, the devices were placed in a plasma cleaner and plasma cleaned for 30 seconds. After plasma cleaning, the devices were bonded while alignment was done using a stereoscope. After plasma cleaning and bonding, the devices were placed in a sterile 100 mm polystyrene petri dish and placed under UV in a laminar flow hood overnight. In the initial experiments with this device, channels were not wetted prior to sterilization, and the channels had to be debubbled. This was done by placing tubing connected to a syringe in one port, closing off the other port, and adding fluid with the syringe and pressurizing the device. However, in later stages of experiments with this device, sterile water was added to the bonded channels immediately after bonding, to allow for continuity and eliminate bubble formation once medium was later added to the channels for cell culture.

Various methods were attempted for loading cells into this device, other than adding the cell mixture to the inlet port while leaving the device on a flat surface. One was to tilt the dish containing the device. Another method was to insert a cylindrical piece of PDMS with a diameter similar to the diameter of the inlet port (as the PDMS piece was made by removal of cored PDMS from the inside of a 4 mm cork borer) into the inlet port, after adding cells to the inlet port, to drive the cell mixture into the device, and then remove the PDMS piece from the port after the devices had been incubated for approximately an hour. Neither method was consistently more successful at increasing the cell number in the devices.
3.2.4 Design 4 – Connected 4-Channel Design

**Design**

For a more basic study of cell viability and hepatocellular function in microfluidic channels, the wells in the third design were eliminated in the fourth microfluidic device design (Figure 10). This would allow the cells to be cultured in a channel instead of a cylindrical area. In addition, the cells were cultured on collagen I–coated glass instead of tissue-culture-treated polystyrene. This method was used because the collagen I coating on glass is another standard method of culturing HepG2 cells.

**Dimensions**

Port diameter: 4 mm (1.2 mm in design below but cored with larger cork borer)

Channels: 500 µm W x 100 µm H

![Figure 10. Top view of Design 4.](image)

**Device Operation**

To prepare this device for use it was first cored with a 4 mm cork borer, plasma cleaned for 30 seconds, bonded to glass, and then immediately wetted with sterile deionized water. Then collagen I was run through the channels by placing a
drop of collagen over one port and using suction from a 1 cc syringe without a needle to pull the collagen through the channels. The devices were then placed with the collagen in the channels under UV in a laminar flow hood for one hour, after which the channels were rinsed with PBS and sterile H₂O and then HepG2 medium. Wells of a 48-well plate were coated alongside the devices with the same set of steps.

Cells were loaded by reducing the fluid volume to a minimum in both ports and adding cells to one port, allowing fluid flow to drive the cells from the port into the channels. This was done by first removing fluid from both ports with a pipette. Then the cell mixture was suctioned into the pipette and pipetted out into the inlet port. The difference in fluid height drove the flow of the cells from the inlet port into the channel and the outlet port.

3.3 Cell Culture

3.3.1 Hepatic Progenitor Cells

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were maintained on a rodent chow under a constant day/night cycle. Six to eight week old mice were used in all experiments. All care and use of animals was approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
3.3.1.1 Isolation

A modification of the two-step collagenase perfusion technique described by Seglen et al.[35], slightly modified from a protocol for isolation of HPCs described by Wang et al.[36] was used to isolate the liver cells from adult C57/BL6 mice. All initial isolation steps were conducted on ice to keep cells metabolically dormant. The cells were stored in HPC isolation medium (described below) prior to seeding and cultured in routine HPC growth medium (described below).

The isolated HPCs were initially stored on ice prior to seeding, in medium consisting of L-15 (Leibovitz) medium plus 0.20% bovine serum albumin (BSA), 20 mM Hepes, 2 µm dexamethasone, 1X antimycotic-antifungal (AA), 0.5 mg/L insulin-transferrin-selenium (ITS), and 0.3 g/L L-glutamine. Cells were then cultured in medium consisting of low glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen Corp., Carlsbad, CA) plus 10% fetal bovine serum (FBS), 20 mM Hepes, 10 mM nicotinamide (niacinamide), 30 mg/L L-proline, 1 mM L-ascorbic acid 2-phosphate (Asc2P), 2 µm dexamethasone, 1X AA, 0.5 mg/L ITS, 10 ng/mL epidermal growth factor (EGF), 1% dimethyl sulfoxide (DMSO), and Gentamicin in a ratio of 1:1000 by volume. In all experiments, the cells were cultured in an incubator at 5% CO₂ and 37°C.

3.3.2 HepG2 Cells

HepG2 cells were obtained from stocks from various passages that were stored in liquid nitrogen. The cells were first cultured in a flask and then trypsinized to detach the cells from the flask surface. In some experiments the cells that were seeded in devices were ones that were then divided from this stock into separate
vials that were then stored at -80°C. In other experiments cells were seeded directly after this first round of plating of the liquid-nitrogen-frozen stock and trypsinization. The cells were trypsinized and reseeded as described below.

3.3.2.1 Cell Preparation

The HepG2 cells plated in the flask were first rinsed with 1X phosphate buffered saline (PBS). The PBS was removed and trypLE, or trypsin in initial experiments, was then added and the cells were incubated until detachment of most of the cells from the surface was observed under the microscope. PBS was then added and the entire cell mixture was transferred to a centrifuge tube and centrifuged at 1100 rpm for 5 minutes. The supernatant was then removed and medium was added and the mixture was agitated with pipetting to break up the cell pellet. A sample of the cell mixture was then combined in a 1:4 ratio with trypan blue and the cells were counted using a hemacytometer to obtain the cell concentration. The cells were recentrifuged and resuspended for the desired cell concentration for cell seeding. The medium used with HepG2 cells was HepG2 medium containing 10% FBS. The HepG2 medium consisted of Eagle’s Minimum Essential Medium (EMEM) (Invitrogen Corp., Carlsbad, CA) plus 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 5 mL antimycotic-antifungal (AA) solution (Sigma Cat. # 5955) per 500 mL medium.
3.4 Assays

3.4.1 Cellular Viability

Three assays were used: MitoTracker, Trypan Blue, and Live/Dead. MitoTracker™ probes (Molecular Probes, Eugene, OR) are a series of mitochondrion-selective dyes. The MitoTracker Red™ dye, once it enters an actively respiring cell, is oxidized to the MitoTracker Red™ fluorescent probe once it is sequestered in the mitochondria. The dye used was 90 nM MitoTracker Red™.

