Fundamental Studies of Liver Cell Culture Microtechnology and Techniques for the Purpose of Designing a Liver Model

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Abstract

Fundamental Studies of Liver Cell Culture Microtechnology and Techniques for the Purpose of Designing a Liver Model

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An authentic human liver model has the potential to be a revolutionary development in the field of biotechnology, and the progress of microtechnology has made this idea a reality. This technology enables the construction of micro-environments that can closely mimic the architecture and environment of human liver tissue in vivo, which is the key to building a physiologically relevant model. To this end, novel platforms have been produced with the goal of eventually building an authentic human liver model. A simple, single channel has been used for preliminary work with primary rat hepatocyte culture. Despite some successes, this system has shown several flaws, which include leakage and inconsistent viability. In order to rectify the leakage issue, a novel clamping system was devised, and cell culture tests have illustrated its capability and reliability in preventing leakage. Additionally, an initially open channel system was devised as an attempt to solve viability issues. We have also developed a novel dual channel system for co-culture of both types of liver cells, and initial tests are promising. This platform is capable of supporting this co-culture, although not consistently. Primary hepatocyte cultures in this system also led to an accidental discovery about the improved viability and cell contacts when cells are in tight confinement. Another important aspect in developing a liver model is obtaining physiologically relevant oxygen levels. To this end, oxygen diffusion simulations were constructed in COMSOL to determine the potential levels in the

novel dual channel system. These results are promising and need to be validated through an oxygen fluorescence experiment. A perfusion system has been constructed for this experiment, and initial tests have been run to determine the proper amount of dye for an adequate fluorescent signal.

CHAPTER 1. INTRODUCTION & MOTIVATION

1.1 . MOTIVATION AND BACKGROUND

The liver is the largest solid organ in the body and is involved in a myriad of metabolic processes required for body homeostasis as well as the detoxification of harmful chemicals[1]. The liver also plays a significant role in the body's immune response by inducing antigen response or removing certain immune cells from the blood. While much is known about the functions of liver cells, there is still much to be learned about the molecular mechanisms that control the activities of liver cells. This gap in our knowledge underlies the need for a physiologically relevant human liver model. Current fundamental liver biology studies predominantly rely on *in vitro* tissue culture models, but in these set-ups, the cells do not maintain their normal liver functions due to a lack of appropriate cellular microenvironments[2]. There are several interesting liver models that more closely mimic in vivo conditions, and one major example is the packed-bed reactor, in which hepatocytes are perfused into a silicon scaffold that allows for the cells to organize into a 3D structure. However, cells cultured in this arrangement have been shown to be distinct from in vivo liver tissue[3].

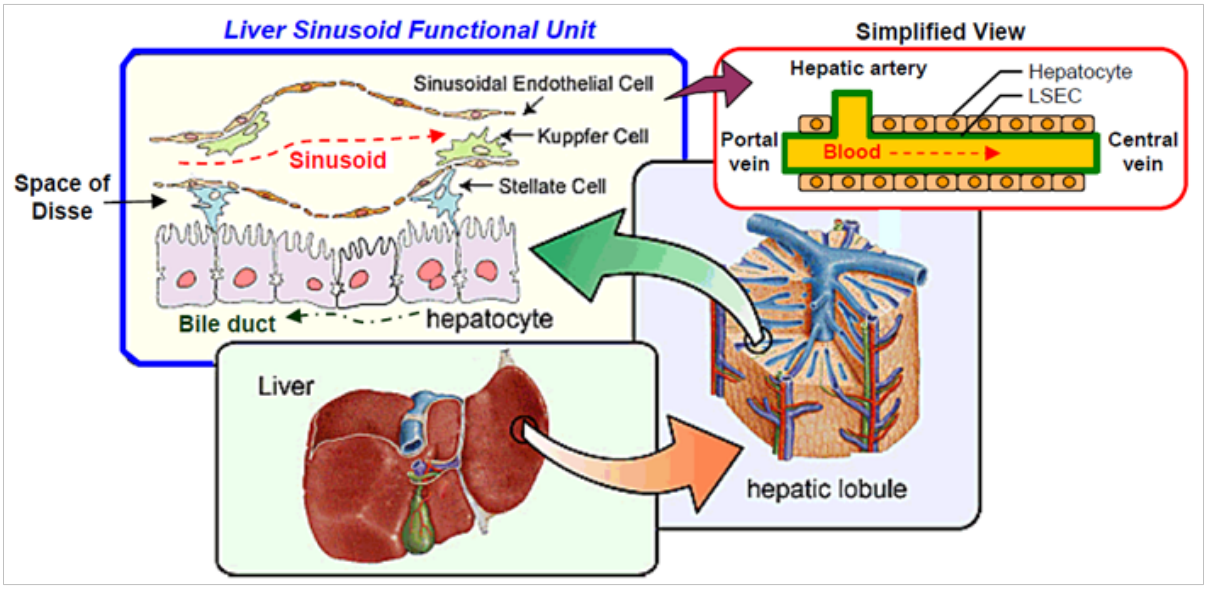

Figure 1.1. Images of liver and liver sinusoid. (Adapted from www.akaike-lab.bio.titich.ac.jp)

The main goal in constructing an authentic liver model is to duplicate the in vivo architecture of the liver, which requires mimicking the structure of the fundamental unit of the liver, the liver sinusoid, as can be seen in **Figure 1.1** and **Figure 1.2**. This fundamental unit consists of the hepatic sinusoid, the neighboring cells, and bile ducts. The hepatic sinusoid is an approximately 10-micron wide capillary that is between 225 and 475 microns long, and it receives blood from the intestines, pancreas, spleen, and gallbladder[4,5].The flow of blood through the sinusoid has a flow rate of approximately 6 µl/s, and it's flow profile is that of a parabolic flow with a flattened profile in the center [6].This capillary is lined by liver sinusoidal endothelial cells (LSEC) with minority cells present. Moving radially outward, the LSECs are surrounded by hepatocytes, and these two cell types are separated by the extracellular-matrix-protein-enriched Space of Disse through which the hepatocytes extend microvilli that expose the hepatocytes to higher levels of oxygen and important nutrients. The hepatocytes are typically

about 18 microns thick, and the Space of Disse is usually around 1 micron in thickness[7,8]. Hepatocytes naturally secrete bile, which is removed from the liver through bile ducts[9]. The hepatocytes are also the source of urea production through ammonia metabolization, and this results in a urea concentration in the blood of 3-6.5 mmol/L[8]. The hepatocytes are also responsible for the synthesis of many transporter proteins, the most significant of which is albumin; hepatocytes synthesize enough albumin to result in a blood concentration of 3.4-5.4 g/dL[10]. Also, the hepatocytes consume oxygen, creating a decreasing oxygen gradient along the length of the sinusoid; this results in partial pressures around 65 mmHg near the inlet and 35 mmHg near the outlet[11].

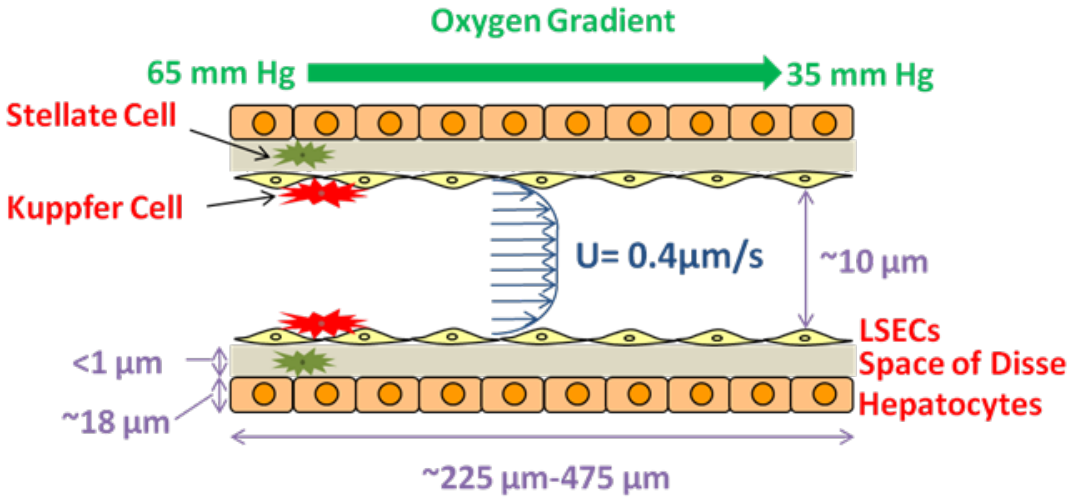

Figure 1.2. Architecture of Liver Sinusoid.

1.2 . RESEARCH OBJECTIVE

The research objective of this project is to fabricate an authentic liver model that closely mimics the human liver sinusoid unit, the location of most liver activities. Novel microfabrication and microfluidic technology will be combined with liver cell culture techniques to develop this

human liver model, which will enable the liver cells to retain the phenotypes that occur in the body. This model will enable the study of the underlying molecular mechanisms that dictate the morphology and behaviors of the cells. Specifically, there are three aims for this project, as listed below.

1. *To build a liver sinusoid functional unit with primary rat cells using microfabrication and cell culture technology due to their relative availability*

The liver sinusoid functional unit will be developed in a systematic process using the two main cell lines that the sinusoid is made of: primary rat hepatocytes(PRHs) and liver sinusoid endothelial cells(LSECs). This process includes the construction and testing of three separate configurations, as is shown in **Figure 1.3**.

The first configuration consists of a single polydimethylsiloxane(PDMS) channel laid on a collagen-coated tissue culture dish, in which the different cell types are layered. In this channel, the PRHs are first cultured directly on the surface of the dish and allowed time to settle down and attach. After the cells have attached, collagen is perfused over the hepatocytes so that a second layer is formed over the PRHs, which mimics the Space of Disse. Finally, the LSECs are pipetted over the separating layer and allowed time to attach. The theoretical architecture of the cells in this configuration is intended to mimic the architecture of the liver sinusoid functional unit, and the health and function of these cells will be tested through specific cell markers. The health and function of the hepatocytes can be tested through the production of transferrin, albumin, and hepatocyte nuclear factor 4, while the LSECs can be tested through markers such as the von Willebrand factor. Once this layered co-culture has been successfully tested under static and dynamic flow conditions, then the second configuration can be tested.

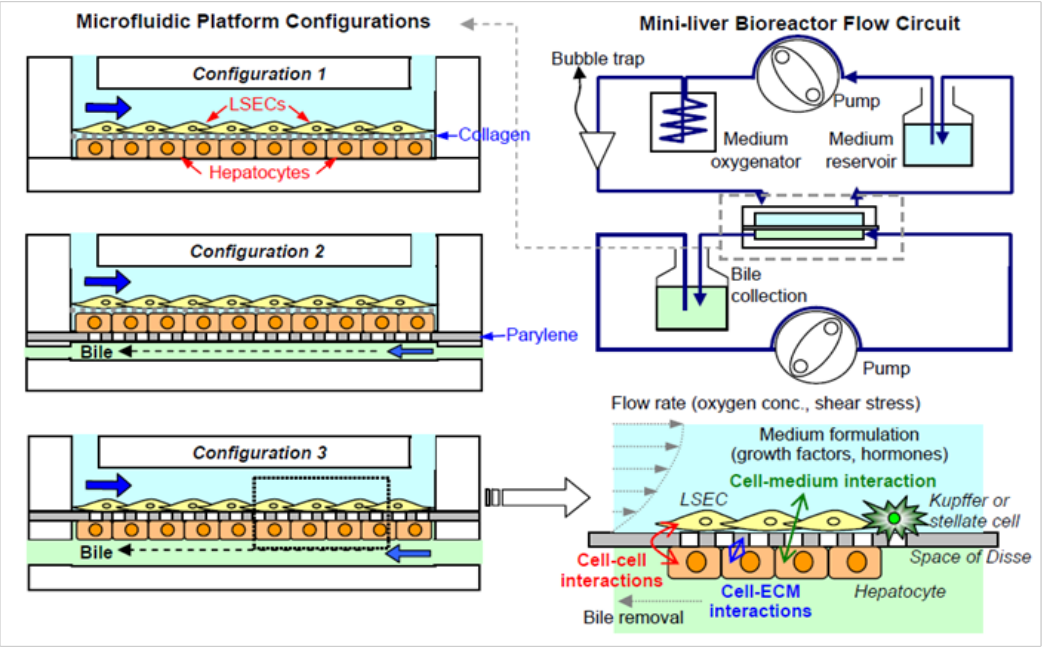

Figure 1.3. Three different culturing platforms for co-culture of hepatocytes and LSECs along with flow circuit used to provide media flow to the cells.

The second configuration adds to the utility of the first configuration by adding a bile removal mechanism, which requires the addition of a second PDMS channel that is bonded to the other channel. These two neighboring channels are separated by a microporous membrane made of either parylene or polyester. In this configuration the cells are layered in the top channel as they were in the single channel of Configuration 1, but the difference is that the cells are cultured on a microporous surface that allows for the diffusion and removal of bile through the bottom channel. Once the cells have been successfully cultured in this arrangement, they can be tested under static and dynamic flow conditions, using the same cell markers as before to test the viability and functionality of the cells.

It is possible that the previously described configurations will not be successful cell culture models, and in this case, a third configuration is proposed. This configuration consists of

the same arrangement of dual PDMS microchannels as is described in Configuration 2. In this case the cells are cultured on opposite sides of the microporous membrane, which can mimic the Space of Disse in the liver sinusoid. This allows simple control of the separation distance between the cells, and this distance can be varied to determine the optimal cell culturing conditions. Once a co-culture of both liver cells is obtained in this system, it can then be tested under static and dynamic conditions, measuring the same cell markers as before.

2. To construct a human liver model using the experimental conditions from Aim 1.

Due to the limited availability of human liver cells, the liver sinusoid model is going to be fully developed using primary rat cells. However, once the utility of the model has been fully tested with rat cells, the model will then be tested using primary human cells. It is difficult to say at this point what changes will need to be made to the system, but the system will be adjusted accordingly to better fit the needs of primary human cells.

3. To test the model through a focused study on the effect of Hepatitis B replication on hepatocyte signaling pathways.

Once the human liver model has been developed, the utility of it will be tested through a study of the effect of HBV replication on human hepatocytes. Our model should provide us an effective platform for this viral replication study because it duplicates the normal architecture of the liver sinusoid and it enables direct infection of human hepatocytes, which avoids the pitfalls of more laborious processes. The first step in the viral replication study is determining the optimal concentration of purified virus to add to the channel, and this is done by varying the amount added. Once this has been determined, the liver model will be used to test if certain kinases, such as Pyk2, FAK and Src, are activated by HBV replication. This result has been observed in other experimental configurations and is hypothesized to occur in this configuration as well.

Only limited progress has been made toward the aims of this project, and the focus of the current thesis is work done towards to goal of achieving Aim 1. The specific aims of this thesis are enumerated below.

1. To develop and improve the design of single channel microchannels to overcome the current challenges

Our cell culture results in single PDMS microchannels have proved to be inconsistent so much of our efforts have been dedicated to solving the issues that have been faced. One of the most significant problems is that we have not been able to consistently achieve a confluent layer of PRHs in a single channel, and this could be due to a variety of reasons. In order to fix this issue, several tests have been performed. One test was varying the initial concentration of the cell suspension. Another test that has been performed is varying the time post-cell seeding that culture media is first changed for the cells. Also, we have tried a new method of cell culturing, in which the cells are first cultured in an open channel environment, and then each channel is later covered with a microchannel. Another major challenge that has arisen is leakage from the non-bonded PDMS channels. When we perform single channel cell culture, the PDMS channel is just laid down on a collagen-coated tissue culture dish, and the sealing of the channel to the dish is achieved by the natural adhesive properties of PDMS. However, in this arrangement, significant cell leakage has been observed recently. Experiments have been performed to ascertain the reasons for this leakage, and novel clamping devices have been designed and built to seal the channels to the plate.

