An In vitro Model for a Cell Monolayer using a Microfluidic Device

Andrew Hartman, Materials Science and Engineering

Mentors: Brendan Leung, Joseph Labuz, & Cameron Yamanishi

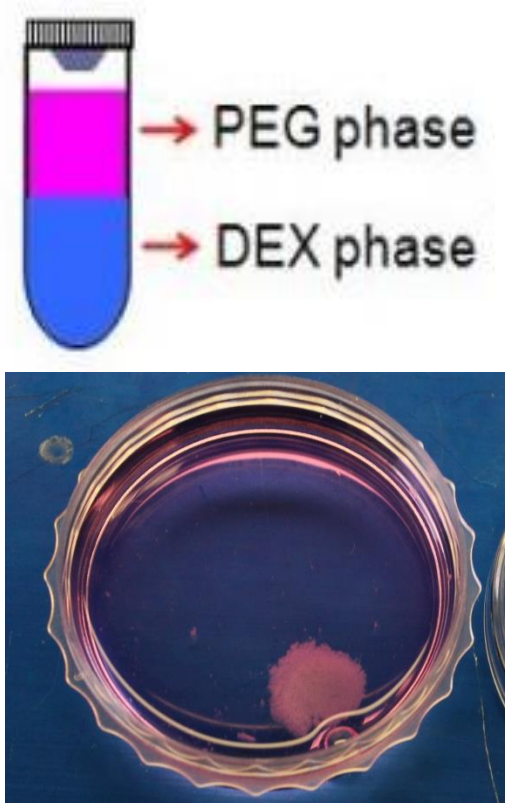
Advisor: Shuichi Takayama

Introduction

Epithelium exist throughout the human body with varying purposes. These include the alveoli of the lungs where gas exchange occurs, as well as the endothelial linings of the capillaries for further gas exchange and waste transport. Monolayers also exist throughout the gut for nutrient uptake as well as in the kidney for waste removal. Although single cell layers serve many vital functions in the body, an *in vitro* model of the same conditions experienced *in vivo* has not yet been designed. Monolayers are present in normal cell culture when growing cells on a dish, but nowhere in the human body do cells exist on plastic. At all natural occurrences, monolayers exist with fluid on either side, or fluid on one side and gas on the other. An *in vitro* design of cell monolayers that is able to model the conditions found *in vivo* would therefore be incredibly useful. Such a design would allow for a more realistic model for drug testing for pharmaceuticals to be used where monolayers are present as well as in a larger project being worked on with the end goal of modeling the human full metabolism *in vitro*.

Currently, cell monolayers, or sheets as we call them, can be formed with liquid on both sides, although this is within a plastic tube. To accomplish this, an Aqueous Two-Phase System (ATPS) is utilized [1]. In our lab we accomplish this with a concentration of a sugar, Dextran (DEX) for one phase, and the other with a high concentration of a polymer, Polyethylene Glycol (PEG). When these two solutions are put in the same container, they separate, like oil and water (**Figure 1)**, and cells in the upper PEG layer will settle to the interface. With nothing at the interface to bond to but themselves, the cells will form junctions with surrounding cells to form a sheet. The issue with this set up is that once the cell sheet is formed within a tube, it is too fragile to use. It is possible to empty the tube into a dish and remove the sheet, though the sheet is too fragile to be moved from the dish. Furthermore, a high concentration of PEG will kill the cells if

left for more than 24 hours, so long term culture within the tubes is impossible. Therefore, another system must be designed which can not only allow cell sheets to form, but keep them alive long term and be easily accessible. Such a system would allow for further testing, staining, and characterization.



