A STUDY ON THE EFFECT OF MEDIA VOLUME MANIPULATIONS ON
GLOBAL HUVEC COLONY PROLIFERATION WITHIN A MICROFLUIDIC
CHANNEL

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Abstract

Microfluidic-based cell culture is a rapidly developing subject used in studying behavior and external influences on cells on a micro-scale. A majority of modern techniques utilize a perfusion-based culture model where fresh media is continuously passed over growing cells in order to keep them replenished. However, such techniques require intricate setups that have to incorporate syringe pumps, the growth platform, and a culture incubator. To add to the complexity, sterility has to be maintained so as not to contaminate the cells. Unlike perfusion-based techniques, this work demonstrates a novel method to culture cells utilizing a culture media reservoir and nutrient diffusion. Two micropipette tips fixed on the inlet/outlet ends of a Polydimethylsiloxane (PDMS) microfluidic culture channel are filled with media that passively diffuses throughout the channel to sustain the cells. Optimal times and techniques to replenish the media reservoir were determined through 3D computer simulation and wet bench experimentation. Cell growth on a glass, PDMS, and a Matrigel platform were explored. Ultimately, it was shown that replenishing the culture media within the reservoirs in increments of 5µL allowed for a confluent Human umbilical vein endothelial (HUVE) cell colony to grow in 32 hours of culture. Replenishing the media in 20µL or 50µL increments did not foster global colony growth. Further computational image analysis of cell culture images showed the 5µL culture technique led to roughly two times greater cell colony coverage throughout the growth channel.
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Introduction

Microfluidic techniques have long provided scientists the opportunity to study complex scenarios quickly and efficiently on a small scale. With the relative low cost of fabrication, and use of the highly biocompatible material PDMS, much work has been performed. The major attraction of microfluidic setups involve low reagent volume use, increased reaction times, and the ability to perform otherwise complex operations (pumping, mixing) on smaller platforms with low energy consumption. Microfluidic techniques have been utilized to perform common techniques such as cell separation[1], liquid chromatography[2], and on-chip PCR [3] in shorter time than their macro-scale counterparts.

Polydimethylsiloxane (PDMS) is a widely used material due to its unique material characteristics. Its chemical inertness makes it a good platform for work with almost any sort of reagent. Being translucent, it is very easy to observe experimentation on, in, or under its surface. Its hydrophobicity makes it a good platform for cell culture or protein adsorption, and simple surface treatments can render it hydrophilic. Its gas permeability allows for molecular gas exchange with the surrounding environment. Finally, its viscoelastic properties gives it good structural integrity. Formulation of PDMS is very straightforward. After mixing its two chemical components, it will crosslink and form its structure either overnight or even sooner when heated. Further, the fact that it can be molded into any shape or configuration makes it very appropriate for simple or complex microfluidic structures [4-6].

Matrigel is a gelatinous hydrogel extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma that is very rich in structural proteins found specifically within a cell's extracellular matrix. Such proteins include collagen IV, laminin, and entactin. Further, it contains a multitude of cellular growth factors that promote growth and adhesion [7, 8]. Tumor cells coinjected into mice with Matrigel exhibit increased growth rates and overall size[9]. Endothelial cells grown in matrigel have also been shown to undergo complex differentiation mimicking angiogenesis in
vitro [10]. Though its effects on cell growth have been widely observed, the exact mechanisms behind its efficacy are yet to be fully understood.

Human umbilical vein endothelial cells (HUVEC) are directly derived from newborn babies’ umbilical cords and further passaged into repeatable cell lines [11]. HUVE cells grown within fibrin-laced hydrogels have shown vessel-like growth [12, 13] and capillary formation[10]. Further, HUVEC monolayer cultures have been utilized in determining the specific growth factors and proteins that mediate vascular growth and proliferation.

Microfluidic technologies have been utilized for cell culture application and have generated tremendous results. A team working under Luke Lee from UC Berkeley utilized microfluidic culture for assay application[14]. Two cell culture arrays were fabricated out of PDMS using soft-lithography techniques, a 1x5 array and a 10x10 array, seen in figure below.

![Figure 1: 10x10 culture chamber microfluidic cell culture array. Hung, 2005.](image-url)

Each individual culture chamber was circular with four different channels leading into and out of it. There were inlet and outlet channels for culture media perfusion, a cell loading channel, and a waste extraction channel. The basic mode of operation involves loading the HeLa cells, providing a constant perfusion of media to promote growth, the introduction of a study element for an assay, and finally removal of cells
for analysis. More specifically, cells were loaded into the chamber roughly 40-50 at a time, and allowed 2 hours to settle. Following this, the perfusion channel valve was opened and a syringe pump was used to flow fresh media through the channel. Cells were grown under various flow rates to determine the optimal growth conditions. The group ascertained optimal growth occurred at 0.12μL/min. To test the assay capability of the set up, calcein AM was introduced into cell growth chamber. As calcein only presents a fluorescent response within viable cells, the assay demonstrated regular cell functionality was present. Such a cell culture array can be utilized for high throughput experimentation of multiple assays.