Trypan blue is used to selectively color dead cells blue. In a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell.

The calcein AM stain is based on the conversion of the virtually nonfluorescent cell-permeant calcein AM to fluorescent calcein. The dye is well retained within live cells, producing a green fluorescence (excitation/emission ~495 nm/~515 nm). In contrast Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes an enhancement of fluorescence upon binding to nucleic acids, thereby producing a red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells.

3.4.2 Cell Number

The MTT assay is a colorimetric assay used to measure cellular proliferation. The assay measures absorbance from formazan, based on the reduction of yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) to purple formazan in the mitochondria of living cells.
3.4.3 Albumin Secretion Assay

Albumin secretion was measured using an ELISA (enzyme linked immunosorbent assay). The ELISA is a biochemical technique for measuring the presence of a certain antigen, e.g. albumin, in a given sample. The antigen is immobilized on a surface, e.g. a 96-well plate, and one then washes a particular antibody over the surface. The antibody is linked to an enzyme that reacts when activated, e.g. by light, as with a fluorescent enzyme. The brightness of the fluorescence then indicates the amount of antigen in the sample.
3.5 Experiments Conducted

3.5.1 Design 1

Three experiments were performed with this design, each with HPCs isolated from C57BL/6 mice.

3.5.1.1 Experiment 1

Cells were seeded in four devices. Cells were observed for five days and the medium was changed at one hour and one day post-cell seeding.

Assay - Viability

At Day 4 post-cell seeding, the cells were assayed for viability with the MitoTracker Red™ stain. After dye addition to four devices and control dishes and a wait period of a minimum of 5 minutes, the results were analyzed using a Zeiss LSM 410 inverted microscope. The same microscope was used in all microscopic analyses discussed.

3.5.1.2 Experiment 2

Cells were seeded in one device at 2000 cells/µL.

Assay – Cell Number

An MTT assay was conducted on Day 0 to measure the cell number in each channel. A standard curve was generated by seeding approximately 100 to 20,000 cells per well in a 96-well plate. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution was added to the channels and the cells were incubated for 3 hours in an incubator at 5% CO₂ and 37°C. The cells were then incubated with MTT solvent (0.1 N HCl in anhydrous isopropanol) until the purple
formazan crystals were dissolved. The absorbance was read at 570 nm using an ELISA plate reader. The cell number was calculated from the standard curve.

3.5.1.3 Experiment 3

Cells were maintained in culture in four devices for five days total with medium changes at one hour and one day post-cell seeding and at Day 4 post-cell seeding the spent medium was collected for an albumin ELISA. An error to be noted for this experiment is that the cell culture medium contained double of each of the components, i.e. the components were added twice to the same bottle of medium. The only other cell culture to which this error applies is the cell culture used in Experiment 4 with Design 1 (Section 3.5.1.4 below).

Assay – Albumin Secretion

Medium was collected from both ports of the devices on Day 4 and assayed for albumin with an ELISA.

3.5.1.4 Experiment 4

Four cells were seeded and of these three devices were maintained in cell culture for eight days total.

In the three devices maintained in culture, medium was changed at one hour, Day 1, Day 2, Day 3, Day 4, and Day 7 post cell-seeding. Samples were taken from the spent medium from the Day 4 and Day 7 medium changes for two albumin ELISA assays.

Assay – Albumin Secretion
Medium was collected from both ports of the devices on Day 4 and assayed for albumin with an ELISA. The medium collected was from a 24 hour sample as medium was changed on Day 3. The medium was replaced and the devices were returned to the incubator.

Medium was then collected on Day 7 and assayed for albumin with an ELISA. The medium collected was from a 72 hour sample as medium was previously changed on Day 4.

**Assay – Cell Number**

The fourth device was seeded as were the other three and was used for an MTT assay. A standard curve was done concurrently with the fourth device. The protocol followed for the MTT assay was the same as that used for the MTT assay described in Section 3.5.1.2.

### 3.5.2 Design 2

Two experiments were conducted with this design, both with HepG2 cells thawed from storage in liquid nitrogen.

#### 3.5.2.1 Experiment 1

Cells were thawed and plated in a T25 flask in HepG2 medium, cultured for 11 days, and then seeded in devices on Day 11 post-cell seeding. Medium was changed in the flask on Days 4, 7, and 10. Devices were seeded at 5000 cells/µL. Controls were seeded with 125,000 cells/500 µL HepG2 medium in a 48-well plate. This seeding concentration was higher than that specified by the standard protocol.
for the lab which is 19,000 cells/500 µL in a well of a 48-well plate for a 25,000 cells/cm² cell seeding density. This seeding density used was based on a protocol specifying 250 cells/µL. Seeding of the first of three devices was semi-static; i.e., the cells were allowed to settle in a pipet tip while observing the uncapped dish under a microscope. Cells were injected into the other two devices and the cell seeding was conducted under sterile conditions in a laminar flow hood. The volume of the cell mixture injected based on observation of the device under the microscope; cells were added until the cell culture chamber was observed to have been moderately seeded. The volume of the medium used to humidify the dishes (pipetted on the dish area surrounding the device) was 0.5 mL. The cells were imaged and the medium around the devices, used for humidification, was emptied and refreshed with a pipette on Days 1, 3, 4, and 5 of the cell culture.