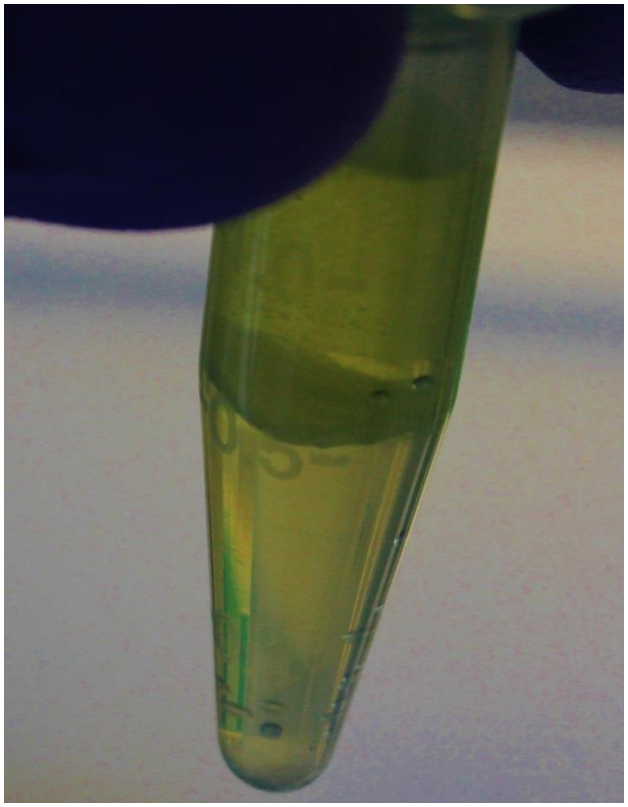


Figure 1: *top left* a diagram of ATPS set up in a tube can be seen. *Right* an actual ATPS set up with a cell sheet grown at the interface can be seen. *Bottom Left* the cell sheet from the image on the right can be seen after it was removed from its tube. At this point it is too fragile for any further usage.

My research goal was to design a system that could grow cell sheets as well as keep them alive for an extended period of time and characterize sheets grown within this system. Extended culture means that the PEG and DEX need to be removed from the sheet, and because the sheets are too fragile to move out of their environment after growth, I would have to design a system that can change the environment and medium around the cells. To do so, I designed a microfluidic device.

Chip Design

Microfluidic devices are typically made of Polydimethylsiloxane (PDMS), a transparent, flexible polymer. My device consists of three individual pieces which are plasma bonded together into one single chip **Figure 2.** Plasma oxidation removes the methyl group from a PDMS chain and replaces it with a hydroxyl group. The resulting PDMS surface creates strong bonds with other PDMS, creating one solid device