A group led by Stokol investigated how well HUVE cells conformed to different growth channel geometries[15]. In addition, they aimed to characterize cellular elements such as the number of focal adhesions and intracellular junctions, as a large number of such intercellular bridges denotes healthy colony growth. Two different channel geometries were studied, square and semi-circular channel structures. The square channel had dimensions of 50μm x 50μm and the rounded, 60μm x 45μm. The channels were fabricated via soft lithography of PDMS off an SU-8 master mold. Prior to cell loading, the channels were sterilized with ethanol and treated with fibronectin to promote cell adhesion. Cells at a density of 5*10^5 cells/cm^2 were subsequently loaded into the channel and allowed one hour to settle and adhere. Whereas culture media is actively pumped through growth channels/chambers in many microfluidic culture set-ups, this group took a different approach. The entire channel structure was first submerged in culture media. A syringe pump was connected to the channel with silicone tubing, and media was drawn out of the channel to induce flow. Cells were grown under three different negative flow rate conditions (0.5, 3, 7.5μL/min), to examine which yielded the most successful colony growth. After allowing growth for six days, cells were subjected to immunostaining. Cells were stained for VE-cadherin, a protein primarily expressed within intercellular junctions, and vinculin, a cytoskeletal protein present at focal adhesion points.
Cells that were grown under 7.5μL/min of negative flow exhibited highest expression of such transmembrane proteins. Further, confocal microscopy revealed that these seeded cells had conformed to the channel shape better than the cells grown under lesser flow and the control group. Their results suggested that HUVE cells need to be exposed to a certain level of shear stress to properly develop.

A fully automated microfluidic cell culture system was developed by members of Stephen Quake’s group[16]. The focal point of the device is an array of 96 culture chambers, each about 2mm² in area.

Surrounding the culture chambers is a network of features that fulfill mixing, pumping, perfusion, and waste removal roles to support cell growth. A custom electronic unit controls miniature solenoid valves that drive fluid flow throughout the channel. The culture chip is mounted on an automated microscope, within an environmental chamber for specific temperature and gas control. The hallmark of this setup is that the process is fully automated. For the initial loading step, a cell
suspension was injected into the device. After allowing 5 minutes for the cells to settle, the microscope would take phase contrast images of the channels, software would count the number of cells within the field of view, and either continue to introduce new cells, or cease loading depending on whether a desired concentration was reached or not. There on out, the controller would dictate pumping of fresh culture media throughout the array every hour. Since the individual chambers only hold a volume of 60nL each, less than 2mL of media would be used in maintaining cell culture every day. Their work demonstrated that fixing and staining the cells could also be performed automatically, with reagents being deposited individually into each growth chamber. Finally, to demonstrate cells grown within this chip were able to grow and proliferate as if in macro-scale culture, they performed key experiments using human mesenchymal stem cells (hMSCs). The proliferation study was straightforward. Cells were cultured for 168 hours, with the automated microscope taking images and performing a cell count every three hours. The results clearly showed that the cell population steadily grew. In order to test if the hMSCs were differentiating into osteogenic and adipogenic cells, cells were stained for major enzymes that should be present in each respective state. Staining results over time exhibited increased expression of the target enzymes.

The motive of my work was to develop a microfluidic-based cell culture system without a perfusion-based arrangement. My design allows for continued cell culture all while reducing the set up footprint, numbers of materials used, and overall cost. The main culture chamber is fabricated out of PDMS using soft lithography, and then bonded to a glass slide. Two micropipette tips are attached to the inlet and outlet to act as a standing reservoir for culture media. As cells grow and consume the nutrients, the media passively diffuses throughout the culture chamber. Such a reservoir-based system allows for a simpler set up that doesn’t require a syringe pump, tubing, or specific microscope/incubation set ups to observe the cells. The media exchange procedure is simple, and only needs to be performed every 10-12 hours to promote healthy cell growth. How much media is exchanged at a time greatly affects cell viability and is a focus of this study. Cell growth on glass, PDMS, and within a Matrigel matrix was studied during the course
of this work. The formation of a confluent HUVEC colony provides an accurate model of the endothelial lining within blood vessels. This design can further be utilized to study interactions between blood cells, foreign parasites/bacteria, or even drugs, and the vessel endothelial lining.
Materials and Methods

This chapter describes the materials and techniques utilized in the fabrication, assembly, and execution of the cell culture experiments.

Channel Fabrication

Cellophane tape was applied onto a 150 x 15mm petri dish and cut into desired dimensions to act as molds for soft lithography. As the shaping of the devices was performed by hand, each varied slightly in overall size. On average, the channel structures had a length of 15.1mm, a width of 7.19mm, and a depth of 100µm. They are trapezoidal in structure and contain an average volume of 14.1mm³. 50g PDMS (Sylgard 184) was mixed at a 10:1 base:curing agent ratio and degassed for 30 minutes at room temperature. The mixture was then poured over the tape molds and baked for 30 minutes in an 80°C oven. The structures were cut out with a razor blade, and inlet/outlet holes were punched with a 21G luer stub (BD). They were then submerged in isopropyl alcohol and sonicated for 5 minutes. This was followed by an acetone wash and nitrogen dry. The channels were then bonded to glass microscope slides (Corning). The slides were cleaned by sonication in a solution of 140mL deionized water, 70mL isopropyl alcohol, and 3.5g KOH for 15 minutes. Bonding surfaces of the channels and slides were subjected to four seconds of corona treatment. The PDMS channels and glass slides were then bonded and subjected to an overnight bake in an 80°C oven. A flowchart of the channel assembly process can be found in figure 1 below.
Figure 4: Channel fabrication flowchart. Cellophane tape is layered on a Polystyrene petri dish and cut into the required channel dimensions. PDMS formulated at 10:1 base:curing agent ratio is deposited on top and cured for 40 minutes in an 80°C oven. The channel is then lifted and a 21G luer stub is used to create inlet and outlet holes. Finally the channel is bonded to a glass slide.

Figure 2 shows top and side views of the channel bonded to a glass slide, with the pipette tips fixed to the inlet and outlet.
A similar procedure was utilized when fabricating the PDMS on PDMS setup, where a flat PDMS slab replaced the bottom glass slide. 6g of PDMS cured at a 10:1 ratio was poured into 60 x 15mm polystyrene petri dishes. This was allowed to cure for 30 minutes at 80°C. Once cured, the cleaned channel structure was bonded onto the PDMS surface. Figure 3 shows top and side views of the channel bonded to PDMS.

Figure 5: Side and top views of glass-bonded channel assembly.