2. To develop and test a layered co-culture of liver cells in a dual channel system

In order to achieve Aim 1 of the project, each configuration that was detailed needs to be tested. In order to make progress towards this goal, preliminary cell culture tests were done with Configuration 3 in order to determine the viability and optimal conditions of these configurations.

Additionally, an interesting observation was made during the testing of Configuration 3 about the viability of PRHs in tighter micro-environments.

3. To optimize the oxygen levels in microchannels seeded with liver cells

One of the important aspects of the liver sinusoid is the oxygen concentration. In the liver, there is an oxygen gradient that exists along the length of the sinusoid, and this is important to the morphology and function of the liver cells. In order to achieve a physiologically relevant liver model, this oxygen gradient must be duplicated in our liver model. To this end, a dual channel simulation was performed with the geometry of Configuration 3 to determine if physiologically relevant oxygen levels can be achieved with it. In order to verify the oxygen levels found in the simulations, a perfusion system was assembled, and preliminary work was performed to prepare for the validation experiments.

1.3 . CURRENT LIVER MODELS REVIEW

1.3.1. Overview of Types of Liver Models

There are three general categories that the existing liver models can be broken down into: two-dimensional, layered, and three-dimensional. The two-dimensional models are defined by a monolayer of one or more types of cells. The three-dimensional models are defined by cells organized in a three-dimensional manner, in which a singular cell type is structured over all three dimensions. The layered models can be considered three-dimensional, but in this review, they are considered a different group of models because they are just several two-dimensional layers stacked on top of each other. All three groups of liver models can be tested under static and dynamic flow conditions, and they all offer the opportunity for mono- or co-culture of cells.

1.3.2. 2-Dimensional Models

One of the simplest set-ups for culturing liver cells is a monolayer culture of hepatocytes in a single microchannel. In general, these channels are bonded or sealed to a glass slide or a

tissue culture dish that is coated with extracellular matrix, which is typically collagen. Hepatocytes cultured in this arrangement are able to survive a few weeks in static culture and at least a few days in dynamic conditions.

The two-dimensional culture has several advantages over other systems. One advantage is that it is a simple method of achieving a homogeneous cellular environment. Also, the system allows for contact between the cells and the media, which removes concerns about diffusion of important nutrients to the cells. Additionally, when connected to a perfusion system, this set-up allows for the easy formation of oxygen and hormone gradients along the length of the channel, phenomenon that are known to occur along the liver sinusoid. Dr. Allen and Bhatia were able to achieve physiologically relevant levels of oxygen using a flat-plate culture with PRHs; the oxygen partial pressure decreased from 76 mmHg at the inlet to 5 mmHg at the outlet. After inducing an oxygen gradient by controlling the flow of oxygenated media through the channel, they were also able to induce heterogeneous distribution of certain enzymes, such as phosphoenolpyruvate carboxykinase and cytochrome P450 2B, and this distribution correlates well with the liver conditions in vivo. They were able to achieve measurement of this distribution of enzymes by performing Western Blot analyses at 4 separate sections of the bioreactor[12].

Despite its advantages, one of the main disadvantages to a two-dimensional cell culture is the high shear stress that cells are exposed to when media is flowing through the channel[13]. A research group at Harvard Medical School performed some experiments to determine the effect of shear stress on the viability of hepatocytes. They performed a co-culture of PRHs with 3T3-J2 fibroblasts in a flat-plate bioreactors and provided media flow over the cells. The flow was varied for different channels, and the albumin and urea synthesis rates were measured for each channel to measure the effect of shear stress. The results of this experiment displayed that the hepatocytes exposed to lower shear stress(0.01 to 0.33 dyn/cm^2) produced albumin and urea at a rate 2.6- and

1.9 times greater than those experiencing higher stress(5 to 21 dyn/cm^2)[14] One group's idea to overcome the problem of shear stress while being able to use a wider range of flow rates was to introduce micro-grooves into a microchannel, and the schematic for this system can be seen in **Figure 1.4**[15]. In this set-up, the hepatocytes are cultured in between the grooves, which are 55 micrometers tall. In this way the cells do not experience the full shear stress because they are only directly facing a greatly reduced flow. This group performed a co-culture of PRHs with 3T3-J2 fibroblasts in this system and also performed the same culture in a microchannel without the pillars. Media flow was provided to both channels, and the albumin and urea production rates were measured over a five-day period. The results of this experiment were that the hepatocytes synthesized urea and albumin at stable rates when they were protected by the pillars, but these same rates decreased when the cells were not protected. This experiment shows the potential of microgrooves in reducing the detrimental effect of shear stress on the health and viability of hepatocytes.

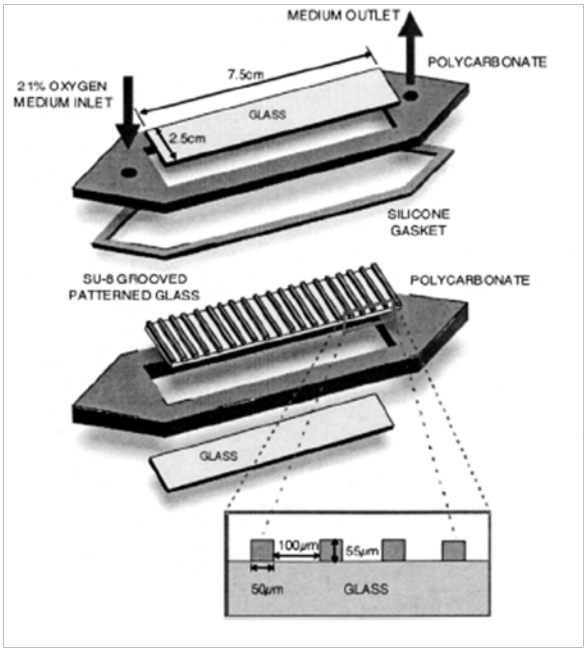

Figure 1.4. Schematic of the micro-grooved substrate[15].

Despite the advantages of two-dimensional cell culture, it is not a viable option for a physiologically relevant liver model. The main issue is that it does not accurately capture the architecture of the liver sinusoid. The liver sinusoid is a capillary lined with LSECs, and the LSECs are bordered radially by an extracellular matrix-enriched Space of Disse and then a layer of hepatocytes. In order to capture this structure, at least two layers of the two different cell types are necessary. Also, there needs to be a layer in between that mimics the Space of Disse. Additionally, when flow is applied in monolayer systems to simulate the blood stream, the hepatocytes are directly exposed to the flow conditions, which is not the case in the conditions in vivo. The hepatocytes are actually shielded by the LSECs and Space of Disse.

1.3.3. Layered Models

In order to more accurately re-create the in vivo environment for hepatocytes, many groups have attempted a layered, or “sandwich,” configuration, in which the hepatocytes are

cultured in between two layers of extracellular matrix, typically collagen or Matrigel. As opposed to the typical flat-plate monolayer configuration, the hepatocytes are in an environment that more closely resembles the tighter micro-environment these cells experience in the body.

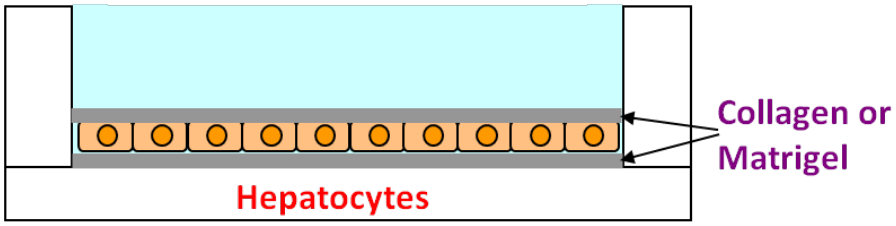

Figure 1.5. Schematic of hepatocytes in a sandwich configuration.

The results of several groups exhibit the great potential of this method of culturing hepatocytes. When hepatocytes are cultured in between two layers of collagen, a plate-like structure is formed that can retain its viability for months[16,17]. This method of hepatocyte culture also results in retention of albumin secretion and organization of the hepatocytes into a more in vivo-like structure. For example, in terms of in vivo-like structure, this method is able to promote the proper polarization of F-actin, cell adhesion molecules, and lateral and apical membrane polarity markers[18]. Additionally, it has been shown in these sandwich cultures that when hepatocytes are exposed to drugs, certain cytochrome P450 enzymes, such as CYP1A2, CYP2B1/2 and CYP3A4, are induced[19,20]. It also has been shown through certain experiments that the sandwich configuration is an optimal method for studying gene expression patterns for liver cells exposed to carcinogens, such as methapyrilene. Additionally, sandwich cultures performed in between both collagen and Matrigel have shown to have certain advantages over homogeneous sandwich configurations. For example, hepatocytes cultured in this configuration have been observed to express connexin 32, an epidermal growth factor receptor [21].

Many research groups have also extended this sandwich configuration by culturing a layer of endothelial cells on top of the monolayer hepatocytes, as many studies have shown the benefit of co-culturing these two liver cell types. Previous work had shown that co-cultures of endothelial cells and hepatocytes were able to maintain viability for several weeks and had resulted in many benefits, such as increased expression of cytochrome P450 enzymes[22]. Harimoto et al. developed a novel method for co-culturing endothelial cells directly on top of hepatocytes. This was achieved by culturing the endothelial cells on a thermo-responsive culture dish grafted with poly (*N*-isopropylacrylamide). The endothelial cells attach to the thermo-responsive dish at a lowered temperature of 32 degrees Celsius, and these cells can then be transferred onto a layer of PRHs because the endothelial cells release from the dish when the temperature is raised to 37 degrees Celsius. From this layered co-culture, it was observed that the PRHs in the layered culture maintained proper morphology and significant rates of albumin secretion much longer than PRHs in a monolayer culture. Additionally, it was shown that, in the layered configuration, the cell junctions were well-maintained and that the secretion of extracellular matrix was much more pronounced[23,24]. Another group developed a different method for layering LSECs and PRHs, in which a chitosan–hyaluronic acid polyelectrolyte multilayer (PEM) ranging from 30 to 55 nanometers is introduced between the two layers of cells. PRHs and LSECs were cultured in this and other similar configurations in a series of experiments. One part of their experiments focused on comparing homogeneous cultures of PRHs. The results of this part of the experiments exhibited that much higher albumin and urea rates are observed in PRHs in a sandwich configuration of just collagen or collagen and PEM than PRHs cultured in a monolayer. Further experiments were carried out with co-cultures of PRHs and LSECs in a couple of configurations: LSECs cultured directly on top of a monolayer of PRHs and LSECs cultured on PRHs sandwiched by PEM. Albumin secretion rates were highest

in co-cultures with a PEM and in monocultures in a collagen sandwich, and urea production rates were very similar among all the different co-cultures but were greater for PRHs in a collagen sandwich. Addtionally, bile caniculi were well-formed in the PRH-PEM-LSEC and PRH collagen sandwich but were sparse in the PRH monolayer and the PRH-LSEC co-culture with no barrier. This indicates that the differentiated function of liver cells is much better in cell cultures where the hepatocytes are sandwiched by collagen or PEM. Finally, CYP1A1/2 isoenzyme activity, which is important in metabolism of toxins, was significantly higher in the PRH-PEM-LSEC culture than in any of the other set-ups, which may indicate the interactions of the two cell types in an in vivo-like environment is key to the induction of these enzymes[25].

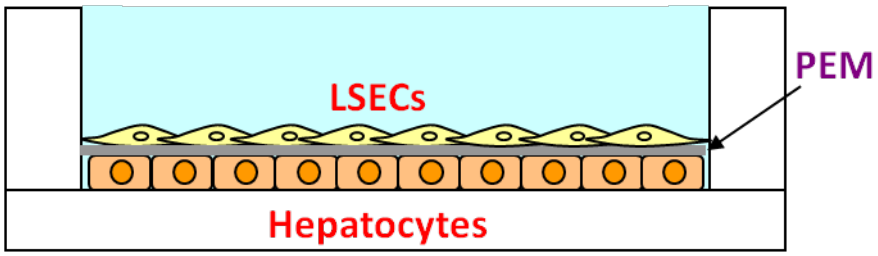

Figure 1.6. Schematic of hepatocytes in a sandwich configuration.

The potential of layered co-cultures of hepatocytes and LSECs is promising for the development of a biomimetic liver model due to the retention of in vivo cell morphology and function. However, the aforementioned models need to be reduced to a controlled environment on a smaller scale in which flow can be easily introduced. The flow is important so that the blood stream in vivo can be duplicated; it is also important for the removal of bile. Additionally, the size of the surrounding environment is important in achieving the in vivo architecture of the micron-scale liver sinusoid. The use of an intermediate PEM between the two cell types is promising because of its expression of enzymes key to detoxification; however, in our

configuration, we use an intermediate layer of collagen or Matrigel. The work done by Kim et al. does show higher expression of these enzymes for co-culture with PEM in between, but they are only comparing it to a collagen sandwich of simply hepatocytes. They clearly show that LSECs play a role in the induction of these enzymes and to neglect the LSECs in the collagen sandwich leaves the superiority of either method in question. Additionally, in their experiments, PRHs in the collagen sandwich do produce albumin and urea at slightly higher rates than co-culture with PEM in between.

1.3.4. Three-Dimensional Models

1.3.4a Three-Dimensional Scaffolds

A major development in the area of three-dimensional liver models is the three-dimensional scaffold, which can be made from a variety of materials. These scaffolds are used to obtain a more three-dimensional morphology of the cells, similar to the three-dimensional conditions in vivo.

One example of a three-dimensional culture of liver cells is the micro-array bioreactor developed by Powers et al. They developed a cell culture platform in which holes are etched into a silicon wafer, and this wafer is attached to a similarly-cut steel plate with a micro-filter in between; a schematic of this platform can be seen in **Figure 1.7**. In these etched holes, spheroids of PRHs are cultured and this platform is enclosed in an environment in which media flow can be provided to simulate the blood stream. Hepatic spheroids cultured in this platform showed positive results that exhibited the cell culture potential. The PRHs were able to maintain stable urea and albumin synthesis rates throughout the two-week period of culture, and microscopy revealed that tight junctions and bile caniculi were formed amongst the cells[26,27].

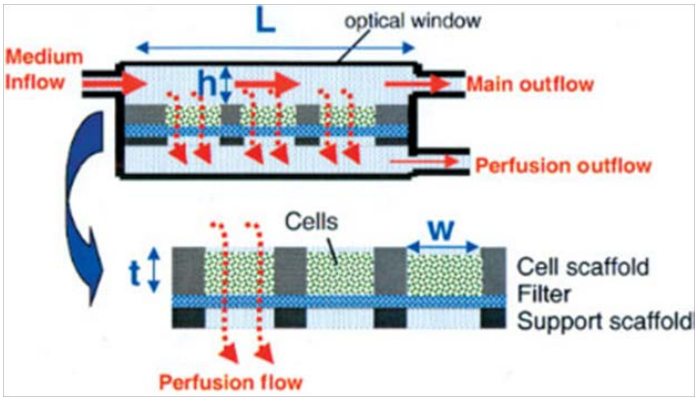

Figure 1.7. Schematic of the micro-array for 3D cell culture[13].