Assay - Viability

To characterize how the cells would respond to static culture over an extended period, HepG2 cells were assayed for viability on Day 6 with a trypan blue stain.

A solution of 10% trypan blue in 1X PBS was used for the stain. To stain the cells the medium in the channels was replaced with the stain by emptying the device ports with a pipette and filling one of the two ports of each device with the stain and allowing it to flow into the cell culture area. To stain cells in the control wells, the cell culture medium was removed and replaced with the stain. After a minimum of 10 minutes, the trypan blue was replaced with PBS and the cells were imaged.
3.5.2.2 Experiment 2

Cells were added to six devices at 1000 cells/µL. The controls were three wells of a 48-well tissue-culture-treated polystyrene plate, seeded with 19,000 cells (in 19 µL medium) added to 481 µL medium for a surface cell density of 25,000 cells/cm² per the lab protocol. The volume of the medium used to humidify the dish area was 1 mL, as opposed to 0.5 mL in the previous experiment, to ensure adequate humidification. The cells were imaged on Days 1, 2, and 5. The medium around the device was refreshed on Day 2 of the cell culture. Also, whereas 35 mm dishes were used to contain the devices in Experiment 1 with Design 2, 60 mm devices were used in Experiment 2 with Design 2 (due to unavailability of the dishes).

Assay - Viability

As viability was difficult to determine with the trypan blue stain, the cells were assayed for viability on Day 6 with the Calcein AM/Ethidium homodimer-1 (EthD-1) Live/Dead cell stain (Invitrogen Corp., Carlsbad, CA).

The cells were rinsed with 1X PBS prior to addition of the stain. A solution of 5 µL of 4 mM Calcein AM and 20 µL of 2 mM EthD-1 in 10 mL of 1X PBS was then added to the devices and control wells. The cells were incubated with the stain for 30 minutes prior to imaging.

3.5.3 Design 3

Four experiments were performed with Design 3, each with HepG2 cells thawed from storage in liquid nitrogen.
3.5.3.1 Experiment 1

Cells were seeded in three devices at 5000 cells/µL. Approximately 19,000 cells were seeded in three control wells of a 48-well tissue-culture-treated polystyrene plate; 485 µL HepG2 medium were added for a total volume of 500 µL. The cells were kept in static culture for four days after cell seeding.

Assay – Viability

A Calcein AM/Ethidium homodimer-1 live/Dead cell stain was done on Day 4 post-cell seeding to test viability. A solution of 2 µL (instead of 5 µL per protocol, due to unavailability of solution) of 4 mM Calcein AM and 5 µL of 2 mM EthD-1 in 10 mL 1X PBS was used for this experiment. The cells were rinsed with 1X PBS prior to addition of the stain and incubated for a minimum of 30 minutes after addition of the stain prior to imaging.

3.5.3.2 Experiment 2

Cells were seeded in four devices at 1,375 cells/µL. Three control wells of a 48-well plate were seeded with 19,000 cells in HepG2 medium for a total medium volume of 500 µL. One deviation from the protocol is that a temperature of 4°C was used during centrifugation during the protocol for cell trypsinization from the T25 flask in which the cells had been cultured prior to device seeding.

In this experiment the cells were kept in static culture for four days. The cells were kept in static culture to minimize the alterations between this experiment and the previous one, while increasing the concentration of the live/dead cell stain used to assess viability.

Assay - Viability
A solution of 60 µL of 1 mM Calcein AM and 60 µL of 2 mM EthD-1 in 3 mL 1X PBS was used. Cells were incubated for 45 minutes prior to imaging. The cells were also re-imaged, although these images are not shown, prior to which they were flushed with medium instead of PBS.

3.5.3.3 Experiment 3

Cells were seeded in 5 devices at 10,000 cells/µL. Three wells of a 48-well plate were seeded with 19,500 cells per well with 500 total µL of culture medium. Note that this is a deviation from the standard protocol, which specifies a seeding concentration of 19,000 cells in 500 µL of medium per well of a 48-well plate. Devices 1, 3, and 4 were seeded by inserting a PDMS piece in the inlet port (see Materials and Methods Section 3.2.3) after the cell mixture had been added to the inlet and removing the PDMS piece only after incubating the devices for 64 minutes. This method was not used for Devices 2 and 5 as a large number of cells was observed to have entered the channels on their own.

Assay - Viability

In this experiment as with the previous two described with Design 3, the cells were kept in static culture. However in this experiment, the cells were assessed for viability earlier to see how viability would differ earlier in the cell culture.

A stain solution of 40 µL of 1 mM Calcein AM and 40 µL 2 mM EthD-1 in 2 mL HepG2 medium was used. Cells were incubated 45 minutes prior to imaging.
3.5.3.4 Experiment 4

In this experiment, two different sets of devices were used: one set which consisted of the Design 3 channel-and-well devices, and one set which consisted of the Design 4 (see Section 3.2.3) devices.

The cells used were from Passage 109 and were initially plated in a T25 flask prior to seeding in devices and controls. A note regarding the cells used in this experiment is that they had initially looked non-viable when, based on qualitative observation under a microscope, but after incubation for six days with no medium change, the cells looked viable based on observation under a microscope. At this point the medium used for the cells was changed. The cells were then seeded the next day in devices and controls.

Two Design 3 devices and three Design 4 devices were seeded at 2000 cells/μL. Four control wells of a 48-well plate were seeded with 19,000 cells in 500 μL HepG2 medium.

In this experiment a medium change was done on Day 1 in addition to the medium change at one hour post-cell seeding. The medium change for the devices was done by nearly emptying the ports with a pipette, adding warmed medium to one port and re-incubating the devices, allowing the fluid to flow from one port to the other. Medium was also changed in the controls.