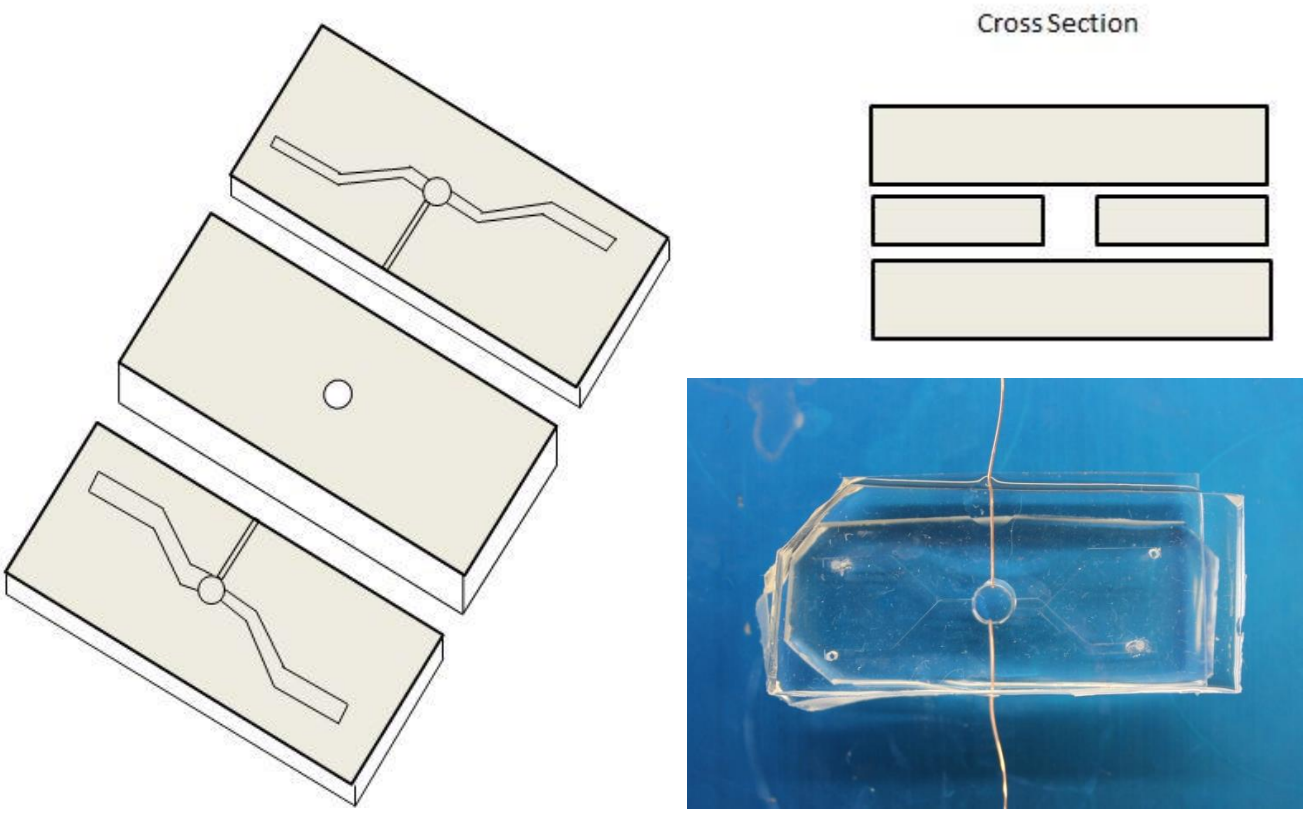


Figure 2: *left* the three initial pieces of PDMS can be seen. The two top and bottom pieces are identical with a microchannel molded into their surface. The middle slab has a 4mm hole punched through it, which is where the cell sheet will form. These outside pieces are bonded to the middle slab, with the channel on the inside, using a Femto Science Covance, with the round area of the channel matching with the hole punched through the middle layer. *Top Right* a cross section of the finished chip. The molded channels sit directly above the middle slab. The middle chamber and top and bottom channels are now entirely inside the PDMS device. *Bottom Right* a finished device.

The design of the chip went through many different prototypes before the design seen above was decided upon. This design allows for an ATPS to set up in the round middle chamber after the

PEG and DEX solutions are flowed in through the top and bottom channels respectively. Cells are suspended within the top PEG layer so that they can settle to the interface and form a sheet. The channels also allow for medium changes after the sheet has formed, so that the ATPS can be removed. The wires seen in **Figure 2** serve as electrodes to measure transepithelial electronic resistance (TEER), which will be discussed in more detail later. The design of this device allows for the environment to be changed around the cell sheet; as opposed to moving the sheet between environments, which is difficult due to how fragile the sheet is.

Device Loading

To fill the device, a great amount of precision is needed. Ideally, the interface of the ATPS would fall exactly in the middle of chamber. Top accomplish this, PEG would have to flow into the top channel at the exact rate, starting at the exact same time as the DEX flowing into the bottom channel. Because the liquid volume within the chamber is so small, approximately 0.0528 mL, the margin for error is very small. A syringe pump allows for constant, low flow rates for multiple syringes, so that the interface can form as close to the middle of the chamber as possible. Two syringes are used, one loaded with the DEX solution, and the other with the PEG and cells. These syringes are connected via medical tubing to 18 gauge tips which can fit into the access holes punched into the channels. This set up can be seen in **Figure 3.**