Figure 6: Side and top views of PDMS-bonded channel assembly.
**HUVEC Media Formulation**

Culture media was formulated by mixing F-12K media with 1% P/S, and 10% FBS. In addition, Endothelial Cell Growth Supplement (ECGS, BD) at a concentration of 100 μg/mL media was added.

**Cell Culture**

Frozen HUVE cell pellets were obtained as a generous gift from Dr. Cheng Dong’s group and stored in liquid nitrogen at -196°C. To get them into culture, cells were defrosted at room temperature and added to a 50mL cell culture flask containing 5mL of HUVEC media pre-warmed to 37°C. They were then placed in an incubator with an internal temperature of 37°C and 5% CO₂ saturation. In 24 hours, after the cells adhered to the flask, the media was replaced with fresh HUVEC media. Thereafter, the media was refreshed every three days until cell confluence was reached.

**Channel Loading**

The assembled device was submerged under water in a petri dish, placed within a vacuum chamber, and degassed for 10 minutes. While keeping the device submerged, a 100μL micropipette tip (VWR 1-100μL sterile Aerosol Pipet Tips) loaded with water was fixed at the inlet. The same was repeated at the outlet. A flat edge was used to push out any bubbles that still remained within the channel. The device was then removed from under water and dried. After ensuring there were no leaks or bubbles within the channel, sterilization of the interior of the channel was performed.

50μL of 70% ethanol was added to the inlet micropipette and 25μL aliquots of fluid were removed from the outlet. Fluid levels on each micropipette were then given time to level out, ensuring there was flow through the channel body. This
process of adding ethanol to the inlet followed by removal from the outlet was repeated until only ethanol filled the pipette tips and channel. The channel was then left to sit for 5 minutes to ensure all interior surfaces of the channel were exposed to the ethanol.

Following sterilization, the channel was flushed three times with 50μL sterile 1x DPBS to remove the ethanol. The channel was then filled with 50μL fibronectin (BD) dissolved in sterile DPBS at a concentration of 50μg/mL. 50μL of DPBS at the outlet end was removed in 25μL aliquots to ensure the fibronectin introduced at the inlet traveled throughout the channel. It was then left undisturbed for 45 minutes so the fibronectin could have time to adhere to the channel base. Finally, the fibronectin solution was flushed out by fresh cell culture media.

Prior to loading cells into the channel, they needed to be removed from culture. Media present in the culture flask was aspirated out and discarded. 4mL sterile 1x DPBS was added to the flask, swirled about, and discarded. 1mL of 0.1% trypsin-EDTA was introduced to the flask, and the flask was placed in the incubator for 5 minutes to allow the trypsin solution to lift the cells into suspension. After, 5mL of fresh HUVEC media was added to the flask to deactivate the trypsin effect. A Hycor Kova® Glasstic® counting slide was utilized to estimate the overall cell concentration. Appropriate dilutions were performed to reach a concentration of 1 * 10^6 cells/mL. Such an aliquot was centrifuged for 8 minutes at 600 g. The supernatant was removed, the pellet re-suspended in 50μL of media, and finally introduced to the inlet. This process is displayed in step 3 within figure 4 below. 20μL aliquots were removed from the outlet and re-applied at the inlet to “pull” the cells into the growth chamber, as shown by step 4 within figure 4. Each pipette tip was then topped off with 30μL media to act as a media reservoir (steps 6-7) and further layered with 10μL of mineral oil to prevent excessive media evaporation (step 8).
Figure 7: Diagram of the cell loading process. Step 1 is what results after fabrication and channel construction. Step 2 encompasses micropipette fixation and sterilization. Step 3 is cell loading. Step 4 is "recycling" the cells within between the two micropipettes to get even distribution within the channel. Steps 6 and 7 concern filling the micropipette tip media reservoir. Finally, step 8 involves topping off the reservoir with mineral oil to prevent evaporation.
Matrigel Channel Loading

Prior to performing the experiments, special care had to be taken with handling matrigel. It was imperative to keep it on ice outside the refrigerator, as it gels at room temperature. Further, any tubes or pipet tips that were to come in contact with matrigel had to be pre-chilled to 4°C.

The channel structure, bonded to glass, was sterilized and flushed with DPBS. The fibronectin dynamic coating step was skipped for this portion, as matrigel itself contains many ECM proteins that promote cell adhesion and growth. The channel was then placed in a 4°C refrigerator for at least 10 minutes, to allow it to chill.

After the cells were lifted from culture and spun down at 4°C, the pellet was re-suspended in 50μL of matrigel. This suspension introduced into the channel, and the whole device incubated for 5 minutes to allow the liquid Matrigel to solidify. Afterwards, the pipet reservoirs were topped off with fresh culture media.

Channel Media Refresh

In order to maintain a healthy environment to promote cell growth, the media within the channel had to be replaced every 10-12 hours. This was performed by removing small aliquots of “old” media at a time until the channel emptied, then re-applying aliquots of fresh media. Performing this step with different removal/replacement volumes had a big effect on not only colony growth, but also whether or not cells survived. This process is illustrated below in figure 5.
Figure 8: Media refresh overview. The first step is mineral oil removal. Following this (steps 3-4) old media is removed in specific aliquots, alternating between micropipette tips. After the channel is emptied, media is re-added in the same aliquot volumes, alternating between micropipette tips (steps 6-7). Finally, the reservoirs are topped off with mineral oil (step 8).
**Microfluidic analysis**

Simulations were performed on COMSOL to estimate the volumetric flow rate and velocity magnitude of media flowing through the channel. A 3D model of the channel was created within the program. The dimensions of the model channel were the averaged dimensions of the multiple fabricated devices. A number of simulations were run at varying inlet pressures to characterize the flow.