Assay - Viability

The stain solution used for the Day 2 stain consisted of 2.5 mL HepG2 medium containing 50 μL of 2 mM EthD-1 and 12.5 μL of 4 mM Calcein AM.
3.5.4 Design 4

Four experiments were conducted with Design 4 (including the experiment discussed in section 3.5.3.4). Each was conducted with HepG2 cells thawed from storage in liquid nitrogen.

3.5.4.1 Experiment 1

HepG2 cells were seeded in three Design 4 devices and 2 control wells. The devices were seeded at 5000 cells/µL and the controls were seeded at 19,000 cells were well in 500 µL HepG2 medium. A medium change was conducted at one hour post-cell seeding.

**Assay - Viability**

The stain solution used for the Day 2 EthD-1 stain consisted of 1.5 mL HepG2 medium with 30 µL of 2 mM EthD-1.

A live/dead cell ratio was obtained using bright-field phase contrast images and dead cell stain fluorescent images taken of each of four channels of each of the devices. The number of dead cells in a given image was subtracted from the total number of cells in that area, counted from the bright-field image. Assuming the number of live cells in that area was the difference between these numbers, the live to dead cell ratio was calculated.

**Assay – Albumin Stain**

The albumin stain was done as follows. The devices and control were first rinsed with 1X PBS. Then a solution of 4% paraformaldehyde was run through the devices and control for fixation. After a 20 minute wait, a solution of PBS containing 0.1% Triton was run through the devices and control as a rinse. After the rinse, a
solution of PBS containing 0.1% Triton and 10% horse serum was run through the devices and control as a blocker. After this step, the devices and control were again rinsed with a solution containing 0.1% Triton. After the rinse, the primary antibody was added, in a 1:500 dilution in PBS (Sigma Inc., Catalog # A6684, Lot 03K348). After a 2 hour wait at room temperature, a PBS rinse, and addition of the secondary antibody in a 1:750 dilution, the devices and control were left at room temperature for an hour. The devices and control were then rinsed with PBS and imaged. Imaging was done in the following order: red fluorescence, green fluorescence for the albumin stain, and bright-field phase contrast.

### 3.5.4.2 Experiment 2

Cells were seeded in four devices at a concentration of 2000 cells/µL and in two control wells at 19,000 cells/well in 500 µL HepG2 medium. Cells were cultured for a total of four days.

**Assay – Albumin Stain**

An albumin stain was conducted on Day 3 post-cell seeding. The albumin stain was done as described above, with a change to the dilutions of the primary and secondary antibodies. Instead of 1:500 and 1:750 dilution of the primary and secondary antibodies, respectively, the dilutions used were 1:250 and 1:200 of primary and secondary antibody, respectively, in PBS.
3.5.4.3 Experiment 3

In this experiment, only viability was assayed, using an EthD-1 dead cell stain. Cells were seeded in four devices at 1000 cells/µL and two control wells at 19,000 cells/well in 500 µL HepG2 medium. Medium was changed on Day 1 and the cells were then imaged.

Assay - Viability

The stain solution used for the Day 2 Ethidium homodimer-1 stain consisted of 30 µL of 2 mM Ethidium homodimer-1 in 1.5 mL HepG2 medium. A live to dead cell ratio was calculated using the same method discussed in section 3.5.4.1.
4 Results

4.1 Design 1 – 4-Channel Design with Varied Channel Width

4.1.1 Experiment 1

Viability Stain

Below are representative images (Figures 11-15) from the cells in Design 1 devices with the MitoTracker Red™ dye on Day 4 post-cell seeding. As viable cells fluoresce when stained with MitoTracker Red™, this assay demonstrated the viability of cells in the microchannels. Based on a qualitative assessment of the images, a difference in viability was not observed within the range of channel widths (100 µm to 400 µm) tested.

The cells were also assessed for aggregation or lack thereof. Cells were observed to aggregate in all the channel widths. Cells were also observed to form clusters that spanned the width of the channel and had collected during cell seeding. Aggregation was not observed in control dishes. Cell debris was also observed in both the channels and control dishes. Also bubbles had entered some of the channels during cell seeding. However these channels would not have been able to be stained with MitoTracker Red™ and were not included in the representative images below.
Figures 11 a-b. (a) Bright-field and (b) fluorescent images of MitoTracker-stained cells on Day 4 post-cell seeding in a 100 μm wide microchannel.

Figures 12 a-b. (a) Bright-field and (b) fluorescent images of MitoTracker-stained cells on Day 4 post-cell seeding in a 200 μm wide microchannel.

Figures 13 a-b. (a) Bright-field and (b) fluorescent images of MitoTracker-stained cells on Day 4 post-cell seeding in a 300 μm wide microchannel.
Figures 14 a-b. (a) Bright-field and (b) fluorescent images of MitoTracker-stained cells on Day 4 post-cell seeding in a 400 µm wide microchannel.

Figure 15. Bright-field image of cells on Day 4 post-cell seeding in a collagen I-coated 35 mm polystyrene dish control.
4.1.2 Experiment 2

Cell Number Assay

The MTT assay for cell number, conducted in a Design 1 device on Day 0, generated non-linear results (Figure 16) in the assessment of cell number vs. microchannel width after cell seeding. It is to be noted that a qualitative observation was recorded that the cell density was similar between channels. Data are also shown after removal of the outlier (Figure 17). The experiment was not done in multiples. A possible explanation for the outlier is that cells were washed out of the 200 µm wide channel when MTT solution was added to the channel.

The purpose of this assay was to assess the number of cells seeded in each of the channel widths, immediately after cell seeding. However, as cell number in the channels was a function of the volume of the cell seeding mixture and other factors affecting the cell number, conclusions regarding the effect of channel width on cell number seeded cannot be made from the MTT assay alone. No control dish was tested in the MTT assay.
4.1.3 Experiment 3

**Albumin Secretion Assay**

The albumin ELISA conducted on three Design 1 devices from a Day 1-4 sample (the sample was taken on Day 4 after static culture on Days 1, 2, and 3)
indicated an increase in albumin secretion by the HPCs with microchannel width.