Another three dimensional culture of hepatocytes was performed using scaffolds of hyaluronic acid that were enriched by deposition of extracellular matrix. Initially fibroblasts were cultured on the scaffolds over a period of three weeks, and, during this time, the fibroblasts secreted collagen, laminin, and fibronectin onto the scaffolding. Once this process occurred, PRHs were cultured on the scaffolding, and they attached in large numbers. In this experiment, after the PRHs attached, they exhibited a normal polyhedral shape, and they were well-integrated into the interstices of the scaffold. Additionally, the cells re-established contact and formed aggregates and cords along the length of the fabric of the scaffolding. The hepatocytes also secreted significant amounts of albumin and urea over a two-week period, but these rates were observed to be continuously decreasing[28].

A hexagonally-meshed scaffold has also been developed, and these scaffolds are made of either of one of two biodegradable polymers, PLGA, poly-DL-lactide-co-glycolide, or PLLA, poly-L-lactide. These scaffolds, which are displayed in **Figure 1.8**, are coated with an alginate film to protect the cultured cells from mechanical stress and to enhance nutrient diffusion. HepG2 cells, an immortal hepatocellular carcinoma cell line, were tested on these scaffolds, and the experiments showed that the HepG2s in these scaffolds produced albumin and urea at similar

rates to monolayer cultures. Also, unlike the cells cultured in a flat monolayer, the cells cultured in the scaffolds were able to maximize cell-cell interaction and cell density [29].

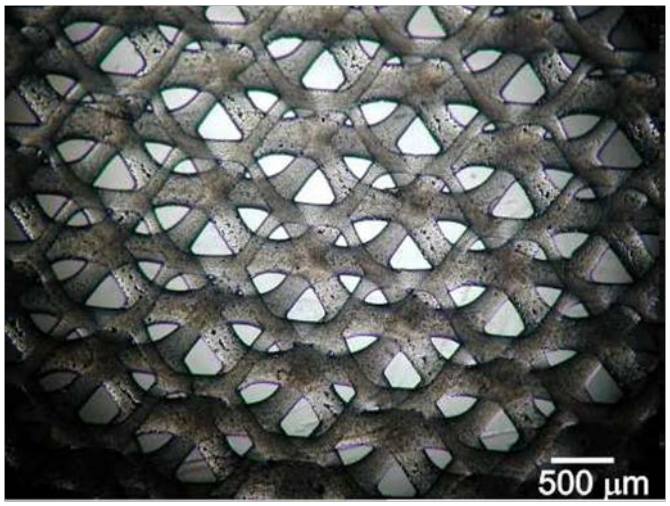

Figure 1.8. Image of the biodegradable and hexagonal polymer scaffold[29].

Three dimensional culture of hepatocytes was also achieved by culturing on an array of PDMS pillars, serving as a type of scaffold. The PDMS was patterned so that micro-pillars are formed on the bottom surface of a two-piece PDMS microchannel, and a schematic of the device can be seen in **Figure 1.9**. When cultured in this configuration, PRHs were able to attach to the pillars and maintain typical three-dimensional morphology. The PRHs were also proven to be able to achieve functional membrane polarity, which was shown through the active gap junctions and bile canalicular network[30].

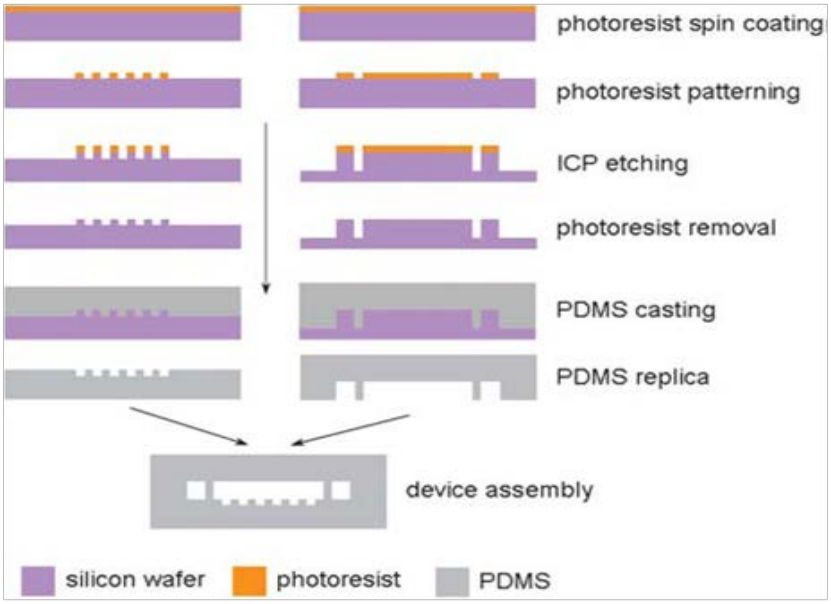

Figure 1.9. Schematic of the PDMS micro-pillars for 3D cell culture[30].

A scaffolding platform has also been developed for co-culture of PRHs and LSECs, and it was designed to fit into the wells of a 12-well plate. In each well there are two open wells; one well contains the scaffold where the cells are cultured and the other is a reservoir well that holds cell culture media. The media is circulated between the two wells via a micropump, as can be seen in the schematic in **Figure 1.10**. The scaffold on which the two liver cell types are cultured are ECM-coated and contain an array of 769 channels. The success of this cell culture was determined by the albumin synthesis rate of the hepatocytes and the marker SE-1 for LSECs. Both markers were observed to be maintained for at least one week in the co-culture configuration[31].

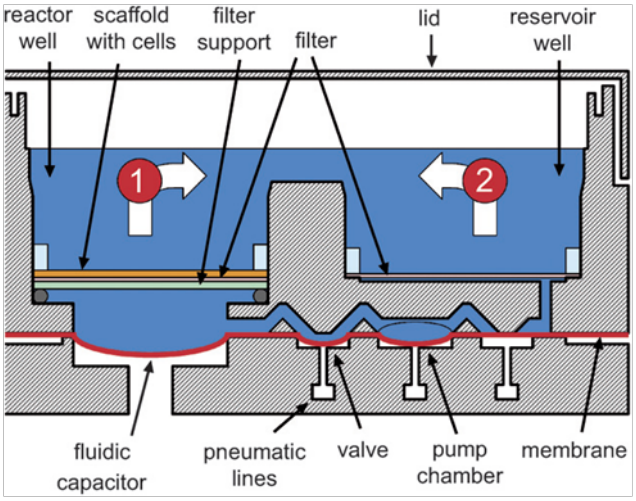

Figure 1.10. Schematic of a single well for the 3D culturing 12-well plate[31].

All of these three-dimensional methods for culturing are viable methods for achieving a three-dimensional morphology and achieving significant cell-cell junctions and bile canicular networks. However, there are still some issues with these models that need to be addressed to achieve a true liver model. These models do not achieve the level of organization of the liver sinusoid where there are layers of LSECs and hepatocytes separated by an intermediate layer of protein-enriched extracellular matrix. Additionally, these models expose the hepatocytes to unnecessarily high rates of shear stress because they are left open to the passage of flow, and it is a major challenge to provide uniform perfusion to these cells to achieve the oxygen and nutrient gradients that exist in the liver sinusoid in vivo.

1.3.4b Photopatterning of Three-Dimensional Hepatic Tissue

Another major development in the area of three-dimensional biomimetic tissue models is the photopatterning of cells in hydrogels. This process involves mixing the cells with a pre-polymer solution of the hydrogel and a photoinitiator, among other materials. When this solution is exposed to UV light at certain intensities, it can be solidified, forming a solid three-dimensional

structure of the cells in the hydrogel. When this UV light exposure is combined with a photomask, these layers can be selectively patterned. One of the most widely used hydrogels for this process is poly(ethylene glycol)-, or PEG, based because of its biocompatibility, hydrophillicity and ability to be chemically altered. PEG-based hydrogels have already been proved to be capable of immobilizing a variety of cells, and the addition of different materials has shown to improve its cell culture viability. For example, the addition of adhesion domains of ECM proteins have been proven to promote cell adhesion, and growth factors have been able to control cell function[32,33].

It has been shown by a few groups that PRHs can be successfully co-cultured with 3T3-J2 fibroblasts using three-dimensional microscale patterning techniques. Underhill et al. were able to successfully pattern healthy PRHs in circular structures, which were then surrounded by patterned fibroblasts[32]. The schematic for the photopatterning process can be seen in **Figure 1.11**. Tsang et al. were also able to

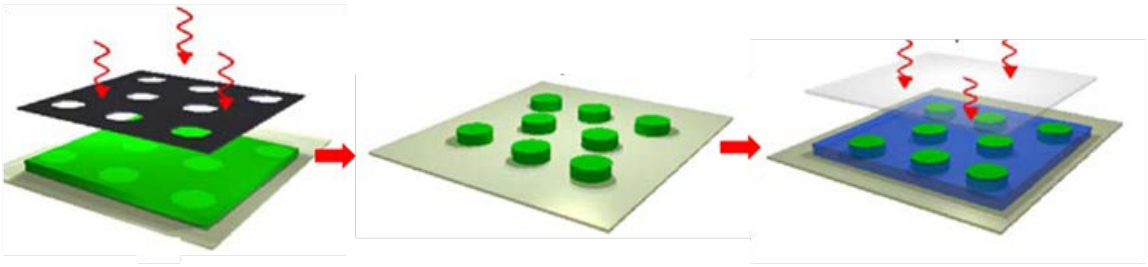

Figure 1.11. Schematic of the photopatterning process[32].

fabricate patterned structures of primary hepatocytes in co-culture with fibroblasts but in a more complex pattern that allowed for enhanced convective diffusion. This arrangement involved multiple layers of hepatocytes in a three-dimensional arrangement, and the schematic of its structure can be seen in **Figure 1.12**. These photopatterned hepatocytes were then maintained in a bioreactor, and the cells survived for a period of two weeks under the application of flow. Urea

and albumin rates were measured for this configuration, and the results were mostly positive. The albumin rates became stable after several days but were lower than comparable albumin rates in a monolayer co-culture of the 2 cell types; however the urea rates were stable throughout and were similar to the rate in flat plate culture[33].

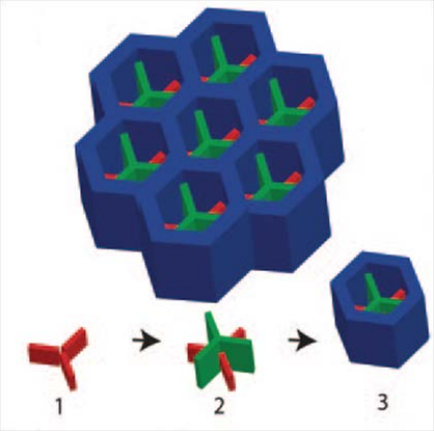

Figure 1.12. Schematic of the multilayer photopatterning process[33].

The photopatterning of liver cells offers great promise for the future. It enables the precise control of a three-dimensional model of liver cells and has shown to be able to maintain strong viability when flow is applied. However, there are several current problems with this configuration. The current models still do not achieve the precise layered architecture of the liver sinusoid, which includes layers of primary hepatocytes and LSECs separated by a Space of Disse. Additionally, this configuration can create significant issues with oxygen and nutrient diffusion to the cells. Tsang's model does allow for additional convective diffusion due to its more open design, but it creates a more complex situation that increases the difficulty of achieving the oxygen and nutrient gradients that result in a structured differentiation of the hepatocytes.

1.3.5. Advantages of Proposed Model

The proposed liver model in Configurations 2 and 3 provides several advantages over the aforementioned models. First of all, these configurations enable well-controlled structure of hepatocytes and LSEC's. These configurations build upon the layered models, in which there are clearly defined layers of both types of cells but also add a well-defined microenvironment that enables proper flow conditions that more closely replicate the in vivo environment. The two- and three-dimensional models do not have such well-defined layers of both types of cells, which create a distinct difference from the in vivo architecture. These models also do not provide a membrane between the co-cultured cells to mimic the Space of Disse in the sinusoid. Our model also provides an extra channel to duplicate the bile ducts that are present in vivo. In addition to the well-replicated in vivo structure, the proposed model also provides a simple channel environment to re-create a single liver sinusoid and its subsequent molecular gradients. The three-dimensional models do provide a three-dimensional structure that is close to in vivo conditions, but it creates a environment that complicates the re-creation of key molecular gradients in the liver sinusoid. Our proposed model involves straight channels with a monolayer of each cell type, which means that there is only one clearly defined layer of cells affecting the molecular gradients.

CHAPTER 2. MONOCULTURE IN SINGLE CHANNEL

2.1 . CURRENT SET-UP

The fabrication and assembly methods for the current single channel cell culture is a simple process. First, sets of four PDMS microchannels must be fabricated through basic lithography techniques. This first requires SU-8 structures to be patterned in the shapes of the channels on a silicon wafer through a multi-step process. A silicon wafer is first kept in a bath of 2% hydrofluoric acid for 10 minutes to remove its oxide layer. After dehydration, SU-8 is spincoated on the wafer to the proper thickness, which is followed by a soft bake. Then, with a photomask placed on top of the wafer, the SU-8 is exposed to the UV light. Finally, following a post-exposure bake and developing, the SU-8 structures are formed on the silicon wafer, and the wafer is exposed to a silanization process that allows easy removal of material. With the completed SU-8 mold, PDMS resin is mixed in a 10:1 ratio with a curing agent and poured over the wafer. The PDMS is then degassed to remove bubbles and placed in an oven at 65 degrees Celsius for an hour to cure. The curing is the final step in the fabrication of the microchannels, which have been formed by the SU-8 structures casting an imprint in the PDMS. Once the channels have been made, they are cut out together in sets of four. These channels are then laid down on a 10-centimeter, plastic tissue culture dish that has been coated with Type IV collagen. The dish is coated by pipetting one milliliter of collagen on the surface and agitating the dish until it is completely covered; the dish is then left open overnight to allow the collagen solution to dry out and adhere to the surface of the plate. The PDMS channels adhere to this surface thanks to the interaction of Van der Waals forces, and in the past, this force was enough to seal the channels. With the PDMS adhered to the surface, the channels then need to be washed with 10X PBS, and once this is completed, the channels are ready for cell culture.