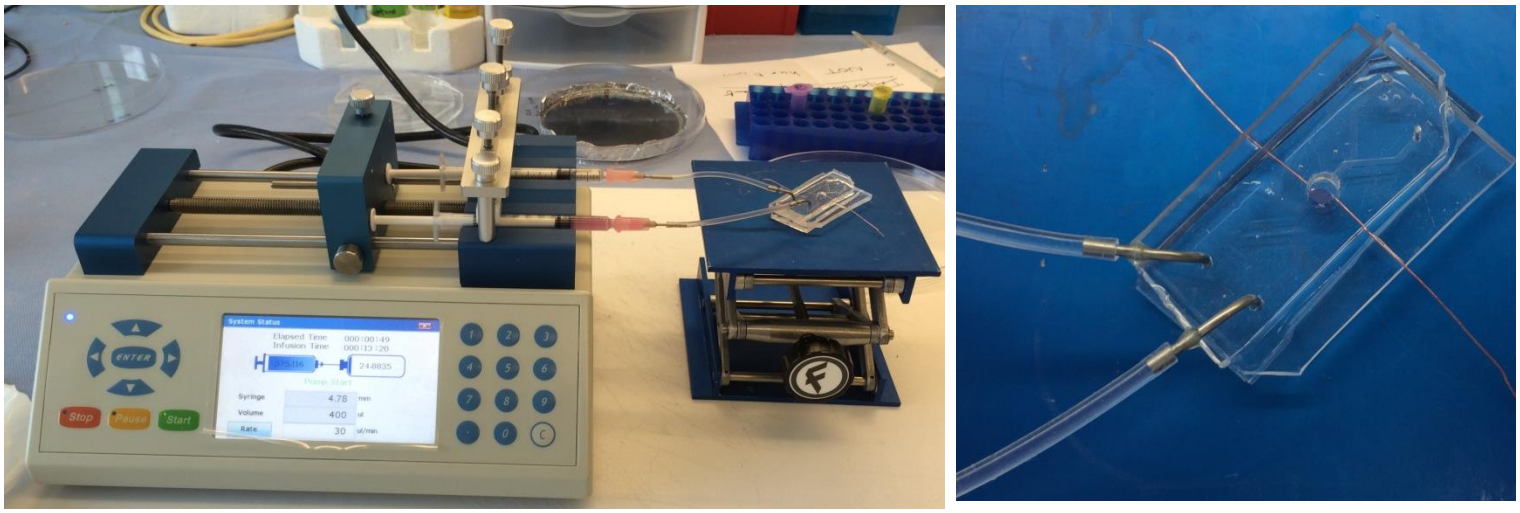


Figure 3 *left* the full set up for loading, and *right* a close up of a chip as the PEG and DEX is being flowed into the chamber by the pump.

Once loaded, the access holes to the channels are covered with a drop of water to prevent any evaporation and the devices are placed in a dish with wet paper towels within an incubator overnight to further prevent evaporation. As the volume is so small within the devices, any

amount of evaporation will lead to air bubbles within the chamber that can interfere with sheet formation and imaging. As the devices sit overnight, gravity leads to the cells falling to the interface and forming a sheet.

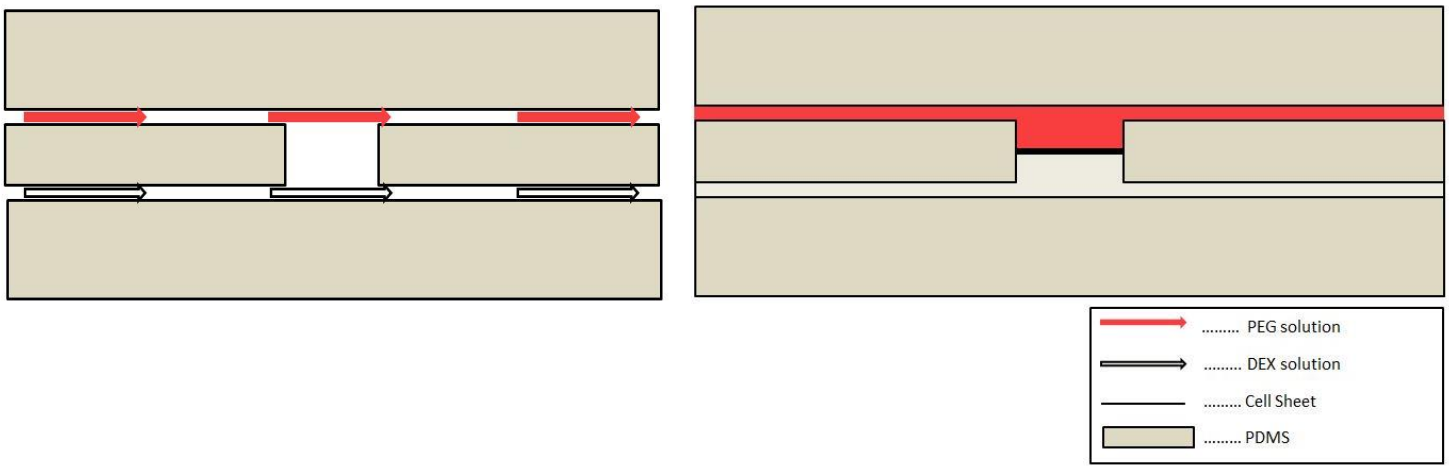


Figure 4: *Left* schematic of the chip being loaded with PEG and DEX flowing through the device. *Right* cross section of a loaded chip with cells settled to form a cell sheet at the interface.