The flow rate within a channel depends on the channel geometry and a driving force. In this case, there is a driving pressure. This pressure manifests from a difference in fluid column height between the inlet and outlet pipette tips, as described by the hydrostatic pressure equation \( P = \rho g \Delta h \). In these experiments, different volumes of fluids were extracted from the pipette tips. Removal of 5 \( \mu \) L resulted in a height change of 0.2mm. Removing 20 \( \mu \) L or 50 \( \mu \) L reduced the height by 5.5mm and 14mm, respectively. These values were determined by physically measuring the change in fluid column height as certain aliquots of fluid were removed. Using the pressure equation, these height differences translated to pressure changes of 1.961, 24.52, and 53.95 Pa, respectively, throughout the channel. These singular pressure values were applied as the boundary condition at the inlet. The pressure value distributions across a channel, for each individual case, are shown in the false color maps below.
Figure 9: False color maps showing pressure distribution through the channel. Images are of 1.961, 24.52, and 53.95 Pa input pressures, respectively.

The appropriate driving pressure was applied at the inlet hole on the left, and the pressure at the outlet was set as 0. Though the overall pressure distribution profiles are the same, the values are very different. Not surprisingly, the pressure is highest at the inlet, and it lowers to 0 in the outlet direction.

Given a single driving pressure, COMSOL was also able to simulate a flow velocity profile for each case. An assumption of laminar, incompressible flow was made, with a no-slip boundary condition at the walls. COMSOL then applied the Navier-Stokes equation across this channel geometry to arrive at velocity magnitude values presented in the false color maps below.
Figure 10: False color slices mapping flow velocity distribution throughout the channel. Images are of 1.961, 24.52, and 53.95 Pa input pressures, respectively.

These projections reveal important information about the characteristics of fluid flow throughout the channel. First, it shows that there is no flow “behind” the inlet and outlet, as the fluid only proceeds in a direction toward the opposite outlet. Further, the highest flow velocity is present at the center of the channel, closest to the inlet/outlet.

Through COMSOL, further information could be extracted from the velocity magnitude distribution data. The volumetric flow rate of the fluid through the inlet was calculated by performing a surface integration was performed at the inlet. Equation 1 below from a paper by Bharani et al. [17] was also used to estimate the volumetric flow rate throughout the channel:
\[ Q = \frac{h^4 \Delta P}{12 \eta \alpha} (1 - 0.63\alpha) \]

Where:

\( Q = \text{volumetric flow rate through rectangular channel} \)

\( h = \text{channel height} \)

\( \Delta P = \text{driving pressure} \)

\( \eta = \text{dynamic flow viscosity} \)

\( \alpha = \text{ratio of height to width of channel} \)

<table>
<thead>
<tr>
<th>Volume Change (μL)</th>
<th>Column Height Difference (mm)</th>
<th>Driving Pressure (Pa)</th>
<th>Calculated</th>
<th>Simulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.2</td>
<td>1.962</td>
<td>7.71 \times 10^{-11}</td>
<td>7.28 \times 10^{-11}</td>
</tr>
<tr>
<td>20</td>
<td>5.5</td>
<td>24.52</td>
<td>9.64 \times 10^{-10}</td>
<td>9.09 \times 10^{-10}</td>
</tr>
<tr>
<td>50</td>
<td>14</td>
<td>53.95</td>
<td>2.12 \times 10^{-9}</td>
<td>2.00 \times 10^{-9}</td>
</tr>
</tbody>
</table>

Table 1: Relationships between reservoir volume change with the fluid column height, driving pressure, and internal volumetric flow rate.

The calculated values came very close to the values derived by the simulation model. The above data, combined with experimental cell growth results provide us with information on what conditions cells are able to grow within this given environment. Whereas these simulations provide a glimpse of the fluid behavior within the channel, the results in the following chapter show how cell growth is affected.
Cell Culture Results

The following experiments demonstrated how well cells proliferated under different culture conditions. Growth was attempted on glass, PDMS, and Matrigel platforms. Further, different media exchange techniques were performed to demonstrate how colony growth was affected. Media was exchanged in increments of 5, 20 and 50μL. Though such volumes were small compared to flask or petri dish-based culture, each influenced colony growth to a lesser or greater extent.

Cell growth on glass

Freshly loaded cells in the channel are rounded in shape, as they have not yet adhered. The cells fully adhere by four hours post-loading, and begin growth. The media exchange technique greatly affected how well cells proliferated. Though many trials were performed, each with different parameters, three of them best demonstrated how greatly cell growth is affected by specific volume manipulations. The three reported here involve 5μL, 20μL, and 50μL media exchange.

5μL exchange

Refreshing the media 5μL at a time was the most successful technique in maintaining colony viability.Removing such a small volume had very little effect on the fluid column height within the micropipette tip, giving a height difference of less than 1mm. As demonstrated by the software simulation, a small height difference between the columns resulted in a small pressure differential, and thus, the flow rate through the channel was minimal. Relatively undisturbed due to the low flow rate during replenishment, the cells were able to form large confluent colonies within 32 hours of culture.

The following images of the channel interior were taken at intervals of 11, 21, and 32 hours after initial cell loading. For each time segment, there are two separate
views, pre media refresh and post media refresh. Each group of images highlights a
different position within a single channel.

Figure 11: 5μL media refreshment timeline.
T=11 hours after seeding

Pre refresh:

Figure 12: Images of cells 11 hours post-seeding, pre-refresh. Cells are well distributed throughout the channel.
Post Refresh:

Figure 13: Images of cells 11 hours post-loading, post-refresh. Cells have not been disturbed and are still widely distributed throughout the channel.

11 hours after seeding, we see the cells have settled and begun to form networks with one another. After the media refresh, we see they are largely undisturbed. This is most apparent in figures 7c and 8c where the spider web-like network is still present and intact.
T=21 hours after seeding

Pre refresh:

Figure 14: Images of cells 21 hours post-loading, pre-refresh. Cell population has greatly increased.
Post Refresh:

Figure 15: Images of cells 21 hours after loading, post-refresh. Larger cell population goes undisturbed.