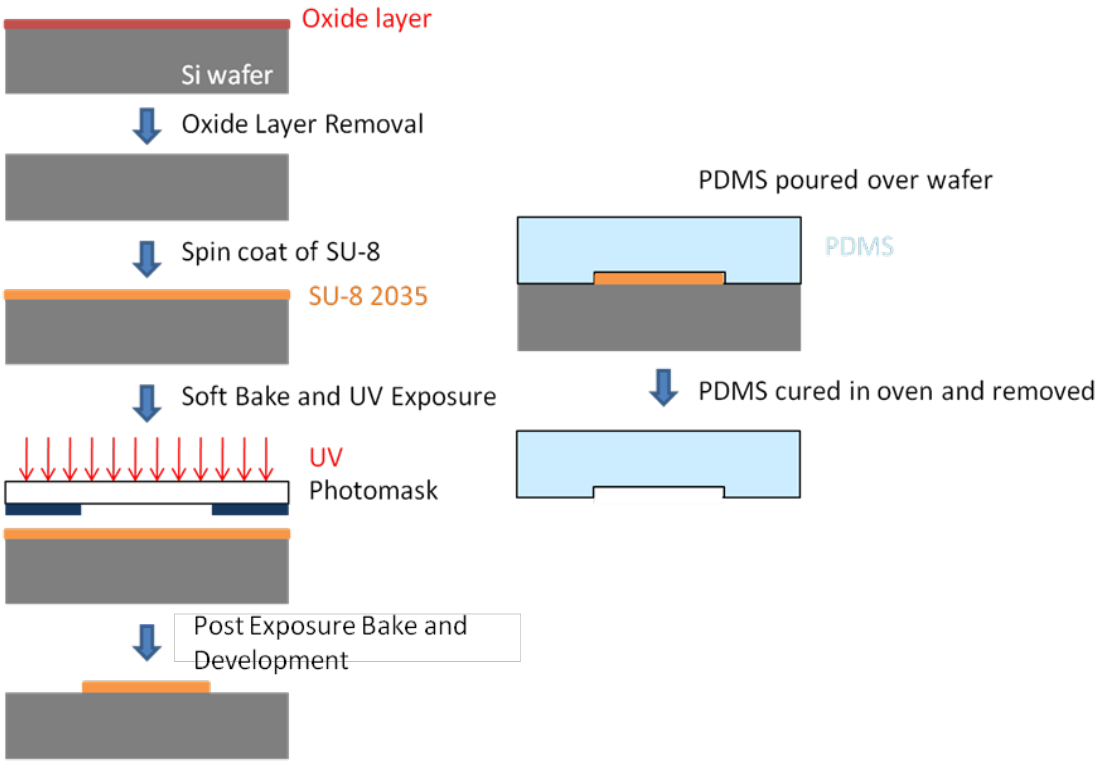

Figure 2.1. Photolithography and Soft Lithography Processes

Previous cell culture results show the capability of these singular microchannels. Temitope Sedunke et al. were able to culture both PRHs and LSECs separately in these channels, and both cell types retained liver specific markers. The PRHs produced both albumin and transferrin as well as HNF-4, and the LSECs showed markers such as KDR, VCAM1, and FLT-1[34]. Images of the cells can be seen in **Figure 2.2**.

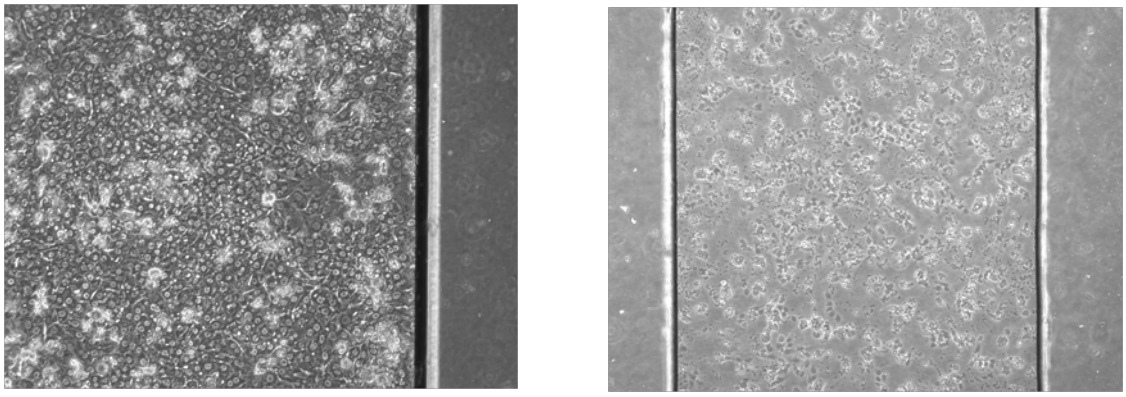

Figure 2.2. Images of primary rat hepatocytes(left) and LSECs(right) cultured in a single microchannel[34].

These same microchannels are also used for Configuration 1 which was outlined previously. Previous cell culture results by Temitope Sodunke show that these channels are capable of supporting a layered co-culture of primary rat hepatocytes and rat adrenal medulla endothelial cells(RAMEC) with a layer of Matrigel in between. The hepatocytes were directly infected with the GFP plasmid to prove their viability after being covered with RAMECs, and images of the cells and the fluorescent image can be seen in **Figure 2.3**.

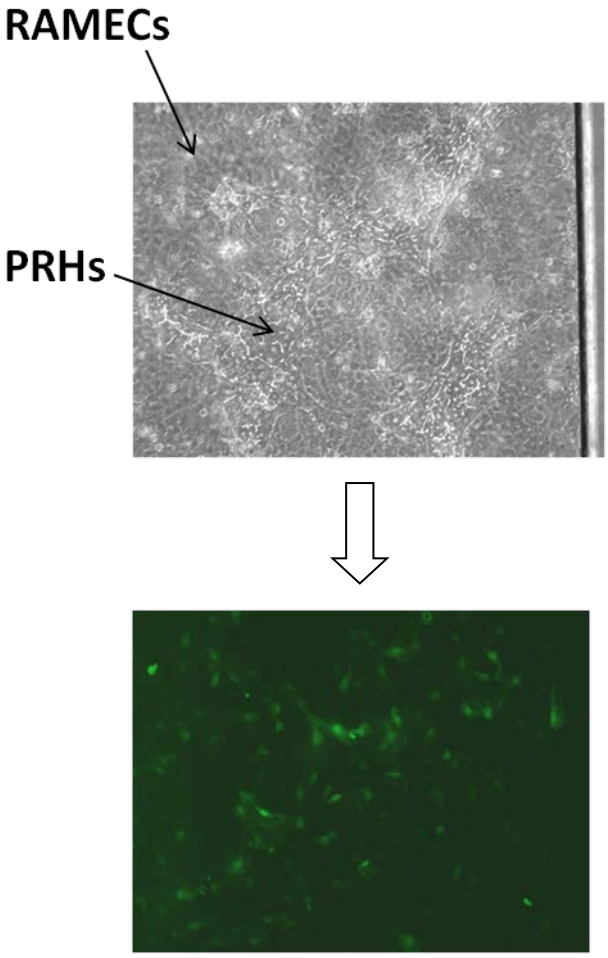

Figure 2.3. Images of primary rat hepatocytes and RAMECs(top) and fluorescent labeling of hepatocytes(bottom).

2.2 . EXPERIMENTAL RESULTS IN TECHNIQUE OPTIMIZATION

Despite the previous successes with culturing liver cells in these channels, a few persistent problems have arisen with continued cell culture in this system. One of these issues is insufficient number of cells attaching and surviving in the microchannel. Oftentimes, a confluent layer of hepatocytes is not being formed in these channels, and the cells are only surviving in patches. This is not adequate if we hope to continue the progress of liver cell culture.

Consequently, a few attempts have been made to optimize the cell culture process so that this problem is avoided.

First, PRHs were cultured in several of these singular channels, and their attachment was monitored via microscopic imaging. Pictures were taken at various times post-seeding to find the optimal time for the cells to attach before the culture media in the channel is changed. The progress of the cells after seeding can be observed in **Figure 2.4**. The PRHs start to attach almost immediately after seeding, and they take some time to spread on the surface. The images show that the PRHs exhibit signs of spreading out as soon as an hour after seeding and that the cells have sufficiently attached and spread out between three and four hours. As a result, it has been decided that the PRHs should be allowed 4 hours to settle and attach before exposing them to the shear stress induced by changing the culture media.

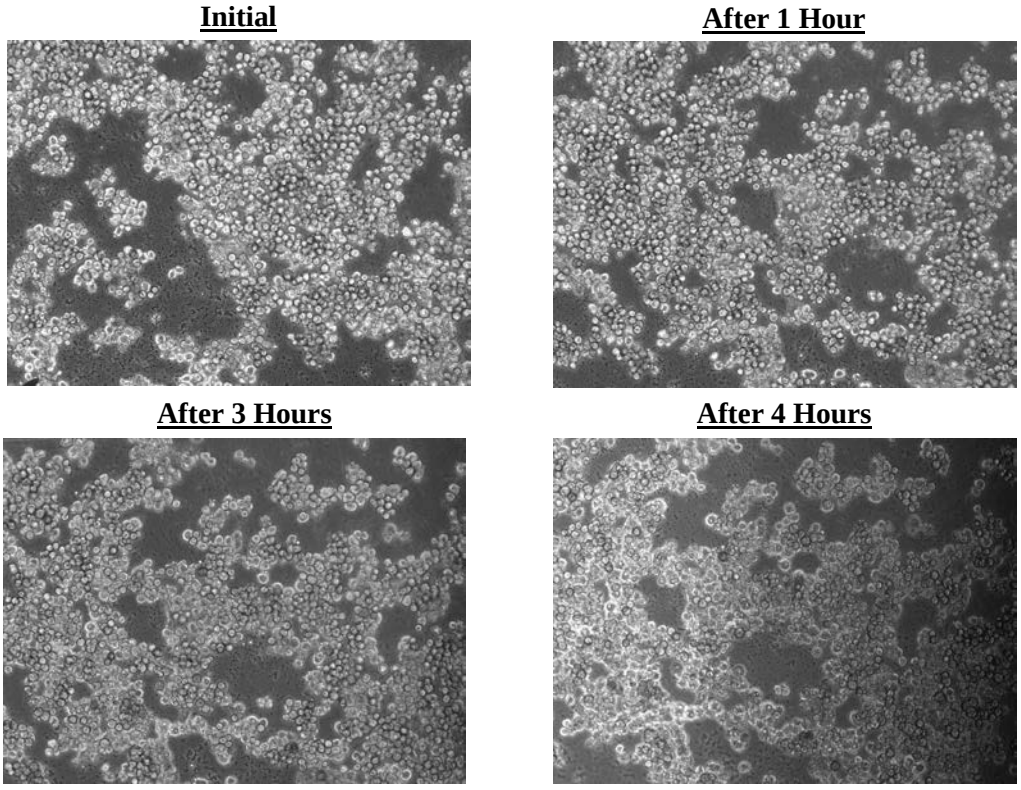

Figure 2.4. Images of primary rat hepatocytes at several time periods post-seeding.

Additionally, PRHs were cultured at a variety of concentrations in the cell suspension because the usual cell concentration was not producing a confluent layer of cells. It was hypothesized that a smaller concentration of cells is needed because cells cultured on open tissue culture dishes are cultured at lower concentrations and still form a confluent layer consistently. The PRHs were cultured at a variety of concentrations that were all a different fraction of the concentration used previously. Images of PRHs cultured at these various concentrations are displayed in **Figure 2.5**. Even at the highest concentration, which was a half of the original concentration, it can be easily observed that insufficient numbers of cells are attaching in the channel. Consequently, the initial PRH concentration must be maintained at a higher level to ensure enough cells are injected into the channel to achieve a confluent layer.

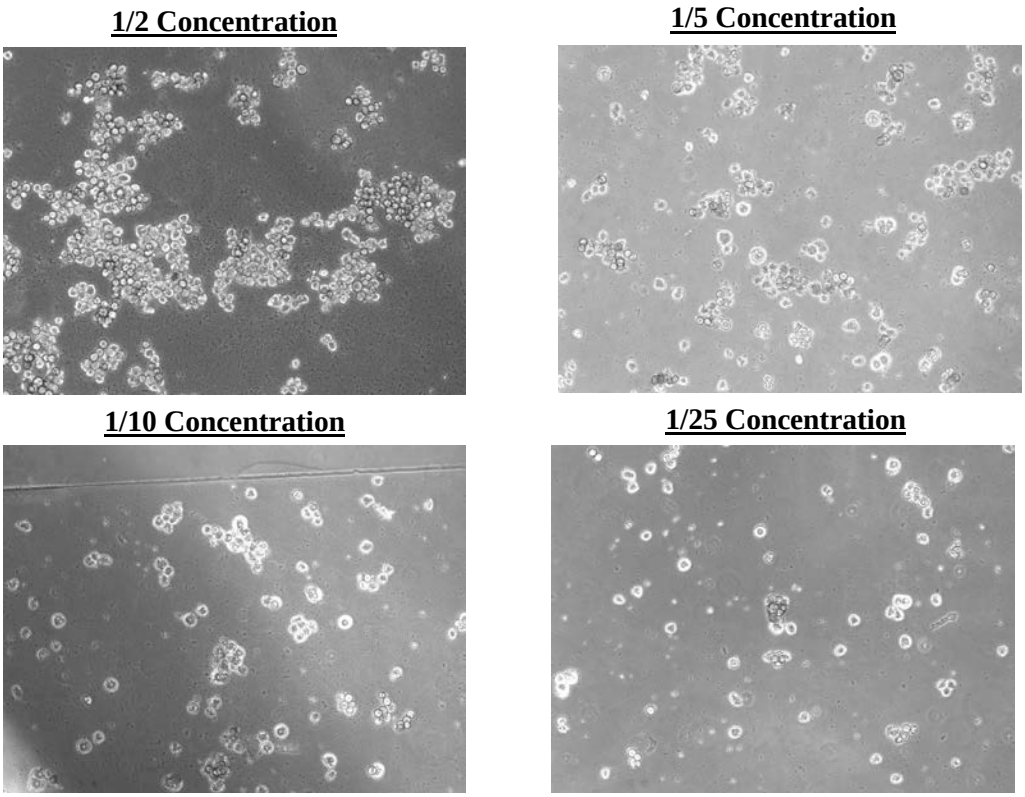

Figure 2.5. Images of primary rat hepatocytes 4 hours post-seeding at different concentrations.

2.3 . COMPARISON OF OPEN AND CLOSED CHANNEL CULTURE

Due to the persistent problems with the aforementioned singular microchannel, other alternatives for cell culture have been considered. One concept focuses on using an initially open channel due to the advantages of open plate cell cultures. PRH cultures performed on collagen coated tissue culture dishes have been shown to be much more consistent and successful than in microchannels. In the tissue culture dish, the PRHs settle down uniformly and are easily capable of forming a confluent layer on the surface, which differs greatly from our PRH culture in

microchannels. In the microchannels, the PRHs often settle down in patches and consequently, often do not form a confluent layer.

Despite the advantages of cell culture in tissue culture dishes, it is still not a viable option for a physiologically relevant liver model. However, the advantages in cell seeding can be utilized in a novel way. The new concept still calls for the microfabrication of PDMS channels, but in this case, the channels are cut out leaving an open channel behind. These open PDMS channels are then laid on a collagen-coated tissue culture dish, and the PRHs are cultured in these channels. After the hepatocytes have been allowed a day to attach to the surface and form a confluent layer, the microchannel that was cut out before is placed back in its original location in order to give the hepatocytes a more physiologically relevant micro-environment. Images of this progression can be seen in **Figure 2.6**.

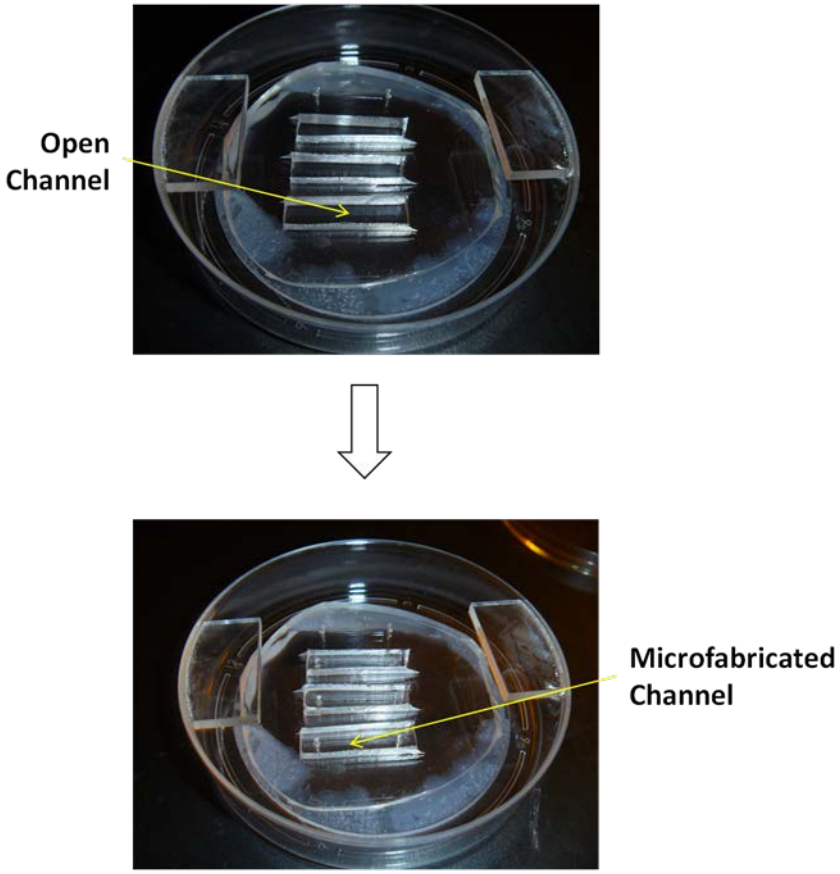

Figure 2.6. Open channels covered by microfabricated channels.