Figure 18 shows albumin concentration versus microchannel width for the devices tested. The cell number was not counted in each channel however, so it is unknown whether the increase was due to an increase in cell number or increased hepatocellular function.

![Mean Albumin Concentration vs. Microchannel Width (hundreds of microns), Day 4 (72 h sample)](image)

Figure 18. Mean albumin concentration (N=3) for four microchannel widths (100 µm to 400 µm) as determined by the albumin ELISA conducted with samples taken on Day 4 after 3 days of static culture.

4.1.4 Experiment 4

**Albumin Secretion Assays**

The first albumin ELISA from this cell culture, conducted on three Design 1 devices from a Day 4 24 h sample (the sample was taken on Day 4 after a medium change on Day 3), indicated an increase in albumin secretion by the HPCs with
microchannel width. Figure 19 shows albumin concentration versus microchannel width for the devices tested. The results show an increase in albumin secretion with microchannel width although the standard deviations vary and do not correlate with this increase in albumin secretion.

Figure 19. Mean albumin concentration (N=3) for four microchannel widths (100 µm to 400 µm) as determined by the albumin ELISA conducted with samples taken on Day 4 after conducting medium changes every day of the cell culture.

The second albumin ELISA from this cell culture, conducted on three Design 1 devices from a Day 7 72 h sample (the sample was taken on Day 7 after static culture from Days 4 to 7), did not indicate an increase in albumin secretion by the HPCs with microchannel width. Figure 20 shows albumin concentration versus microchannel width for the devices tested.
Cell Number Assay

The assay failed, based on a standard curve done concurrently with the assay as well as the previously done standard curve (Section 3.5.1.2). The results showed negative values for the cell numbers for all channel widths. Due to the need for greater sensitivity with the MTT assay to low cell numbers, it was not possible in this assay to correlate the standard curve with positive cell numbers in the device.
4.2 Design 2 – Spheroid “Chamber” Design

4.2.1 Experiment 1

Viability Stain

This exclusion stain conducted on the Design 2 devices showed a predominantly non-viable cell population after six days in static culture. Figures 21 – 22 show images (10X magnification) from all the devices as well as the control 48-well plate. Note that Device 1 was seeded under non-sterile conditions while testing seeding methods under the microscope (see Materials and Methods Section 3.5.2.1). A mixture of cells and debris is seen in the device wells. Cells in the controls were observed to have seemingly greater viability. This could be attributed to the unknown difference in cell seeding densities; whereas the controls were seeded with 125,000 cells in wells of a 48-well plate with a total of 500 µL of culture medium (Section 3.5.2.1), an unknown number of cells settled in the wells of the devices after an unknown volume (enough to moderately seed the cell culture chamber) of cell mixture at 5,000 cells/µL was seeded into the devices.

Device 1

Device 2

Figure 21a

Figure 21b
4.2.2 Experiment 2

Viability Stain

In another experiment, overlap in the green (live) and red (dead) fluorescence seem to indicate autofluorescence in images (Figures 23 a-l) of the Calcein AM/Ethidium homodimer-1 live/dead stain conducted on Spheroid Chamber devices.
on Day 6 post-cell seeding. However cells lying in different planes may express one or the other fluorescence depending on their viability, so autofluorescence is not a direct conclusion.

The following modifications were made to the protocol between this and the previous experiment. The cell concentration used in this experiment was 1000 cells/µL as opposed to 5000 cells/µL in the previous experiment, so that a potentially larger volume of 1 µL could be pipetted for a desired cell number of 1000 cells/well. In addition, the cell seeding density for the control was reduced to 25,000 cells/cm², the value specified by the standard protocol for the lab.

Controls also show overlap in green and red fluorescent areas (Figures 24 a-f). Again, however, this does not indicate total viability or non-viability of the cell culture due to the potential difference in staining of cells in different planes.
Figures 23 a-l. *Calcein AM (green) (live) and ethidium homodimer-1 (red) (dead) cell stain conducted in spheroid chamber devices on Day 6 post-cell seeding.*
Figures 24 a-f. Calcein AM (green) (live) and ethidium homodimer-1 (red) (dead) cell stain conducted in control wells on Day 6 post-cell seeding.

4.3 Design 3 – Connected 4-Channel and Well Design

4.3.1 Experiment 1

Viability Stain

Low expression of calcein AM fluorescence was observed in the devices and the controls on Day 4 post-cell seeding after four days in static culture (Figures 25 a–l). A lower calcein concentration than specified by the protocol (due to lack of
availability) was used in this assay so this may have resulted in less expression of the live cell fluorescence.
Figures 25 a-l. Calcein AM live (green) and ethidium homodimer-1 dead (red) cell stain results with Design 3, conducted on Day 4 post-cell seeding.
4.3.2 Experiment 2

Viability Stain

In another experiment, the HepG2 cells seemingly showed autofluorescence in a Day 4 live/dead stain with the Design 3 devices (Figures 26 a–h). Controls are shown in Figures 26 i–n.
Figures 26 a-n. Fluorescent images of calcein AM live (green) and ethidium homodimer-1 dead (red) cell stain images taken of the Design 3 devices and control wells on Day 4 post-cell seeding.

4.3.3 Experiment 3

Viability Stain

Autofluorescence was observed in both devices and controls (Figures 27 a–p) with this live/dead stain conducted on Day 2 after two days of static culture, as with the autofluorescence observed in both devices and controls on Day 4 after four days of static culture in the previous experiment discussed for Design 3. The results
again indicate that even more brief periods of static culture may still be unsuccessful at maintaining cell viability.
Figures 27 a-p. Day 2 live/dead stain with Design 3 devices and control wells.