After 21 hours, larger, more densely populated colonies of cells are present.
T= 32 hours after seeding

Pre refresh:

Figure 16: Images of cells 32 hours post-loading, pre-wash. With minimal disruption, proliferation continues.
After 32 hours, we see the densest population of cells thus far. However, culture beyond this point proved difficult as many cells started dying. It is speculated that because of the greater number of cells present in the channel, the 11-hour media exchange period itself was too long and the cells consumed the nutrients at a greater rate. If this is true, it is necessary to increase the replenishment frequency as the cell population grows. Images taken at 42 hours are shown below.

Figure 17: Images of cells 32 hours post-loading, post-wash. Though overall growth is positive, the larger population consumes more nutrients in the media, quicker. Maintaining the colony becomes difficult.
Figure 18: Images from within the channel 42 hours post-loading. Many dead, floating cells can be seen. This is most likely due to over-population and nutrient depletion.
**20μL exchange**

Refreshing the media in increments of 20μL proved relatively positive towards the cell culture, though not as effective as with 5μL increments. As the simulations show, there is an order of magnitude difference in the flow rate through the channel when volumes of 20μL are removed, which translated to more cells being lifted from underlying glass. However, cells still managed to organize into colonies with strong ECM networks and survived up to 81 hours. Though the media was replenished at regular intervals between 10-12 hours, only images from time points at 11, 37, 67, and 81 hours are presented below.

*Figure 19: 20μL media refreshment timeline.*
T=11 hours after seeding
Pre refresh:

Figure 20: Images of cells 11 hours post-seeding. They have only settled. Image 7c was taken at the inlet, and thus, shows the greatest cell density.
Post refresh:

![Images of cells 11 hours after seeding, post-refresh. Colonies are not present. Rather, many cells have been lifted into suspension. Even the large population of cells by the inlet has been lifted.](image)

In the first set of images, we see many scattered cells, as they have not had the time to form colonies. In figure 14c we see many more cells around the inlet area (large black spot), as this was the point of entry for all of the cells. Though flow is induced by cycling volumes of media from the outlet back into the inlet, large numbers of cells remain within the pipette tips. Over time, they simply fall into the channel and collect right by the inlet/outlet. Thus there are more cells around the inlet/outlet than towards the middle of the channel. In figure 15c we see the cells pushed away in a semi-circular pattern, caused by the influx of fresh media from the media refresh. In the other images we see less cells scattered throughout the field of view as most have been forcibly lifted and pulled into suspension.
T=37 hours after loading

Pre refresh:

Figure 22: Images of cells 37 hours post-loading, pre-refresh. Because cells were lifted and displaced during the prior refresh, there are few in the field of view.
Post refresh:

![Figure 23: Images of cells 36 hours post-loading, post-refresh. The few cells that were present also got displaced.](image)

At 37 hours, we don’t see as many cells as with the 5μL experiments, but small, isolated colonies continue to survive. Still, we see many cells still being lifted after the wash steps, as made evident by the bright spots in the image which are floating cells. The colonies that are still present are getting larger.
T=67 hours after seeding

Pre refresh:

Figure 24: Images of cells 67 hours post-loading, pre-refresh. Large, isolated colonies present.
Post refresh:

Figure 25: Images of cells 67 hours post-loading, post-wash. Though many cells are still adhering, they are only within small colonies.

After 67 hours the colonies are getting even bigger, though still isolated. After the wash steps many more cells remain adhered while the floating ones are cleared away. Most, if not all of the remaining cells are formed into small colonies with very few singular cells scattered about.
T=81 hours after seeding

Pre refresh:

Figure 26: Images of cells 81 hours post-loading, pre-wash.
Post refresh:

![Images of cells 81 hours post-loading, post-wash. Only isolated colonies of cells remain, no large, confluent colonies.]

After 81 hours we see the biggest colonies. However, these colonies did not expand enough, or merge with other colonies to form a more continuous monolayer. Cells growing in a flask achieve a much better level of confluence after 81 hours than cells grown in this manner. Thus, culture was terminated at this point because the goal of growing a single confluent colony was not achieved with this set of experiments.
50μL exchange

This set of experiments demonstrated that it was impossible to culture cells when refreshing the media in aliquots of 50μL. Unlike the difference between the 5μL exchange and 20μL exchange were there were simply fewer cells left on the channel base; every single cell was uprooted and washed away when refreshing the media 50μL at a time. Though the cells would still be viable if allowed to re-settle, the culture attempt would be futile because the cells do not get enough time to form colonies/networks before getting lifted after another wash.

A summary of the findings from the wet bench experimentation can be found in the table below.

<table>
<thead>
<tr>
<th>Media Refresh Quantity</th>
<th>Effect on Cell Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>5μL</td>
<td>Best culture method. Channel media can be replenished with minimal colony disturbance.</td>
</tr>
<tr>
<td>20μL</td>
<td>Insufficient colony growth. Though some colonies do survive dispersion, overall confluence percentage is too low.</td>
</tr>
<tr>
<td>50μL</td>
<td>No colony growth. At each media refresh iteration, all cells are lifted and scattered.</td>
</tr>
</tbody>
</table>

Table 2: Summary of wet bench experimentation.
Cell Growth on PDMS

Channel sterilization, fibronectin fixation, and cell loading in the PDMS-PDMS environment was identical to that of the PDMS-glass set up. However, after leaving the cells to incubate overnight, large bubbles formed within the channels that were not present immediately post-loading. An external view of the channel in figure 22 depicts the bubble formation.