PRHs cultured in this arrangement showed positive results in a trial run. The cells maintained strong viability after being in the open channel for a period of 30 hours. After this time period, the microfabricated channel was placed over the cells, effectively closing off the channel, and the cells maintained strong viability and maintained approximately 80% confluency in the channel 24 hours after being covered. Images of the PRHs before and after being covered with the microchannel can be seen in **Figure 2.7**.

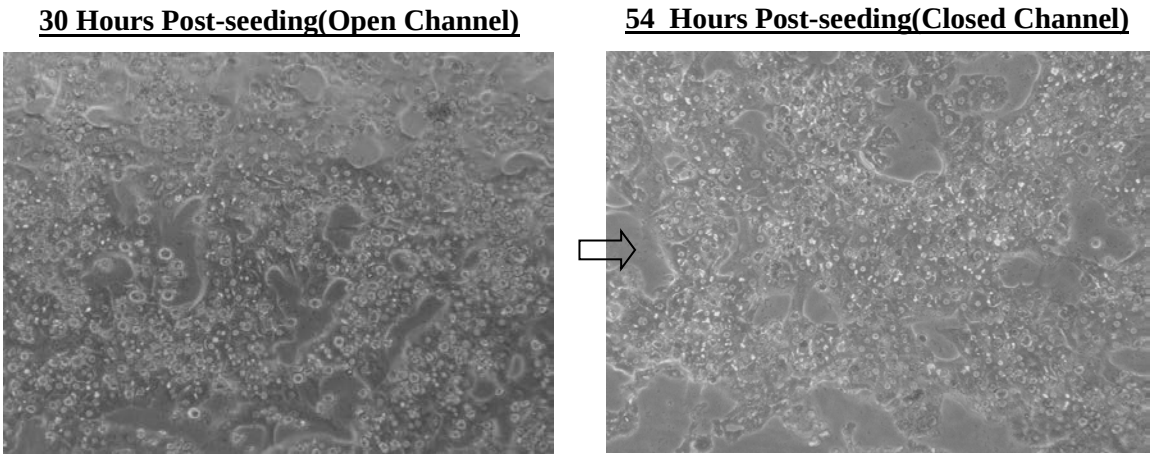

Figure 2.7. Primary hepatocytes first cultured in open channel and then covered by a microchannel.

These results show the potential of this method for single-channel cell culture. By first culturing the hepatocytes in an open channel, it is simple to obtain a confluent layer of viable hepatocytes, unlike when hepatocytes are initially cultured in a microchannel. Then the micro-environment can be introduced by placing the microchannel over the open channel. This method still needs to be refined, however, as some problems still persist. It is difficult to avoid contamination when handling the cells in this manner post-seeding. Additionally, these channels leak significantly. This leakage issue, however, can be solved if a clamping device is introduced to seal the microchannel to the plate. A possible solution for this issue is the clamping mechanism described in Section 2.6.

2.4 . LEAKAGE FROM SINGULAR MICROCHANNELS

Another significant issue with singular microchannels laid on a collagen-coated dish is leakage during the culture of PRHs. Recently, every time PRHs have been cultured in this arrangement, significant leakage of culture media has been observed to occur from the channels

within the first couple of days of culture. This is a major issue for a couple of reasons. First, the media contains many nutrients important for the hepatocytes' survival, and leakage deprives the cells of the media. Also, we intend to layer both major types of liver cells, primary hepatocytes and LSECs, in these channels and if the leakage persists, the second layer of cells will not be contained within the boundaries of the microchannel, nullifying the intent of layering in a microchannel.

In order to ascertain the cause of this leakage issue, a series of simple experiments was carried out. Eight sets of four microchannels, resulting in a total of 32 microchannels, were fabricated, and each was laid down on a collagen-coated tissue culture dish. Initially, water was injected into each channel in the same manner in which culture media is introduced into the channel, and it was left in the incubator at 37 degrees Celsius when not being handled. This water was then changed twice a day for a period of 7 days to mimic the daily media changes in cell culture. In these 32 microchannels, no leakage was observed over the course of the 1-week period. In order to better simulate cell culture conditions, primary hepatocyte media was used for this same experiment instead of water. Except for one out of the 32 microchannels, no leakage was observed, potentially indicating the key role of hepatocytes in causing leakage.

Another simple experiment was later carried out to further understand the role of hepatocytes in causing leakage. In this experiment, PRHs were cultured in a PDMS microchannel laid on a collagen-coated tissue culture dish, and for the first four hours after seeding, images were obtained to observe the behavior of hepatocytes. One of these images, as seen in **Figure 2.8**, revealed that some PRHs were already leaking out of the channel in the first few hours of culture, even before noticeable media leakage was observed. This indicates that the cells may play a significant role in this leakage issue. The presence of the PRHs could be adding an additional pressure that helps break the seal of the PDMS to the tissue culture dish or the cells prefer the

tighter micro-environment and are forcing their way out of the channel. Either way, the hepatocytes clearly play a major role in this leakage issue.

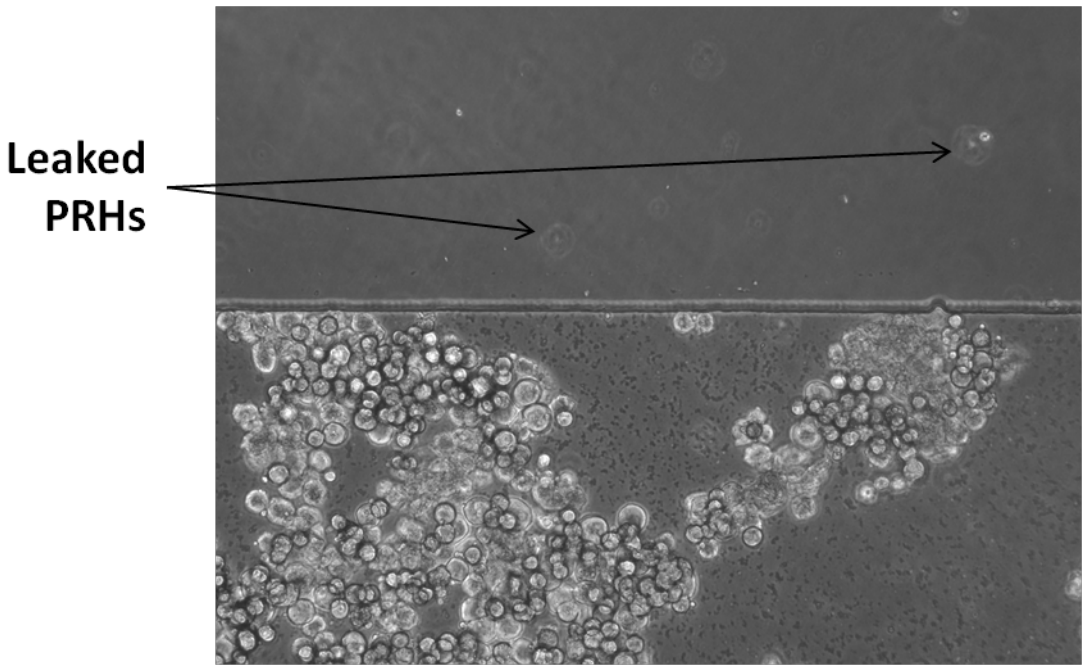

Figure 2.8. Primary rat hepatocytes leaking out of microchannel.

2.5 . INITIAL ATTEMPT AT DEVELOPING CLAMPING DEVICE

Our single channel culturing system consists of PDMS channels laid down on collagen-coated tissue culture dishes, relying on the natural adhesive properties of PDMS for sealing. However, in our recent cell culture experiments, we have experienced a persistent problem, in which the channels begin to leak within the first two days of culture. Derived from experimentation, we have discovered that the presence of the PRHs in the channel contributes significantly to our leakage issue, and we have surmised that an external compressive force is necessary to prevent leakage from the channels.

One method that was attempted to correct this leakage issue relied on compressive force from a poly(methyl methacrylate) (PMMA) plate pressure fit into the petri dish. From manual manipulation of the PDMS, it can be easily observed that little force is needed to hold the PDMS down to the plate, and the idea is that with the PMMA plate tightly fit into the petri dish, it can provide just enough compressive force down on the PDMS to seal the channels off. In order to fabricate this PMMA plate, a laser cutting machine was used that was able to cut the piece to the appropriate dimensions. The diameter of the plate was cut so it would precisely fit into the tissue culture dish, and access holes were cut to allow access to the channels. A picture of this assembled device can be seen in **Figure 2.9**.

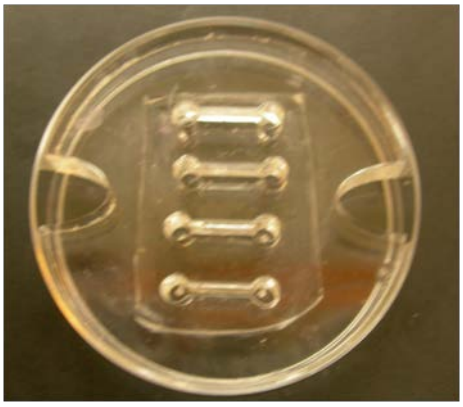

Figure 2.9. Assembled pressure-fit device to seal PDMS channels to dish

This clamping method, however, did not prove to be effective. Due to the force of the PMMA plate on the walls of the petri dish, the bottom of the dish deflected downward, along with the PDMS channels attached to it. This resulted in uneven pressure from the PMMA plate on the top surface of the PDMS, and as a result, leakage still occurred. Additionally, the cut of the PMMA from the laser was not precise enough and often resulted in a diameter of the plate that did not properly fit the tissue culture dish. In each of the twelve sets of four channels that were

tested in primary hepatocyte culture, there was leakage observed. From this, it was surmised that a new method was needed to provide even pressure to seal off the channels.

2.6 . DEVELOPMENT OF CURRENT CLAMPING DEVICE

Another possible solution for the leakage issue is a novel spring-loaded device that applies a compressive force to PDMS channels in a tissue culture dish. The idea behind this device is to load springs in a compressed state in some sort of device on top of the channels so that this compressive force is able to seal the PDMS to the tissue culture dish. A schematic view of this clamping device can be seen in **Figure 2.10**.

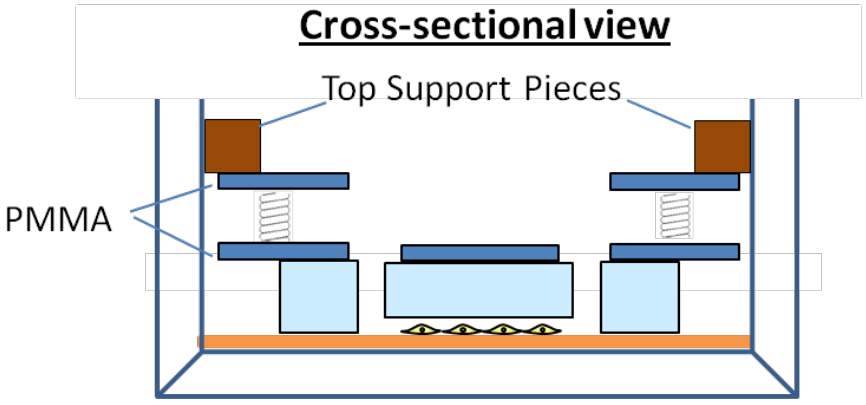

Figure 2.10. Schematic of the assembled clamping device

The key feature of this device is the two laser-cut PMMA plates connected by four or more springs. This part of the device is laid flat on the top of the PDMS microchannels, which means that these plates need to be specially cut for handling purposes. The bottom plate that is in contact with the PDMS is circular with holes cut in the same shape as the channels. This geometry not only allows access to the channels, but it also enables oxygen to diffuse through the PDMS to the cells. The top PMMA plate is also circular and has rectangular access holes, which

are simply there for the purpose of not obstructing cell culture. Also, this top plate has a rectangular piece running down the middle that allows for extra springs to be placed in the middle in case extra support is needed. These plates can be seen in an image of the assembled clamping device in **Figure 2.11**.

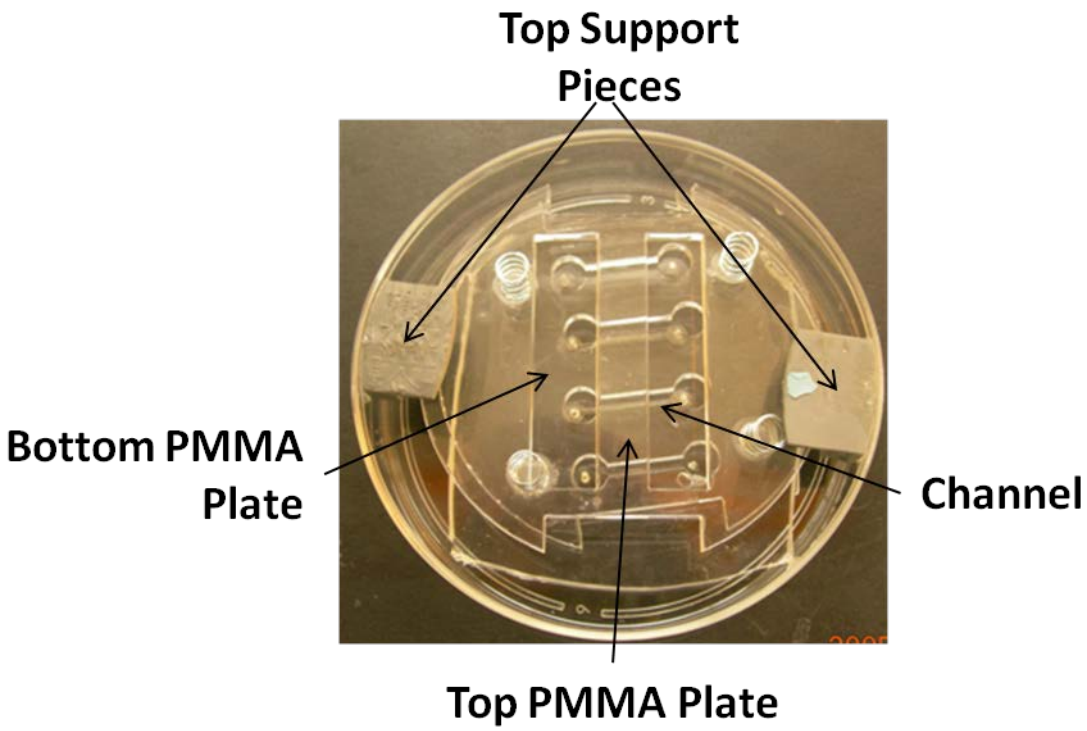

Figure 2.11. Image of the assembled clamping device

In order for the PMMA plates connected by spring to impart a force on the channels, there must be another part that holds the springs down in a compressed state. This is provided by two support pieces that are glued to the sidewall of the petri dish, and these pieces were initially fabricated via Envisiontec's stereolithography machine. This stereolithography required that these support pieces be drawn in ProEngineer or a similar CAD software, in which the drawing can be

saved in an ".stl" file. The software associated with the machine is then able to recognize the file, which can then be built through the machine. There are several different patented materials that can be used in this machine, but the one used for these support pieces was Photosilver. Once all the separate pieces have been fabricated, they are assembled as seen in the schematic side view in **Figure 2.10** and the actual assembled device in **Figure 2.11**.