4.3.4 Experiment 4

Viability Stain

In this experiment both the Design 3 and Design 4 devices were tested. Design 4 devices consisted of the devices bonded to collagen I – coated glass. Neither set of devices was visibly more successful than the other at maintaining cell viability until Day 2 post-cell seeding (Figures 28 a-e). Control wells were also not consistent in the level of cell viability observed (Figures 28 f-i).
Figure 28a

Design 3 Device 1

Design 3 Device 2

Figure 28b

Design 4 Device 1

Design 4 Device 2

Figure 28c

Figure 28d

Design 4 Device 3

Figure 28e
Figures 28a-i. *Design 4 calcein AM live (green)/ethidium homodimer-1 dead (red) cell stain images. Stain done on Day 2 post-cell seeding.*

4.4 **Design 4 – Connected 4-Channel Design**

4.4.1 **Experiment 1**

4.4.1.1 **Albumin Stain**

In another experiment, the presence of albumin was indeterminate as observed with the fluorescent albumin stain conducted with Design 4 devices and controls on Day 2 (Figures 29a–j). Note that a rounded area is shown at the end of
the channel (Figure 29e); this is due to the image being of the end of one of the channels at the rounded junction connecting two channels.

One factor in the indeterminate presence of albumin may have been the short period of time (two days) for which the cells had been in culture.

**Albumin Stain**

**Device 1**

![Figure 29a](image1)

![Figure 29b](image2)

**Device 2**

![Figure 29c](image3)

![Figure 29d](image4)
Figures 29 a-j. *Albumin stain conducted on Design 4 devices and control wells, Day 2 post-cell seeding.*
4.4.1.2 Viability Stain

The EthD-1 dead cell stain, conducted with the same cells on Day 2 (Figures 30 a-g), was used to assess the ratio of live to dead cells by counting the total number of cells in bright-field images and subtracting the number of dead cells stained with EthD-1 to obtain the number of live cells per image. The data show that, on average, the mean fraction of live cells was more than half of the cells in the devices. Fractions were not obtained for the control images due to the high number of cells in the images. The data for each device are provided in Table 1.

<table>
<thead>
<tr>
<th>Device #</th>
<th>Mean Total # Cells</th>
<th>Mean Total # Dead Cells</th>
<th>Mean # Live Cells</th>
<th>Mean Live/Dead Ratio</th>
<th>Mean Fraction of Live Cells</th>
<th>Std. Deviation of Mean Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>143.5</td>
<td>69.5</td>
<td>74</td>
<td>1.065</td>
<td>0.516</td>
<td>0.196</td>
</tr>
<tr>
<td>2</td>
<td>116.75</td>
<td>27.25</td>
<td>89.5</td>
<td>3.284</td>
<td>0.767</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>73.5</td>
<td>45.5</td>
<td>28</td>
<td>0.615</td>
<td>0.381</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Design 4 Experiment 1 - Live/dead cell ratios and mean fraction of live cells for each device

Ethidium Homodimer-1 Dead Cell Stain (red fluorescence) Overlay Images

Device 1

Device 2

Figure 30a

Figure 30b
Figures 30 a-g. *Ethidium homodimer-1 stain of cells in Design 4 devices and control wells, Day 2 post-cell seeding.*
4.4.2 Experiment 2

Albumin Stain

In another experiment, the devices showed greater levels of albumin by Day 3 in an albumin stain conducted on the Design 4 devices and controls (Figures 31 a-l). Note that rounded areas shown are junctions between channels.

The concentration of the antibodies used for the stain had been increased so this is a potential reason for the increase in fluorescence from the albumin stain between this and the previous experiment.

Device 1

Figure 31a

Figure 31b

Device 2

Figure 31c

Figure 31d
Figures 31 a-l. *Albumin stain conducted on Design 4 devices and control wells, Day 3 post-cell seeding.*

### 4.4.3 Experiment 3

In another experiment, an EthD-1 dead cell stain of cells in Design 4 devices, conducted on Day 2, showed lower viability than the previous Day 2 EthD-1 stain. Note that Device 3 data is not listed in the table, as the device had poor flow of the stain through the device, i.e. when the inlet port was filled with the stain (after both port volumes had been reduced to a minimum with a pipette), the stain remained in the inlet port instead of flowing through the device.

<table>
<thead>
<tr>
<th>Device #</th>
<th>Mean Total # Cells</th>
<th>Mean # Dead Cells</th>
<th>Mean # Live Cells</th>
<th>Mean Live/Dead Ratio</th>
<th>Mean Live Cells Fraction</th>
<th>Std. Deviation of Mean Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.75</td>
<td>34.75</td>
<td>15</td>
<td>0.432</td>
<td>0.302</td>
<td>0.153</td>
</tr>
<tr>
<td>2</td>
<td>17.5</td>
<td>12.25</td>
<td>5.25</td>
<td>0.429</td>
<td>0.300</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>103.75</td>
<td>45</td>
<td>58.75</td>
<td>1.306</td>
<td>0.566</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>poor flow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. *Design 4 Experiment 3 - Live/dead ratios and mean fractions of live cells for each device*
Shown in the figures (Figures 32 a-l) are bright-field and dead cell stain images from each device and the control wells.
Figures 32 a-l. *Ethidium homodimer-1 stain conducted on Design 4 devices and controls, Day 2 post-cell seeding.*
5 Discussion

5.1 Experimental Calculations

Surface area to volume ratio, glucose availability to consumption ratio, Reynolds number, shear stress, and oxygenation are five factors that affect cell viability and expression of hepatocellular function. Following are the results of calculations of these values for the designs tested and discussion of the implications for each design.

The following surface area-to-volume (SAV) ratios were calculated for each of the devices. The SAV ratios were calculated by dividing the total surface area of the top, bottom, and side surfaces in each design by the volume of fluid within the area encased by those surfaces.