![Figure 28: Bubbles present within PDMS-PDMS channel construct.](image)

The presence of bubbles within the channel poses a few significant challenges in the culture of cells. If the bubbles are present right at the inlet/outlet of the channel, they completely block all fluid flow and prevent not only the seeding of cells, but also the replacement of the media. Bubbles within other portions of the
channel take up space that cells would otherwise occupy and equally importantly that media would occupy. Also, the high surface tension at the boundary between the bubbles and the fluid can damage the integrity of cells it comes into contact with. Figure 26 shows images of cells left in the channel overnight after seeding. The bright elements in the images are floating cells that failed to adhere to the PDMS base.

![Images of cells unable to adhere to base due to bubble presence.](image)

To determine if the bubbles were forming spontaneously, or if they were a cellular byproduct, a separate channel was loaded solely with culture media and placed in the incubator overnight. No bubbles formed in this empty channel. It is speculated that the porous nature of PDMS contributed to this bubble formation. In the PDMS-glass setup, any gases released by the cells can only travel upwards from the culture and are absorbed/diffuse through the PDMS to the outside environment. In the PDMS-PDMS case, these gases can also enter into the PDMS underneath the
cells and may not fully diffuse into the atmosphere. If the PDMS directly surrounding the cells gets saturated, and isn’t diffusing into the environment, the gases will stay trapped in the channel and form bubbles. These bubbles not only take up space that cells would otherwise grow in, but also occupy a volume that would otherwise contain fresh media, thereby preventing cells in other areas from receiving proper media refreshment.
**Cell growth within Matrigel**

Another method to promote cell growth was attempted, and that was to grow cells within a Matrigel scaffold. Matrigel is most commonly used in cell culture to enhance colony growth as it contains many proteins found in the cell ECM that promote cell adhesion, bonding, and signaling. An image of cells, 5 hours after being suspended in the Matrigel and loaded into the channel can be seen below in figure 27 below.

![Image of cells within Matrigel matrix, 5 hours post-loading.](image)

The morphology of the cells show they have attached to the glass and are healthy. They have the spread out, elongated structure of properly adhered HUVE cells. The bright spheres seen in the picture are likely cells that are within the Matrigel, but not adhered to the glass surface, much like the displaced floating cells seen in previous experiments. The cells were allowed to grow undisturbed for another six hours before the media within the channel was refreshed. As previous experimentation had shown, removing media in increments of 5μL disturbed cells the least, thus, the same approach was taken in refreshing the media for cells grown in the Matrigel. Figure 28 below show the structures of these cells immediately after media removal and replacement.
Figure 31: View of cells 10 hours post-loading, post-refresh. Whole healthy cells are rare, and there are large scrambled masses present which suggests cells were damaged.

From the image, it seems as though the cells got pushed together somehow, if not broken apart altogether. Earlier, cells that were displaced by too high of a flow rate were simply lifted and would later settle. Here, we see some rounded features that belong to lifted cells, but we also see an abundance of grainy regions, which suggests cells may have been destroyed. Unlike previous experiments where cells were exposed to forces from moving fluid, these cells experience stresses from the bulk Matrigel. As media is removed from either pipette tip, and one goes deeper into the pipette tips, some of the Matrigel that overflowed out of the channel into the reservoir is invariably removed. This removal of the Matrigel in the tips causes the Matrigel within the channel to shift and move just as regular fluids do. As the whole bulk moves, cells that are adhering both to the glass below and the gel above experience shear forces that rupture them. Had the channel been completely sealed by the Matrigel scaffold, this may not have occurred. To see if these cells were permanently damaged, their culture was continued. Figure 29 below shows the state of the channel after another 9 hours of culture.
Further culture of cells for another 5 hours (14 hours post-loading total) does not show any new colonies or re-adhered cells.

The cells in figure 26 above have been cultured for a total of 14 hours. In these images we see a few cells have re-settled, but the bulky grainy mass takes up a majority of the space. Had the cells simply been lifted from their position, they would have re-settled. The presence of such few re-settled cells and the continued presence of the grainy mass suggest most of the seeded cells were destroyed when refreshing channel media. Though initially seeded cells do adhere and begin to form networks in the Matrigel, the media change technique utilized does not allow for a successful continued culture.

Despite initial difficulties with loading the cells and properly maintaining the culture, much was learned about microfluidic cell culture. Fibronectin deposition was crucial to promote cell adhesion on glass, but it was not needed within the Matrigel scaffold. Its presence was trivial within the PDMS platform, because bubbles prevented cell adhesion altogether in our experiment. The most important
aspect in maintaining a confluent culture was the media refresh technique. As the results show, increasing the volume of media exchanged by even 15μL completely alters if the cells grow in a single large colony (as observed for 5μL incremental change), isolated colonies (as observed for 20μL change), or prevents long term growth altogether (as observed for 50μL change).
Cell Growth Analysis Via Image Processing

It is important to make quantitative observations to better elaborate on the differences between the two media refresh techniques. Two image processing programs, ImageJ and CellProfiler, were used in tandem to further analyze the cell images. ImageJ [18] is a java-based program developed by the NIH for image analysis. It has many different image manipulation and processing functions built in and is open to free use by the public. CellProfiler is open-source software developed by Anne Carpenter [19] and her team in 2006 for purposes of automatically extracting important phenotype information from captured images. Its hallmark feature is the ability to create a computational pipeline to analyze tens if not hundreds of images in sequence, and pool the data together. It was designed with ease of use in mind to allow persons without programming knowledge to apply image processing algorithms to their work.

Though cells grew into colonies in both cases, the colony sizes differentiated one growth technique from another. Cells grown by refreshing the media in 5μL increments grew into much larger colonies than when media was refreshed in 20μL increments. As previously explained, this was due to the increased flow rates that 20μL aliquot changes instigate. Many cells were displaced throughout the culture period, and thus prevented them from proliferating.

With the use of these two free programs, it was possible to quantify the area in the visual field occupied by cell colonies. Colony area measured in this manner was used to judge how much better one growth technique was than the other. In the future, this technique can be further refined to analyze and classify the colonies in an automated fashion.