Despite the promise of this device, leakage was still observed in eight of the sixteen channels that were tested with culture of PRHs. As was seen previously, the bottom of tissue culture dish underwent bending due to the force being applied. As a result, the PDMS channels bent with the bottom of the culture dish, and the bottom PMMA plate did not lie flat on the top of the PDMS. This resulted in uneven pressure from the spring-loaded device, which still permitted the leakage. In order to prevent this bending issue, a thin PMMA plate was glued to the bottom of the culture dish, which is able to prevent much of the bending; this dish with the additional PMMA plate is shown in **Figure 2.12**. In this arrangement, the PMMA plate remained flat on top of the PDMS, resulting in an even sealing pressure on the channels. This addition to the culture dish has had a 100% success rate for preventing leakage in 24 separate channels when the device remained intact.

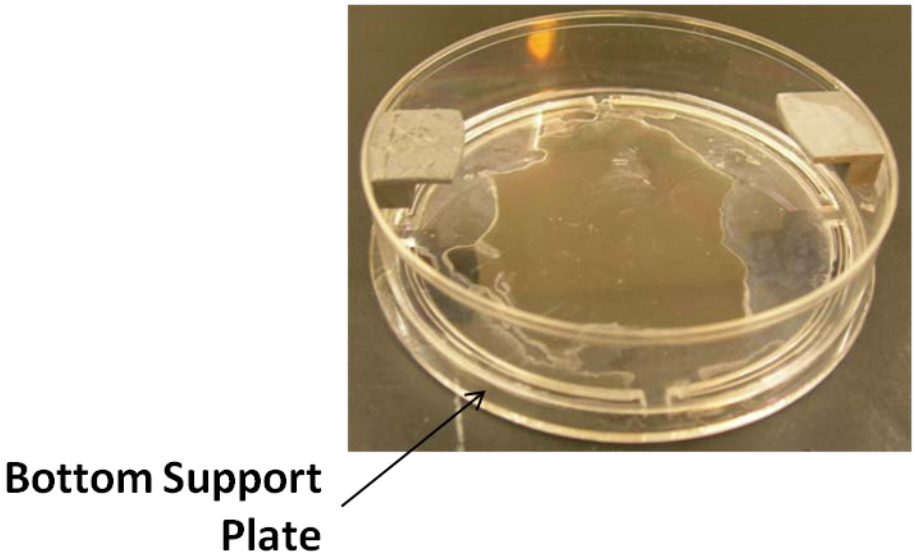

Figure 2.12. Petri dish reinforced with bottom support plate

An issue arose for this configuration of the clamping device, however. The top support pieces that were fabricated through stereolithography are sensitive to the levels of humidity, and when the clamping device is exposed to the humid conditions in the incubator, these support pieces are susceptible to fracture. In order to correct this issue, these top support pieces have also been fabricated with PMMA, as this material is much stronger and less dependent on humidity changes.The assembled clamping device with the PMMA top support pieces can be seen in **Figure 2.13**.In 10 separate clamping devices with the top support pieces made of PMMA, none have structurally failed.

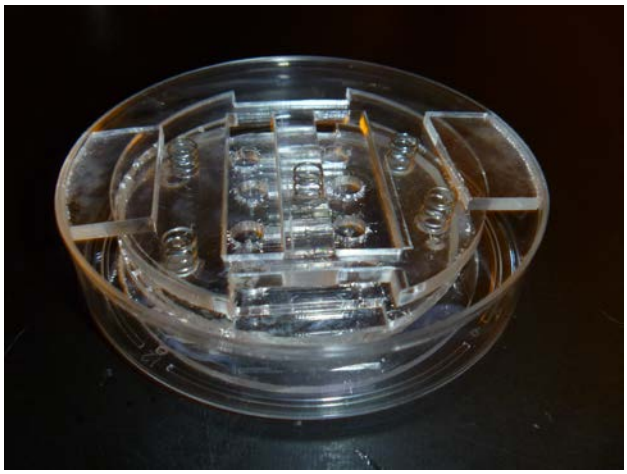

Figure 2.13. Clamping device with PMMA top support pieces

A structurally-sound clamping device has been developed for use with single microchannels on a collagen-coated tissue culture dish. This device has been proven to prevent leakage in this set-up, which should allow future progress in the area of culturing PRHs alone and in a layered co-culture with LSECs. Additionally, this enables the simple application of flow to a single microchannel without risk of leakage. Previously, flow experiments had been attempted in single PDMS channels adhered to a collagen-coated dish, but leakage was commonly observed. However, in this case, the extra clamping force provides sufficient sealing of the channels, adding robustness to this simple configuration.

CHAPTER 3. CO-CULTURE OF LIVER CELLS

3.1 . DESCRIPTION OF DUAL CHANNEL SYSTEM

The dual microchannel design consists of a few components: two layers of PDMS microchannels and a thin partition membrane between the channels. The channels are fabricated through common soft lithography techniques, in which the channels are formed from a PDMS pre-polymer mixed with a curing agent at a 10:1 ratio. The dimensions of the two channels are slightly different from the previous version. The shape of both channels are exactly the same as each other, and the dimensions are the same, except for their length. One channel is slightly longer than the other so that the inlet and outlet of both channels can be accessed from one side. Overall, the channels are approximately 1.5 cm long, 1 mm wide, and 70 μm high. The partition membrane is one of two materials: parylene or polyester(PET), and both membranes are approximately 10 micrometers thick. The parylene membrane is acquired through vacuum deposition onto glass slides, and the PET membrane is a microporous Transwell© membrane manufactured by Dow Corning.

The assembly of these dual microchannels is a simple process, and its final arrangement can be seen in **Figure 3.1**. First, a small piece of the partition membrane is manually cut and placed over one of the channels. Next, four holes are manually bored in the other channel, which enables access to both channels from the top. The two PDMS channels are then exposed to oxygen plasma and pressed together to bond so that the two microchannels are overlapping with only the membrane separating them. This way, PRHs and LSECs can be cultured in separate channels, and two separate media flows can be supplied to the cells, as indicated by the arrows in **Figure 3.1**.

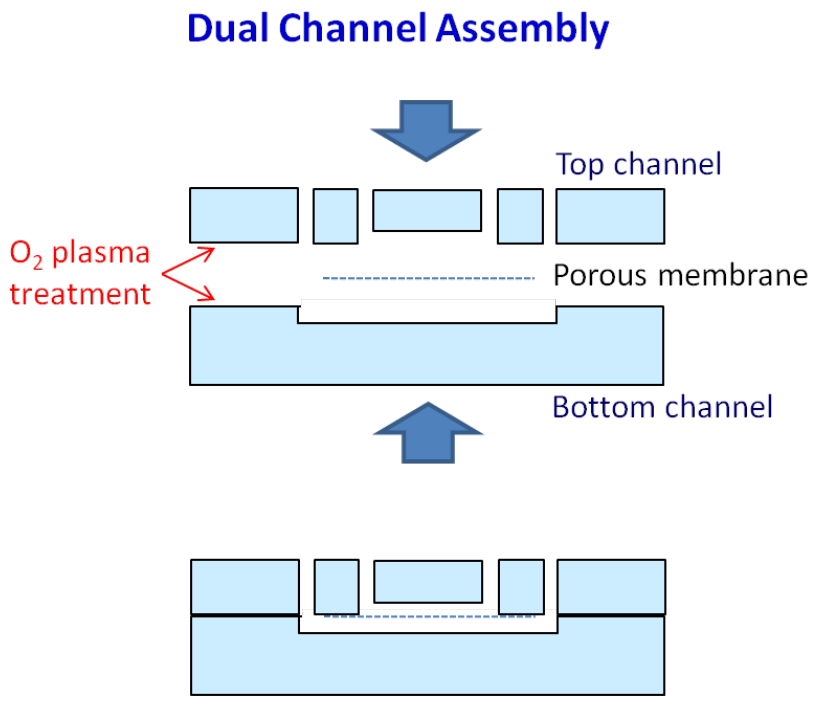

Figure 3.1. Configuration of the dual microchannel set-up. In the top channel, LSECs are seeded, and in the bottom, there are PRHs.

3.2 . PRELIMINARY CELL CULTURE IN DUAL CHANNEL SYSTEM

3.2.1. Dual Channel Seeding Technique

Proper seeding of the two cell types, PRHs and LSECs, into the dual microchannel system requires several hours. First, the liver cells need to be obtained from an adult male rat. After the liver is isolated from the rat, it is enzymatically digested and perfused with collagenase buffer. Then the primary rat hepatocytes can be isolated. After isolation of the hepatocytes, there remains a supernatant that contains the LSECs. The LSECs can subsequently be isolated through centrifugation along a Percoll density gradient. Once the LSECs are obtained, they are injected into the top channel of the dual channel system and supplied with Williams Media E

supplemented with 10x fetal bovine serum, among other essentials. The cells are allowed one and a half hours to settle down and attach to the partition membrane. Once the LSECs are attached, the PRHs are injected into the bottom channel and provided the same media formulation as the LSECs. The system is then inverted for one and a half hours in order to allow the PRHs to attach to the underside of the partition membrane. Once this amount of time expires, the media is replaced in both channels, and the cells are maintained by changing the culture media twice per day.

3.2.2. Dual Channel Co-culture Results

At first, only PRHs were cultured alone in the dual channel system. After testing this set-up several times and ensuring that PRH's could be seeded properly, co-culture of PRHs and LSECs were attempted, and through the cell culture methods described previously, this co-culture was able to be achieved. We were not able to obtain a high confluence of both cell types at the same time, but these preliminary results show that our platform is capable of supporting a co-culture of cells. A picture of the cells in this configuration can be seen in **Figure 3.2**. It was difficult to image the LSECs in this arrangement, but their presence was able to be observed through refocusing the microscope although they could not be clearly imaged. Further work needs to be done to ensure higher confluence, but the current results are promising.

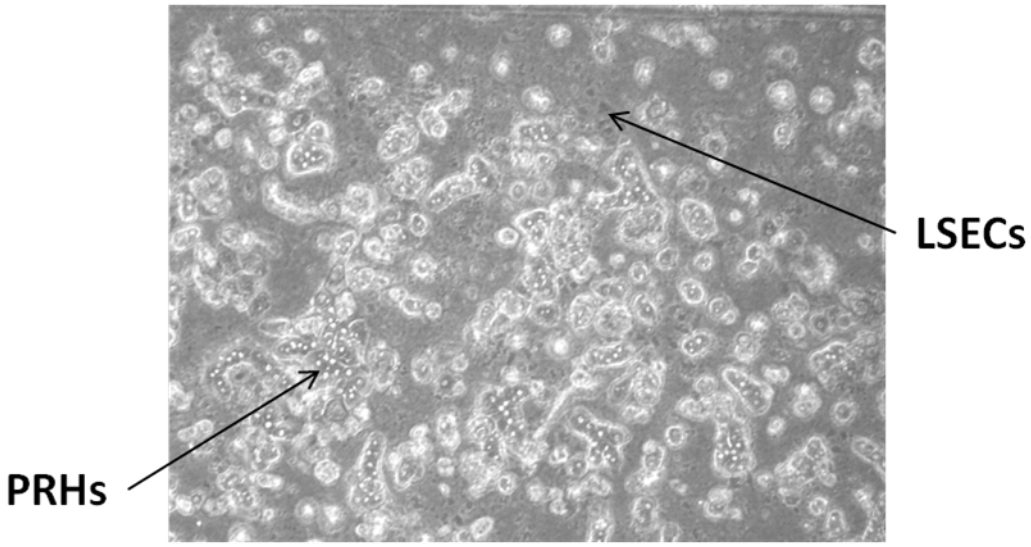

Figure 3.2. LSEC's and PRH's Cultured in Dual Channel Arrangement

3.2.3. Leakage Issue and Viability of Cells in Tighter Micro-environment

Leakage was observed in some of the earliest versions of the dual microchannels. The plasma bonding between the PDMS channels was incomplete due to uneven pressure applied when the dual channels were assembled. Careful attention needed to be taken to ensure the bonding along the edges of the partition membrane. When dyed water was flown through the channels, the leakage was observed to occur along the edges of the membrane. Despite this problem, the cell culture was still carried out with PRHs. Due to the leakage, some of the cells ended up trapped between the partition membrane(PET) and PDMS. Images were taken to observe the health of the cells.

In every one of these instances, the cells consistently formed a highly confluent layer and the cells maintained viability for at least one week. An image of these cells can be found in **Figure 3.3**. These consistent results show us the potential of culturing cells in smaller micro-environments, something we hope to take advantage of in the future.

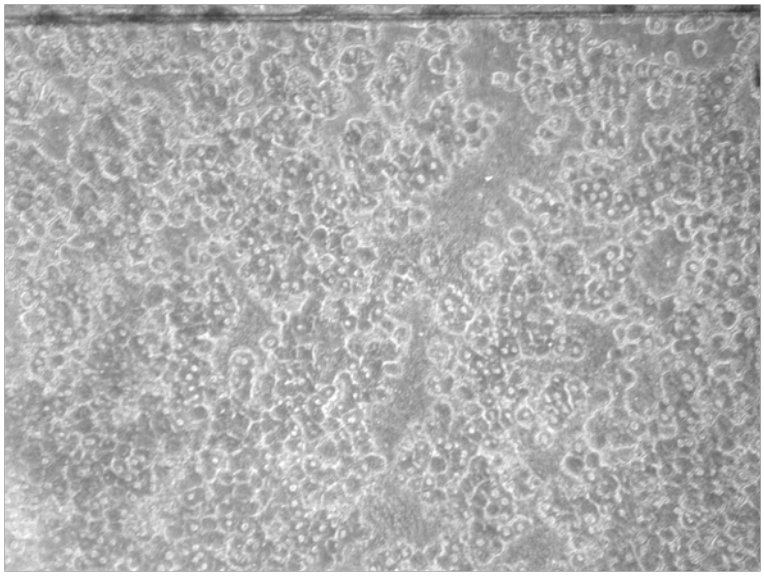

Figure 3.3. PRH's trapped between PDMS and PET membrane

3.2.4. Further Experimentation with Cells in Tighter Micro-environment

In order to further investigate the effect of a smaller micro-environment on the cells, a simple experiment was performed with PRHs. The PRHs were initially cultured on a collagen-coated petri dish and supplied with culture media. After three hours, the media was aspirated and a 6-mm thick slab of PDMS was placed on top of the cells. Images were obtained for the PRHs in this arrangement to monitor their health.

Similar results were observed in this cell culture arrangement. The morphology appeared to be slightly different, but the PRH's formed a highly confluent layer and were able to survive one week consistently. A picture of the cells in this arrangement can be found in **Figure 3.4**.