<table>
<thead>
<tr>
<th>Design #</th>
<th>SAV Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Design 1</td>
<td>400/cm (100 µm width channel), 300/cm (200 µm width), 267/cm (300 µm width), 250/cm (400 µm width)</td>
</tr>
<tr>
<td>Design 2</td>
<td>120/cm</td>
</tr>
<tr>
<td>Design 3</td>
<td>233/cm</td>
</tr>
<tr>
<td>Design 4</td>
<td>159/cm</td>
</tr>
</tbody>
</table>

Table 3. Device SAV ratios.

The glucose availability to consumption ratios were calculated based on a value for HepG2 cell glucose consumption of $10^{-11}$ g/cell/h [37] and a glucose concentration in HepG2 medium (Eagle’s Modified Essential Medium) of 1 g/L. The
Reynolds number for each design was < 1. The amount of shear stress on cells in all the devices was estimated at 0.0445 dyn/cm², using the equation $\tau = -\mu \frac{dv}{dz}$ and assuming $\mu = 8.9 \times 10^{-4}$; $v_i = 0.0005$ m/s; and $z_i = 0.0001$ m. This result for shear stress may be optimal for the hepatocytes or HPCs, based on data from Tilles and colleagues. Tilles and colleagues showed that at lower wall stresses (0.01 to 0.33 dyn/cm²), hepatocyte functions, measured as albumin and urea synthesis rates, were as much as 2.6- and 1.9-fold greater, respectively, than those at higher wall shear stresses (5 to 21 dyn/cm²).[6] The ratio of oxygen available to oxygen consumed in each device was calculated using Equations 2 and 3, where oxygen consumption in mols/device/day $X_{O2\text{consumption}}$ is calculated from the cell density in the microdevice $N_{\text{cells/cm}^2}$, the oxygen consumption of a single hepatocyte per second $X_{\text{onecell}} = 1 \times 10^{-16}$ mols/cell/sec, and the area for the cell culture $S_{\text{device}}$, and where $F_{\text{max}}$ is the amount of oxygen that could be supplied into a device in mols/device/day and is estimated from the permeability of oxygen in PDMS $D_{\text{PDMS}}$, the thickness of the PDMS wall of the device $\Delta z$, and the oxygen concentration gradient across the wall of the device $\Delta C$.

The constants used in the equations above were an $N_{\text{cells/cm}^2}$ value of 5000 cells/cm², an $X_{\text{onecell}}$ value of $10^{-16}$ mol/cell/sec, a $D_{\text{PDMS}}$ value (for 1st, 2nd, and 4th designs) of $4.1 \times 10^{-5}$ cm²/sec, a $D_{\text{H2O}}$ value (for 3rd design) of $3.24 \times 10^{-5}$ cm²/sec[26], a $\Delta C = 2 \times 10^{-7}$ mol O₂/cm, and a $\Delta z$ of 0.3 cm.

5.2 Design 1
We were able to show maintained viability of the adult murine hepatic progenitor cells (HPCs) in microchannels (Figures 3-5) until Day 4 post-cell seeding with Design 1. Prior work has not been done with HPCs in microchannels.

We were also able to show production of a measurable level of albumin in the channels. The albumin production was calculated to be an order of magnitude lower (5.55x10^{-6} ng/cell/h vs. 2.78 x 10^{-5} ng/cell/h) than that of HPCs cultured in a monolayer in a collagen I-coated 35 mm dish.

Difficulties with this design included figuring out the best way to load the cells into the channels. The methods used, leaving the dishes flat after adding cells to one port and tilting the dishes after cell addition, were not consistently adequate in driving cells into the channels. The cell seeding method might be improved by using the method discussed in Materials and Methods Section 3.5.3 of using a cored piece of PDMS to plug the inlet port and move the fluid volume towards the channel.

Advantages of this design include the simplicity of the design of the one-layer microchannel structure, which allows for the elucidation of biological and physical responses of cells to the three-dimensional microfluidic environment with a basic approach.

The MTT assay conducted with a Design 1 device showed an increase in cell number with microchannel width with the removal of the data for the 200 µm microchannel. These results do not however suggest that cells are more viable with an increase in channel width, as the cell seeding number may have also been greater.
The albumin assay conducted with medium collected from Design 1 devices on Day 4 with 72 hours of static culture showed an increase in albumin concentration with channel width. Again this increase cannot be attributed to the dimensions of the channel, as cell seeding density data was not collected for the channels.

In another experiment, ELISA data from HPCs in culture for 4 days demonstrated a slight increase in albumin secretion with increasing channel width. With the same devices, based on a different ELISA experiment, the cells did not generate albumin in proportion to the channel width in a 72 hour sample collected at Day 7 of the cell culture. These conflicting results suggest that no correlation can be deduced between microchannel width and albumin secretion.

Design 1 has the highest SAV ratios (from 400 cm$^{-1}$ to 250 cm$^{-1}$) of all the devices; a feature which is most conducive to oxygen delivery to the cells. This design, along with Design 4, also has the lowest Reynolds number (Re = 0.056). A lower Reynolds number is conducive to enhanced cell signaling. The lower flow rates that correlate with a lower Reynolds number would prevent cytokines from being washed away from a cell’s microenvironment. With regard to oxygenation, Design 1 has the same estimated ratio of oxygen availability to consumption (54.7) as Designs 3 and 4 (higher than Design 2) due to the use of the same cell seeding density and device thickness values for these devices. The estimated glucose consumption of cells in this device design is also below the glucose availability based on the ratio of consumption to availability of 8.33. In addition, the shear stress (0.0445 dyn/cm$^2$) is at or below physiological levels (< 2 dyn/cm$^2$) for the
device channel height and the flow rate estimated from observation under the microscope.

5.3 Design 2

With the Spheroid Chamber design (Figures 6-7) we were able to show that HepG2 cells were low in viability after six days in static culture whereas the control cell culture was seemingly higher in viability. As the control seeding density was specified, however, and the device seeding density was dependent on the cell seeding method, the unknown difference in densities could be a potential reason for the difference in viability.