The primary strategy was to identify what percentage of the total area within the field of view the cells occupy. Since the microscope camera took a digital images, it was simplest to divide the number of cell-pixels by the total number of pixels in the image. Initially, CellProfiler was not able to easily identify which features in an
image were and were not cells. A number of photographic manipulations had to be performed before it was able to identify and count cells accurately. ImageJ was used for the pre-measurement processing. First, the RGB source image taken by the camera was converted to 16-bit greyscale. Next, the “edge-detection” tool was applied to enhance the image. Finally, a median filter was applied over the image to smooth grainy features and make the differences between the cells and the background more apparent. After these changes, the image was loaded into Cell Profiler. First, the image was cropped down to eliminate the black borders and bring focus to the circular field of view. Next, a built in function called “IdentifyPrimaryObjects” was applied to distinguish relevant objects (cells) from the background. Once the cell features were isolated, the program calculated the pixel-area occupied by the features.

A different area-measurement technique utilizing a binary representation of the image was also attempted. The image (post-ImageJ processing) was opened in CellProfiler and converted to binary with the “ApplyThreshold” function. This resulted in the cell features being converted to white pixels and everything else being converted to black. Next, the “MeasureImageAreaOccupied” function divided the area occupied by the cells (white pixels) by the total number of pixels in the image to determine a cell area occupation percentage. Each technique had its own merits and faults. With these two approaches, it became possible to quantify the efficacy of the different culture techniques.

Two images selected from the 5μL and 20μL growth procedures were processed through this image processing pipeline.
5μL sample

Original image and image post-ImageJ manipulation:

Figure 33: Comparison of the original image, and the image after ImageJ processing. After conversion to 16-bit, an "edge detection" algorithm was applied, followed by a medium filter to "smooth" out the image.

The above black and white image was uploaded into CellProfiler and additional computational steps were performed.
CellProfiler object detection:

![Figure 34](image)

Figure 34: Object detection performed by CellProfiler. Green regions are included in further calculations, purple are not.

Applying the CellProfiler function “IdentifyPrimaryObjects” generates a modified image, as seen above. The green lines outline objects that fall under the entered minimum and maximum size specification for the cells, 10-40μm in this case. The purple lines delineate objects detected by the program, but do not meet the user specifications. Only the objects outlined in green were used for area counting. Although the program did a fairly good job identifying cellular growth regions, it failed at certain points inside the colony. In the top-right quadrant of the photo, there are black regions within the colony lacking cell growth. Those areas were still segmented and included in the overall area count by the program, however. The same can be seen at a region at the center of the photo. The program also generated an image where the segmented portions have been filled with different colors, for more visual clarity.
Filled Segments with processed original:

![Filled Segments with processed original](image)

Figure 35: Comparison of the modified original image and CellProfiler-generated image highlighting detected cellular colonies. Some regions devoid of cells in the upper half of the image are missed and counted as having cells.

With this specific process, the program determined that the cells occupied 52.5% of the total area within the field of view.

Below are the findings from the binary-conversion area calculation method.

Binary image:

![Binary image](image)

Figure 36: Binary representation of the previous 16-bit image.

This processing step did an acceptable job of capturing the area occupied by the cells. With this method, it is estimated that the cells occupied 44.0% of the total
area in this visual field. The difference between the previous estimate and this one is due to two main reasons. Firstly, this image has a lot of black spots peppered throughout the white cell space. The program tags these spots to be the background. Closer visual inspection of the original image at many of these regions shows that they are in fact more translucent cellular regions where cells border one another, and so, should have been counted as cells. Secondly, the areas devoid of cells in the top right quadrant were detected by the binary procedure, whereas they were missed by the object detection procedure. These two differences explain why the second technique gave an 8.5% lower estimate of the area occupied by the cells.
20μL sample

Original image and post-ImageJ manipulation:

Figure 37: Comparison of the original image, and the image after ImageJ processing. After conversion to 16-bit, an “edge detection” algorithm was applied, followed by a median filter to “smooth” out the image.

Cell Profiler Object detection:

Figure 38: Object detection performed by CellProfiler. Green regions are included in further calculations, purple are not.
Filled segments with processed-original:

Figure 39: Comparison of the modified original image and CellProfiler-generated image highlighting detected cellular colonies. Some regions devoid of cells in the upper and lower third of the image are counted as having cells.

According to this approximation, the cells occupy 24.4% of the total area. The binary image-based estimation yielded a result of 24.5% area occupied by cells.

Binary image:

Figure 40: Binary representation of original 16 bit image.

Unlike in the previous image set where the binary image included background elements where there were none, in this set, it picked up non-existant cell structures. Though they are small specks individually, they are so widely distributed throughout the image that it alters the overall area count. It is
interesting to note that in this case, the object detection and binary conversion methods both yielded similar area coverage values. However, upon closer inspection of both manipulations compared to the original, it is easy to point out where each technique fell short in identifying cellular regions. It is by coincidence that the numbers ended up close.

More images from the 5μL and 20μL culture techniques were also subjected to image processing. Specifically, those from the 32 hour post refresh and 81 hour post refresh, respectively. A summary of the findings from the processing steps is found in the table below.

<table>
<thead>
<tr>
<th>Cell Culture Technique</th>
<th>Cell Area Occupation Percentage</th>
<th>Image Processing Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photo Sample</td>
<td>Object Detection (%)</td>
</tr>
<tr>
<td>5μL</td>
<td>1</td>
<td>56.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>65.5</td>
</tr>
<tr>
<td>20μL</td>
<td>1</td>
<td>27.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>21.9</td>
</tr>
</tbody>
</table>

Table 3: Image processing results outlining the differences in total cell area occupation between 5μL and 20μL culture techniques.