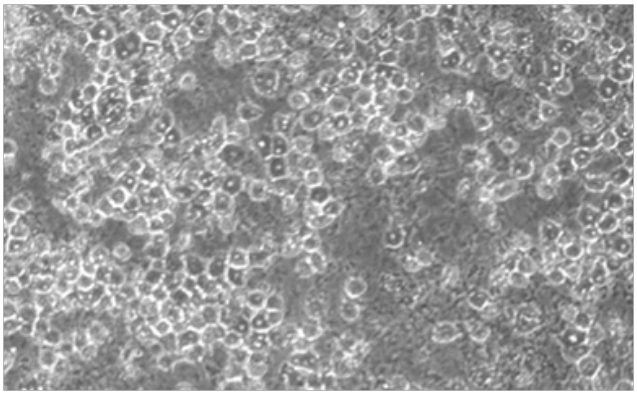

Figure 3.4. PRHs cultured between PDMS and a collagen-coated petri dish.

CHAPTER 4. Oxygen Levels in Liver Model

4.1 . DUAL CHANNEL SIMULATION

4.1.1. Set-up of the Simulation

The purpose of the dual channel simulation is to model the oxygen concentration in the dual microchannel system, taking into account the diffusion of oxygen from the atmosphere and the consumption of oxygen by the cells. In the body, an oxygen gradient is observed along the length of the liver sinusoid, varying from about 65 mmHg to 35 mmHg, which translates to about 0.101 mol/m^3 to 0.54 mol/m^3[11]. The ultimate goal is to achieve a similar oxygen gradient in a microfluidic platform seeded with a co-culture of liver hepatocytes and LSECs.

In order to model the oxygen diffusion, the geometry first had to be specified in COMSOL, and the general layout can be seen in **Figure 4.1**. Two rectangular geometries of the same dimension were defined for the two microchannels; these channels are 68 micrometers in height and 1.5 centimeters in length, which are the dimensions of the channels we typically use. Additionally, two layers of two-micrometer height needed to be specified the cells adjacent to the microchannel. These were required in order to properly model the consumption of oxygen. Finally, in the space between the cell layers, there was a ten-micrometer thick layer defined for the partition membrane, which can be either PET or parylene.

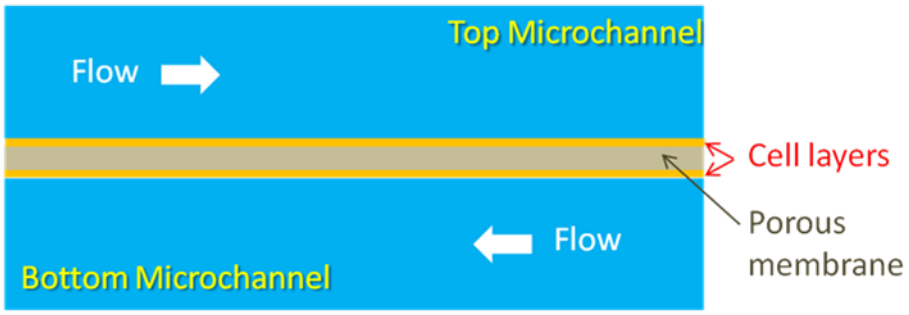

Figure 4.1. Schematic of dual channel simulation geometry

The equation governing diffusion in this simulation is Fick's Second Law of Diffusion. There are two versions of this equation, with and without a convective term and are shown in equations (1) and (2):

$$(1) \quad \nabla(-D\nabla c) = R + u\nabla c$$

$$(2) \quad \nabla(-D\nabla c) = R$$

For these diffusion equations, "D" represents the diffusivity , "c" is the concentration, "R" is the reaction rate, and "u" is the velocity term. Equation (1), which has the convective term, must be used for the microchannel subdomains because of the simulated media flow. The media flow simulated in this case has a parabolic flow profile with a mean velocity of $1*10^{-4}$ m/s and is in opposite directions for the two channels[35]. Otherwise, in the cell and partition membrane layers, there is no flow so equation (1) is used to characterize the oxygen diffusion. Additionally, the diffusivity of oxygen in each of the subdomains must be defined.

One of the controlling factors of oxygen concentration in this dual channel arrangement is the diffusion of oxygen through the top of the channels. PDMS is highly permeable to oxygen so an appropriate flux equation must be used to accurately model the oxygen diffusion through the top boundary of the upper channel. The equation defining this oxygen flux through PDMS is in equation (3).

$$(3) \quad N = K_{la}(c_{sat} - c)$$

In equation (3), "K_{la}" is the mass transfer coefficient whose value is approximately $9*10^{-7}$ m/s, "c_{sat}" is the saturation concentration of oxygen in the media, and "c" is the bulk concentration[36].

Additionally, flux equations must be defined at each boundary at the interface of two layers. In order to define the flux of oxygen between layers, the stiff-spring method was used. This method requires using equation (4) to define the flux of oxygen at the boundary.

$$(4) \quad N = M(c_j - \frac{c_i}{K})$$

For the stiff-spring method, "M" is an arbitrarily high constant that establishes the flux continuity at each boundary, and "K" is the partition coefficient, which represents the ratio of solubilities of a gas between two different materials. A partition coefficient is only needed for the boundaries between the cell and the partition membrane layers because the cell layers are assumed to have the same diffusion properties as the microchannel layers. Hence, "K" is equal to 1 for the boundaries between the cell and microchannel layers.

There are several boundary conditions that needed to be identified for vertical boundaries as well, specifically for the microchannel layers. In the experimental conditions, the media flow that is provided to both channels is assumed to be oxygenated to specific concentrations; consequently, the inlet boundaries for both microchannels must be set to equal that concentration throughout the simulation. Also, the outlet boundaries of both microchannels need to be specified as convective flux. All other boundaries that have not been described are set as insulation because they are assumed to have a negligible effect on oxygen diffusion throughout the system.

Finally, a reaction rate needs to be specified for the bottom cell layer to simulate oxygen consumption by the hepatocytes. The equation that specifies this consumption is defined by Michaelis-Menten kinetics and is defined in equation (5).

$$R = \frac{1}{t}\frac{V_{max}c}{K_m + c}\sigma \quad (5)$$

In this equation, "V_{max}" is the characteristic uptake rate by the cells, "K_m" is the Michaelis Menten constant, "t" is the thickness of the cell layer in the simulation, and "σ" is the density of cells in cells/m^2[36].

The simulation described up to this point assumed the partition membrane to be a solid material. However, in the actual dual channel platform, the membrane has micropores that enhance oxygen diffusion. The effect of these micropores needs to be investigated experimentally to ascertain their exact effect. In order to observe the potential effects of higher diffusion, other simulations were performed for both types of membranes, and in these cases, the diffusion coefficient of the partition membrane material was assumed to be a factor of ten greater than the original value.

4.1.2. Results of the Dual Channel Simulation

The oxygen diffusion simulations for a solid partition membrane did not produce the desired results of a decreasing oxygen gradient along the hepatocytes. Due to the consumption of oxygen and the low permeability of oxygen in both partition membranes, the hepatocytes were shown to not experience the desired oxygen gradient whether the partition membrane was PET or parylene. The oxygen concentration along the hepatocyte layer is observed to decrease to approximately zero about halfway through the channel for both simulations. Due to the low permeability of both membranes, low levels of oxygen are able to reach the hepatocytes in the bottom channel, resulting in an oxygen gradient opposite of what is necessary. The velocity of the

flow could be increased to ensure that the flow reaches the outlet with an appreciable level of oxygen, but the desired gradient would still be the reverse of what is necessary.

The presence of pores in the partition membrane can possibly solve this problem as is displayed in other simulations. Experimentation is required to ascertain the exact effect of the pores in the membrane, but in order to observe the potential of higher rates of diffusion, the diffusivity of the partition membrane was increased by ten-fold. These additional simulations showed that when the membrane is made of parylene, the levels of oxygen provided to the hepatocytes in the bottom channel would still be insufficient. However, when the diffusivity was increased by ten times for the case of a PET membrane, a physiologically relevant oxygen gradient(0.17 to 0.05 mol/m^3) was observed to develop along the hepatocyte layer. The oxygen levels along the hepatocyte layer in the two simulations for both partition membranes can be found in **Figure 4.2** and **Figure 4.3**

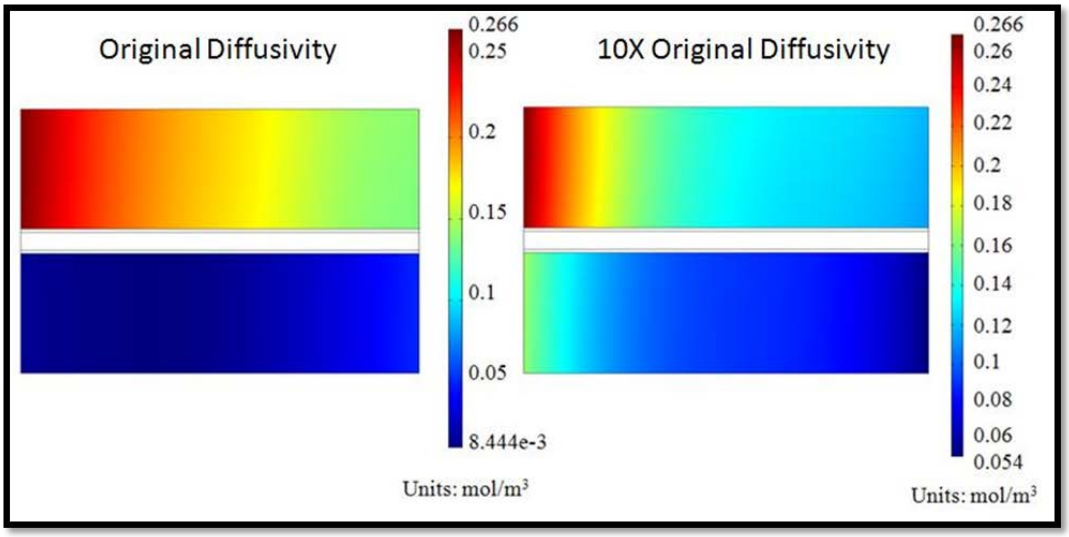

Figure 4.2. Results of the oxygen diffusion model with a partition membrane of PET. These are the results of 2 cases with different values for the diffusivity: the original value and ten times the original value. Some of the values of important parameters are listed below. c_{sat}: 0.266 mol/m^3, D_{media}: 2.1*10^{-9} m^2/s, $D_{polyester}$: 5.6*10^{-13} m^2/s, $K_{polyester}$=4.851[36;37].

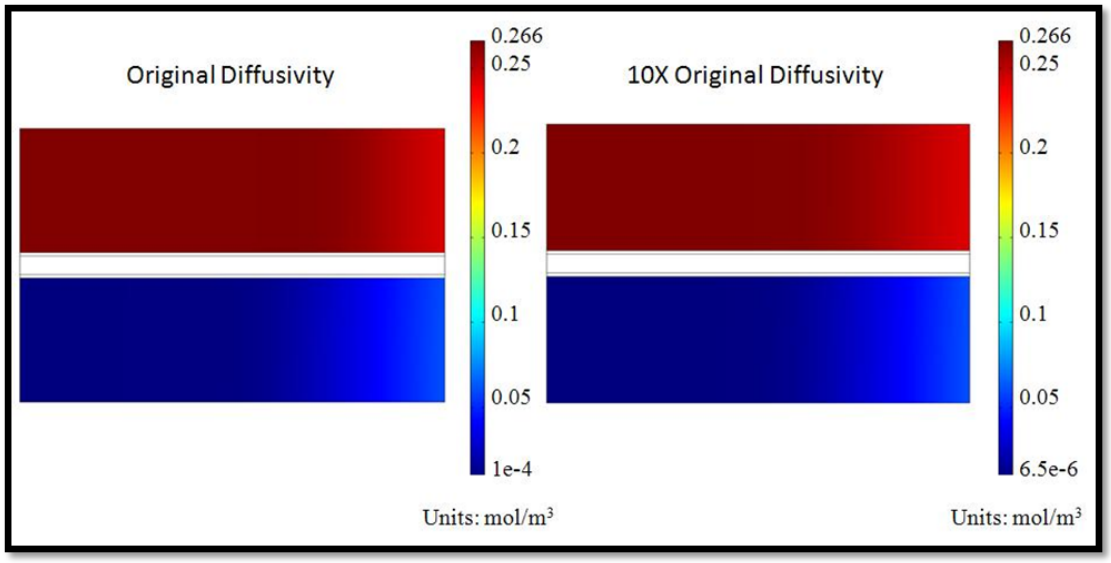

Figure 4.3. Results of the oxygen diffusion model with a separating membrane of parylene. These are the results of 3 cases with different values for the diffusivity: the original value, twice the original value, and ten times the original value. Some of the values of important parameters are listed below.Some of the values of important parameters are listed below. c_{sat}: 0.266 mol/m^3, D_{media}: 2.1*10^{-9} m^2/s, $D_{parylene}$: 3.5*10^{-13} m^2/s, $K_{parylene}$=0.000893 [36,38].

4.2 . OXYGEN CONCENTRATION VALIDATION EXPERIMENTS

In order to validate the accuracy of the simulations, adequate experiments must be performed in a dual microchannel system to determine the oxygen levels. This result can be achieved by introducing an oxygen-sensitive dye over the cells, the fluorescent intensity of which can be used to find the oxygen concentration. In our experiments, the dye tris(2,2 -bipyridyl)dichloro-ruthenium(II) hexahydrate, or RTDP, is used.

In order to properly measure the fluorescence of the RTDP dye, a wide-field microscopy system is required with an excitation source, filter, charge-coupled device(CCD) camera, and

image analysis software. The concept behind this fluorescent experimental system is very simple. The fluorescence of the dye is excited by a light from an excitation source. However, before this light reaches the dye, it is passed through an excitation filter that best fits the excitation spectrum of the dye. Then the emitted signal is passed through an emission filter that removes any extraneous light. Finally, the CCD camera records 2-D images of the fluorescent signal to be analyzed by the image analysis software. In our case, the specific components are the Olympus IX71 inverted microscope, the Nikon CoolSnap HQ CCD camera, the Endow GFP filter, and MetaMorph software. This set-up can be seen in the image in **Figure 4.4**.