In addition to trypan blue viability assays we conducted a calcein AM/Ethidium homodimer-1 live/dead viability assay on the cells and observed both live (green) and dead (red) fluorescence in both devices and controls in an experiment conducted with another cell culture on Day 6 post cell-seeding. The assay was inconclusive at determining the feasibility of a six day static culture at maintaining viability, as the level of background in the device and control images was similar.

In addition to testing the cell culture method we tested cell seeding methods and were able to determine that cell injection is more effective than cell settling at getting high cell numbers in the device.

Advantages of this design include the potential ability, with the proper device dimensions, to encase cells or cell aggregates in an environment conducive to cell aggregation with the ability to perfuse the cells and handle cells in each culture chamber individually. Current drawbacks include the need to manually core an
access port above the culture chamber for cell addition, and the need for relatively large dimensions for this access port, for the purpose of pipetting cells into the well, in the current design.

Design 2 has the lowest SAV ratio (120 cm\(^{-1}\)) of all the designs. In addition, it has the highest Reynolds number (0.669). These characteristics are disadvantageous for oxygenation and cell signaling, respectively. The ratio of oxygen availability to consumption (24.0) is also lower since Design 2 has no PDMS ceiling, and the diffusion coefficient of oxygen through water is lower than that of oxygen through PDMS. However, one advantage of Design 2 is the ratio of glucose available to glucose consumed (250.6); because a large volume of medium is above the cells in the well, a large amount of glucose is available to the cells relative to the amount available in the other designs.

5.4 Design 3

With the two-layer channel and well design (Figures 8-9) we were able to test how cells respond to being cultured in a 300 µm well. We found that the cells were non-viable in Day 4 and Day 2 experiments, based on the amount of background observed in live fluorescence images taken after a calcein AM/EthD-1 live/dead cell stain. One possible reason for non-viability is the accumulation in the medium of cytokines which inhibited cell survival. Another is that the cell seeding density is not conducive to cell survival. From observation of the images, cell seeding density was lower than in the controls. As a result of the low cell seeding density, the amount of cell aggregation may have also been lower.
An advantage of this design is the ability to observe how cells behave when cultured statically in a well below a perfusion channel. A disadvantage is that the design is not conducive to cell settling in a single well in the center of a channel.

The SAV ratio (233 cm$^{-1}$) of Design 3 is lower than those of Design 1 but higher than those of Designs 2 and 4, making it relatively advantageous relative to Designs 2 and 4. Its glucose availability to consumption ratio is greater than that of Design 4, less than that of Design 2, and similar to that of Design 1, making it advantageous primarily relative to Design 4. Its ratio of oxygen availability to consumption (54.7) is the same as Designs 1 and 4 and greater than that of Design 2, making it advantageous relative to Design 2. Its Reynolds number (0.112) is greater than that of Designs 1 and 4 and less than that of Design 2, making it advantageous for cell signaling relative to Design 2.

5.5 Design 4

With the fourth design (Figure 10) we showed viability of the HepG2 cells. In addition, we were able to show the presence of some albumin in the channels as observed with a fluorescent albumin stain on Day 3 post-cell seeding. Albumin is an indicator of hepatocellular function, therefore this indicates the maintenance of this function after seeding of the cells in the microchannels. The Day 2 albumin stain showed indeterminate presence of fluorescence from the albumin. This may have been due to the short period of time in culture and a short amount of time available for albumin to reach a measurable amount. It may have also been due to low formation of intracellular contacts which would promote expression of hepatocellular
functions such as albumin secretion. This could be due to a low cell seeding density, the structure of the channel, or due to the collagen I ECM coating on the bottom surface of the channel. Thus potential improvements would be to increase the seeding density, change the structure of the channel, or test the cell culture without an ECM coating or with other ECM coatings. Viability was shown in the channels using EthD-1 stains alone. In the experiments in which only the EthD-1 stain was done, the number of live cells in a given image was calculated from the total cell count from the bright-field image minus the number of dead cells counted from the EthD-1 stain. The mean ratio of live/dead cells was then calculated for all the devices in a given experiment. For both such experiments with this design, the number of cells that were alive or dead seemed comparable.

Advantages of this design include the ability to seed multiple channels with the same cell seeding density, the ability to perfuse all the channels at once, and the simplicity of the microchannel design, as with Design 1, which allows for a more basic elucidation of cellular responses to the microchannel environment. Disadvantages include the lack of a method to prevent cells from washing out of the channel.

Design 4 has an SAV ratio (159 cm\(^{-1}\)) higher than Design 2 but lower than Designs 1 and 3, making it advantageous for oxygenation only relative to Design 2. Its glucose availability (availability to consumption ratio of 5.26) is the lowest of all the devices, making it disadvantageous in this parameter. Its Reynolds number (0.056) is the same as that of Design 1, making it advantageous for cell signaling,
while its oxygenation ratio (54.7) is also the same as Designs 1 and 3 and higher than Design 2, making it advantageous for oxygenation relative to Design 2.

We were able to show that both HPCs and HepG2 cells can be kept viable and express hepatocellular function in microfluidic environments, in devices based on designs 1 (HPC cell cultures) and designs 2 through 4 (HepG2 cell cultures).

The physical phenomena of the microscale may allow for a more in vivo-like environment for cell culture and the culture of more in vivo-like liver tissue for transplantation, extracorporeal therapy, drug screening, biological studies, and other applications. Here we have studied the effects of placing hepatic progenitor cells and HepG2 cells in microfluidic devices, and shown that the cells do survive and express the hepatocellular function of albumin secretion. This research may assist in future studies with microfluidic in vitro liver cell cultures through having helped answer some basic biological questions and test various methods of operating the microfluidic devices.
References