From the image processing results we see that culturing cells with the 5μL refresh technique is superior to the 20μL technique, based on the bigger percentage of area coverage.

It is important to note these results are a comparison of but six images out of all of the images taken throughout experimentation. Even though CellProfiler allows for batch image processing, there are two main reasons why such group processing could not be performed. First, not enough pictures were taken over the entirety of the channel surface. Though a substantial number of pictures were taken, they were of random spots within the channel. Further, some timestamps had more images than others. Had images been taken of the whole channel for each and every experiment, there would be enough consistency to be able to combine the results and make generalizations. The second reason all of the images could not be analyzed in a uniform fashion is because of the differences in image properties such as
brightness and focus. The microscope used for this study was located in a public space and frequented by many different users throughout the day. At the time, there was no effort made to ensure the source brightness setting was the same as in previous sessions of use. If the brightness of two images is a little different, contrast enhancing which works perfectly for one image may not work for the next. Hence, one would have to manually alter each image. This was impractical given the large number of images. Further, capturing images was a purely manual process. Due to the camera set up, it was difficult to ensure the same focus was applied for each image. For example, the borders of the field of view were slightly blurred in some images. If the camera is not properly focused on a colony, then CellProfiler may overlook it as a relevant object. If this were to happen multiple times over many image sets, important data would be overlooked.

Small differences across multiple images made it difficult to perform batch image processing and obtain accurate results. This experience demonstrated that proper measures need to be followed consistently when taking the images in order to generate better computational results concerning cell proliferation.
Discussion

Degassing the channel prior to loading was crucial. If filling the channel with a micropipette tip in open air were attempted, a small bubble would form at the interface of the micropipette tip and the interior chamber. Because of the high surface tension, it is very difficult to push out that bubble and it blocks fluid flow. Loading the channel while it’s submerged underwater eliminated this, as there was no air present for the bubble to form at the pipette tip. Loading underwater was not sufficient, however. Tiny bubbles still formed at the edges of the channel, most likely a result of the gases that are dissolved in the PDMS. Degassing the channel while it was submerged removed the bubbles.

Initial channel sterilization and subsequent fibronectin introduction was very straightforward, as it only involved direct flow through the channel. Optimizing the cell loading protocol proved challenging, however. After lifting the cells from the culture flask and spinning them down, early attempts involved re-suspending the cell pellet in 150μL of media and introducing 50μL aliquots of the cell suspension to the channel. After the fluid level in the two micropipette tips stabilized, 20μL was removed from the outlet to induce more flow in from the inlet. This process was repeated 2 more times with the remaining cell suspension. Such a procedure was mostly ineffective, as removal of 20μL from the outlet caused cells that had already entered the channel to be sucked out, which resulted in large amounts of cell loss. A more effective procedure was re-suspending the pellet in 50μL of media, and introducing the suspension at once. To ensure the maximum numbers of cells were drawn into the channel, 20μL of media was removed from the outlet, like before, but re-added to the inlet. This not only drew in more cells into the channel, but the re-introduction at the inlet also pushed more of the suspension at the inlet into the channel.

Determining how frequently to replenish the culture media after loading cells took further investigation. Typically, cells cultured in a dish or flask require media to be changed every three days. In this case, because cells are growing in a much
smaller volume, the nutrients in the media are depleted much quicker. Experimentation showed that in order to maintain cell viability, the culture media needed to be changed no later than every 10-12 hours.

With initial experiments, maintaining a continuous culture proved difficult. Early techniques for replenishing the media involved removing anywhere from 50-140μL aliquots from the outlet end, thereby drawing the “old” media out of the channel and inlet reservoir. The same amount of fresh media would then be introduced to the inlet to not only replenish the media, but also to further drive flow of the old media toward the outlet. This technique was not successful, as removing/adding even as little as 50μL from each end induced a high enough flow rate within the channel to uproot and wash out the already adhered cells. A few changes to the refreshing technique rectified the problem of cells getting displaced. Primarily, a much smaller amount of media was removed from each end. Lowering this volume results in a smaller pressure differential between the fluid columns, which results in a lower flow rate of media through the channel. Second, alternating media removal between the inlet/outlet also prevented a drastic column height difference forming between the two micropipette tips. With these improvements, cells were not greatly disturbed and continued to grow successfully.

**Future Work**

Additional testing can be performed on the cells to ascertain colony properties. Cells exhibiting healthy colony growth form intercellular bridges, which consist of adherin junctions, gap junctions, and tight junctions among others. Each type of junction is composed of various proteins and protein complexes that can be immunostained to determine the integrity of the colony. Since single cells won’t have such proteins widely expressed, as they are not communicating/interacting with anything around them, the presence of these proteins is a strong indication of healthy colony formation. Further, cells organized into confluent colony and tissue
structures exhibit an elaborate extracellular matrix. Testing for structural proteins that anchor cells to the ECM can be used as further evidence for confluence.

A cell culture set up as described in this work can be used for a malaria study. Red blood cells infected with malaria undergo changes various morphological changes[20]. These include changes in shape, rigidity, membrane composition, and surface protein expression[21-24]. These changes cause them to create clots and block arteries/capillaries in an otherwise healthy vascular system. An increase in surface protein expression increases their tendency to not only stick to one another, but along vessel walls. With time, these small cell clusters can grow into large blockages that heavily constrict blood flow[25]. As the interior of vessels within the human body are lined with endothelial cells, this culture platform can be used as a model to study the interaction of malaria-infected cells with HUVE cells. Malaria-infected blood and normal, healthy blood (as a control) can be flowed through the cell-coated channel at physiological flow rates. A microscope can be set up to capture the interaction of blood cells as they pass above the confluent cell layer mimicking the interior of the vessel. Information on blood flow rate, clotting rate, and sticking tendency of the infected red cells can be gleaned from experimentation.
Bibliography