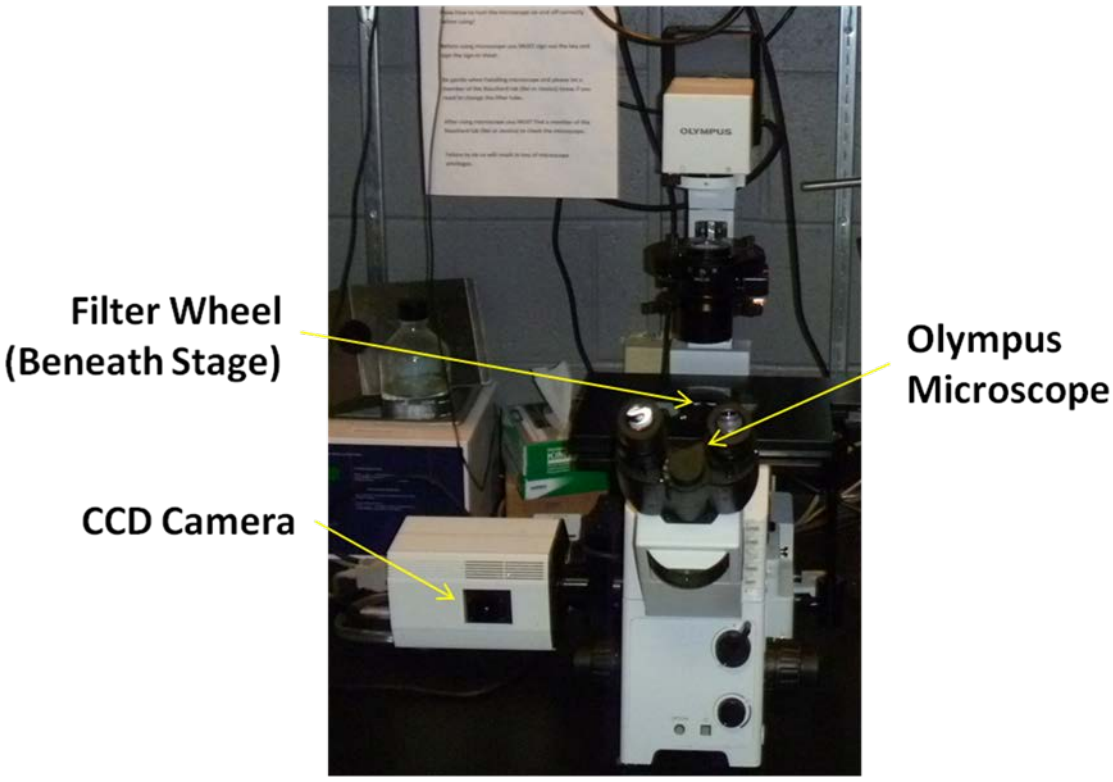

Figure 4.4. Image of Microscopy System

In preparation for the final experiment, a simple experiment was run to determine a proper concentration of dye. For this experiment a single PDMS channel was used in the clamping mechanism described previously, but no cells were seeded in this channel. RTDP dye was mixed with PBS at several concentrations ranging from 0.5 to 3 mg/ml. Also, in order to ensure that each sample would have the same oxygen concentration for this experiment, each had a saturation concentration of sodium bisulfite dissolved in it; this was done because a solution saturated with sodium bisulfite ensures zero oxygen. These samples were each passed through the PDMS channel at a flow rate of 1 ml/minute, and the fluorescent intensity was measured at, at least, 50 different intervals with an exposure time of 200 milliseconds. Graphic results of this experiment can be seen in **Figure 4.5** . From the graph, it can be seen that at this certain exposure time, the concentration of 0.5 mg/ml of RTDP is sufficient for a bright intensity signal. Also, this concentration is large enough to be readily measured while not using excessive amounts of dye. Consequently, this concentration will be used in future oxygen fluorescence experiments.

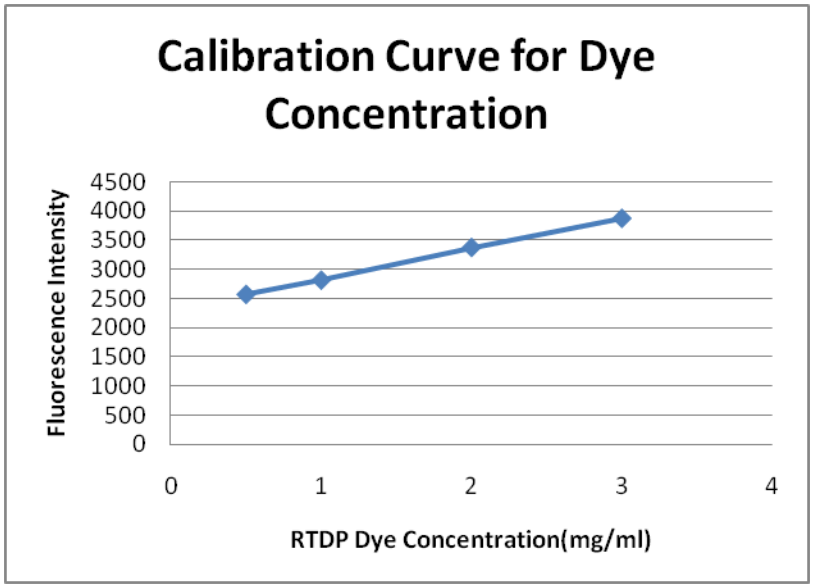

Figure 4.5. Dye concentration calibration curve

In the future, this work needs to be continued in order to validate the oxygen concentration simulations. A systematic approach should be followed, in which single channel experiments are first performed to validate the previous work of other groups. In this case, a monolayer of hepatocytes should be first seeded in a collagen-coated PDMS channel. Once the hepatocytes have been allowed one day to settle and attach in the channel, it could then be connected to the perfusion system pictured in **Figure 4.6**.

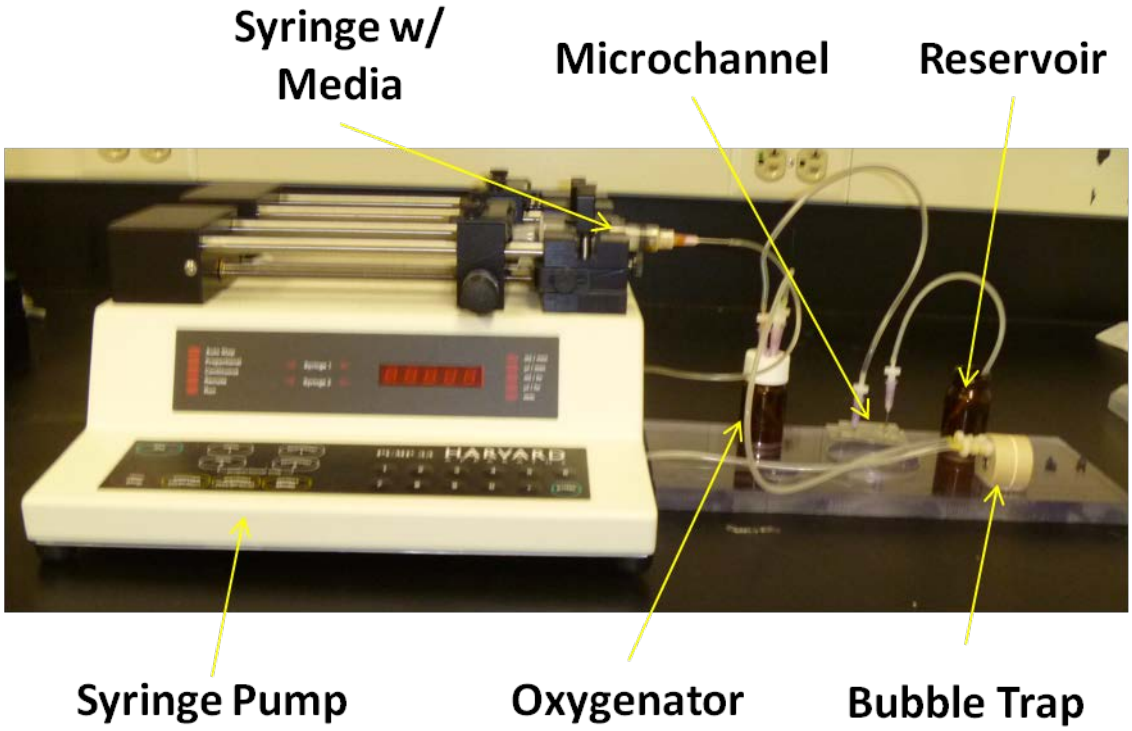

Figure 4.6. Image of the perfusion system

In this perfusion system, media with dye mixed in is pumped from a syringe pump through a bubble trap and then an oxygenator to provide the proper concentration of oxygen. Once the media is sufficiently oxygenated, it then flows to the channel, at which point the oxygen levels can be measured through fluorescence. In order to measure the fluorescence, the channel must be lined up in the field of view of the microscope attached to the fluorescence detection system. Also, the proper filter, Endow GFP, must be used in the microscope to properly filter the fluorescence signal. Once this is all set up, images can be recorded through the MetaMorph software on a time lapse, and the intensity of the fluorescent light can be measured. However, these intensities do not automatically correlate to concentrations; the light intensity is dependent on several other factors as well. Consequently, two known concentrations of oxygen must first be

measured in the system. One known concentration is zero percent oxygen, which is achieved by mixing the media with a saturation concentration of sodium bisulfate, and the other known concentration to be used is saturation, which can be achieved by pumping the media through the oxygenator pressurized with 21% oxygen, 5% carbon dioxide, and 75% nitrogen. Once the fluorescence intensities of these two concentrations have been determined, then the Stern-Volmer equation can be used to establish a fluorescence intensity curve to be used throughout the rest of the experiment:

$$(6) \quad \frac{I}{I_O} = 1 + K_q[O_2]$$

In this equation "I_O" represents the intensity at zero concentration, "I" is the intensity at a certain concentration, "O_2" is the concentration, and "K_q" is the Stern-Volmer constant. Once, the intensities at the two aforementioned known concentrations have been determined, they can be inserted in the above equation to determine the Stern-Volmer constant. This establishes the fluorescence intensity curve, which allows the concentration to be determined throughout the rest of the experiment. In order to complete this experiment, the fluorescence intensities are measured throughout the whole length of the channel to establish quantitatively the oxygen gradient that is formed. Once this experiment has been achieved, it can be then expanded to a dual channel system with both PRHs and LSECs seeded in the two channels.

CHAPTER 5. Conclusions

5.1 . SUMMARY

The liver is the largest solid organ in the human body, and it controls a number of metabolic processes, including detoxification of harmful chemicals. This importance of the liver points to the need for the development of a physiologically relevant liver model. This theoretical model could be used to learn more about the mechanisms of liver activity and to test new drugs for a variety of liver diseases. A novel combination of microtechnology and cell culture techniques provide us this opportunity to develop platforms that mimic the environment that liver cells experience in the human body so that a biomimetic environment is produced. Also, the development of this model could be an even more far-reaching development in the area of biotechnology as it could lead to the development of models for other major organs.

In order to achieve a liver model, single and double channel systems have already been produced. These systems have already been tested with liver cell culture, and while improvement is needed, these platforms could be the precursor to a potential future liver model. Additionally, oxygen levels are essential to the function of hepatocytes cultured in them, and simulations have been performed to ascertain the possible levels of oxygen in these systems. These are only initial steps in the development of novel liver model, but the research thusfar demonstrates its potential.

5.2 . DISCUSSION OF AIMS

There were several aims of this research that were mentioned previously, and the progress on each aim is summarized below.

1. To develop and improve the design of single channel microchannels to overcome the current challenges (50% Completed)

Previous work done on this project had proven the capability of the single PDMS channel platform for monoculture of PRHs and co-culture of PRHs and LSECs. However, recent issues, such as leakage and inconsistent viability had prevented further progress with this platform. In order to rectify the issue of leakage, a novel clamping system was devised, and this clamp was incorporated into the tissue culture dish, enabling us to still use a platform similar to the one that had displayed success in the past. Repeated trials of culturing PRHs within this clamping system has shown to consistently prevent leakage. Inconsistent viability of PRHs has still proven to be an issue, however, and one alternative method was tested. This method involved initially culturing PRHs in open channels cut out of PDMS because of the consistent results of PRHs cultured in open dishes. Then, after the PRHs were allowed a day to settle, microfabricated PDMS channels were placed in the open channels, providing a more physiologically relevant environment. The PRHs showed much higher viability consistently using this method, and if problems persist with the "old" method of cell culture, it could be a viable alternative option, particularly with the use of a clamp.

2. To develop and test a layered co-culture of liver cells in a dual channel system

(20-30% Completed)

In order to properly achieve a biomimetic liver model, a platform is needed in which PRHs and LSECs can be cultured in conjunction in the proper architecture. One possible solution to this issue is a novel dual PDMS channel system, in which the channels are separated by a thin, porous membrane. This set-up allows the two cell types to be in close contact but with some separation to mimic the Space of Disse in the liver sinusoid. It can also allow for diffusion of essential nutrients and molecules, while also enabling easy removal of bile. This dual channel system was tested with cell culture of PRHs alone and also with a co-culture of PRHs and LSECs. The initial results show that this platform is capable of supporting this co-culture, although

improvements need to be made to ensure higher viability of both cells. Additionally, the initial cell culture with PRHs experienced some leakage and led to a very interesting discovery. When PRHs are trapped in a tighter micro-environment, they exhibit high viability and extensive cell-to-cell contacts.

3. To optimize the oxygen levels in microchannels seeded with liver cells (50% Completed)

The levels of oxygen PRHs are exposed to is one of the main factors in their differentiation, an important feature to be considered for a physiologically relevant liver model. Typically, in the human body, there is a decreasing oxygen gradient along the length of the liver sinusoid, a gradient that needs to duplicated in our model. To this end, a COMSOL simulation of oxygen diffusion in the aforementioned dual channel system was constructed to determine the potential of an oxygen gradient. This simulation was based off the work of several other research groups who use PDMS channels in their work, and the results are promising. A simulation of a dual channel system with a porous polyester membrane yielded results that exhibit the potential of an oxygen gradient along the level of the PRHs. However, this result needs to be validated through an oxygen fluorescence experiment. To this end, a perfusion system was constructed with an oxygenator that can control the levels of oxygen in the media flown over the cells. Also, preliminary tests were run to determine the proper levels of dye needed for completion of the experiment.

5.3 . FUTURE WORK

The research for this novel liver model is only a part of the work that needs to be performed in order to recognize the achievement of a complete model. This research has displayed the capability of single and dual channel platforms in culturing liver cells, along with the potential of a physiologically relevant oxygen gradient. However, there are several steps that

need to be completed before the desired final product is achieved. The future work for this project will be outlined along the lines of the three aims mentioned previously.

1. Improve the single channel platform for further research

The single channel system is an important part in the systematic approach to developing a liver model. This system is a simple case that allows the proper conditions for PRH culture to be worked out. Additionally, one proposed model involves co-culture of hepatocytes and endothelial cells in the same channel, further illustrating its importance. To achieve these goals, further cell culture needs to be done with the clamping device. This device has been proven to eliminate the leakage issues, but more cell culturing needs to be done to ensure that the device is capable of supporting a consistent, viable PRH culture. If the current cell culturing method is insufficient, more work should be done to improve the method of first culturing in an open channel.

Once an adequate method has been adopted for PRH culture in a single channel, the culture should be expanded to a layered co-culture of PRHs and LSECs separated by a thin layer of Matrigel or collagen. Different thicknesses of the separation layer should be tested, in order to optimize the environmental conditions for the cells. If this co-culture is successful in exhibiting certain cell markers under static conditions, it should also be tested under dynamic flow conditions to determine if the cells can survive and maintain proper function when exposed to physiologically relevant levels of shear stress. If the cells are able to maintain their viability, this layered co-culture could be a strong possibility for a novel liver model. This platform could then be expanded to include a second channel to allow for removal of bile, a potential toxin to the cells.

2. Improve the dual channel platform for further research

The dual channel platform described previously displayed potential in supporting a co-culture of PRHs and LSECs. However, the viability levels need to be increased on a consistent

basis. If this improvement can be made, there is great potential for this system. Also, the system can be further optimized by attempting different thicknesses and porosities of the separating membrane. The optimization of these factors can be determined through the measurement of certain markers for PRH and LSEC function and viability. Once these characteristics have been optimized, dynamic flow tests can be performed as described for the single channel platform to ensure the hepatocytes can withstand the shear stress of the flow. Cell markers should be measured again to ascertain the health and function of the cells.

3. Run experiments to determine and optimize the oxygen levels

The oxygen levels are essential to the health of the hepatocytes, and oxygen fluorescence experiments, as described previously, are required to determine the potential oxygen levels in the proposed platforms. Whichever systems prove to be successful in supporting a co-culture needs to be tested in this experiment. A physiologically relevant oxygen gradient is necessary in developing a liver model.

4. Test successful rat models with human cells

This research is aimed specifically at developing a human liver model, but human cells are in short supply. As a result, all the proposed future work thusfar has been suggested for rat liver cells. Once all of the conditions have been optimized for rat liver cells, the model needs to be expanded to human cells. The results can not be assumed to be the same for both types of cells, and it is difficult to propose possible changes in the system to accommodate human liver cells instead of those of a rat. However, some changes may need to be made and should be done as is necessary in the future.

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