Transepithelial Electronic Resistance (TEER)

TEER involves an electrical circuit with a cell monolayer as a part of the circuit. The resistance across the monolayer can be used a quantitative measure of sheet quality. The more tightly bonded the cells are, the harder it becomes for the current to pass through the junctions between them. TEER is widely used to evaluate growing monolayers on trans well membranes [2], and measurement devices are made specifically for this purpose. Commercially sold devices are incompatible with our chip, since it required two electrodes to be submerged on either side of the monolayer. Because the chip is a closed environment, we would have to design our own circuit for measuring TEER. This is possible by measuring the voltage drop across the cell sheet compared to a known resistor at different frequencies (an AC current is required as the cellular membrane acts as a capacitor and would build up a charge in a DC circuit). The copper wires seen protruding for the chip in **Figure 2** are used as electrodes to connect the sheet into the circuit. Special thin channels were designed into the chip to allow for the electrodes, which hook the chip into a circuit, to be positioned into the edge of the chamber on either side of cell sheet. We can then calculate the impedance of the cell sheet and in turn the resistance attributed to the junctions between cells, our value of interest. The cell sheet includes two capacitive components from the cell membrane, and two resistive elements from the cytosol as well as cellular junctions. Through much circuit analysis the resistance of the junctions was calculated.

$$R_j = \left(\frac{Z}{K \times f}\right)^{\frac{1}{3}}$$

Where Z is the magnitude of the impedance of the sheet, K is a known constant, and f is the frequency of the input signal at which measurements were taken. The circuit used to model our set up can be seen in **Figure 5.**

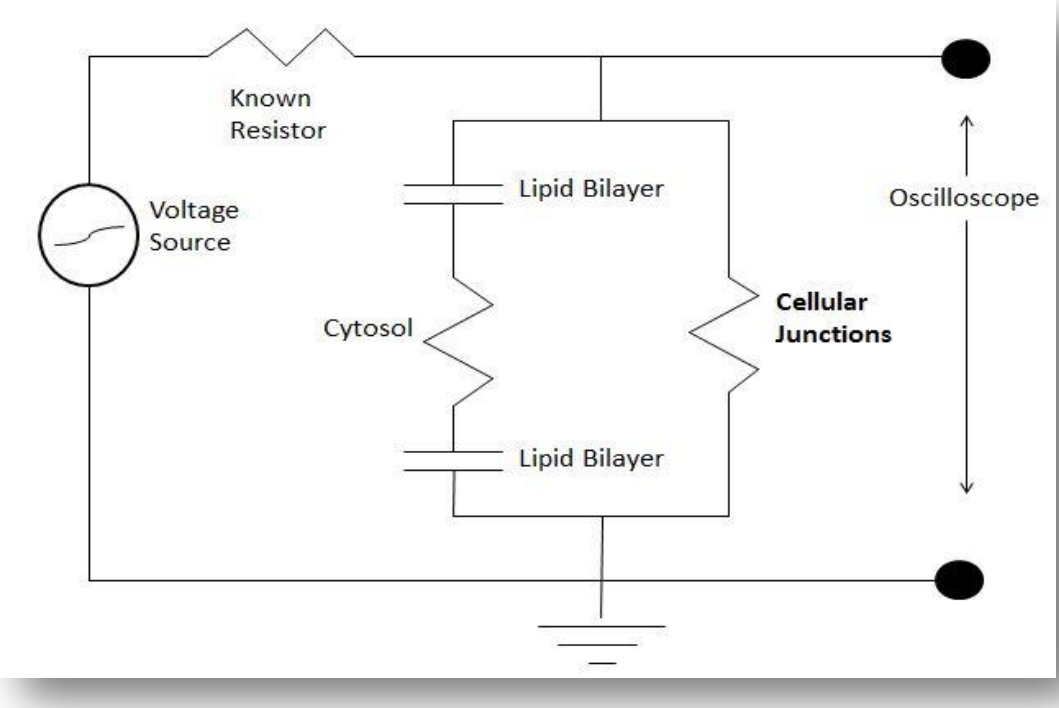


Figure 5 the circuit used for TEER readings.

Results

From the beginning of summer to the end of summer, protocol, chip design, and cell type used all underwent changes. Initially, tests were conducted with MCF10a cells, which are human breast epithelial cells. In the past at the lab, MCF10a sheets had been grown in tubes without issue. Initial tests were done in chips with MCF10a but were never able to form full sheets although control sheets in tubes formed normally. In chips, sheets only partially covered the interface in wisps, seen in **Figure 6**. This sort of morphology is not possible for cells simply settling to the interface; there is no reason for the cells to collect at these locations more than the rest of the interface, as the surface is smooth with constant interfacial tension. It was theorized that what was occurring was a complete cell sheet forming and bonding not only cell to cell, but also cells to the outside of the chamber. The force of the cells pulling to the wall pulls the cells at

the middle of the sheet apart, resulting in the stretched morphology we saw. This was confirmed by a time course of images taken from 1 hour post seeding of cells through 24 hours seen in **Figure 7.**

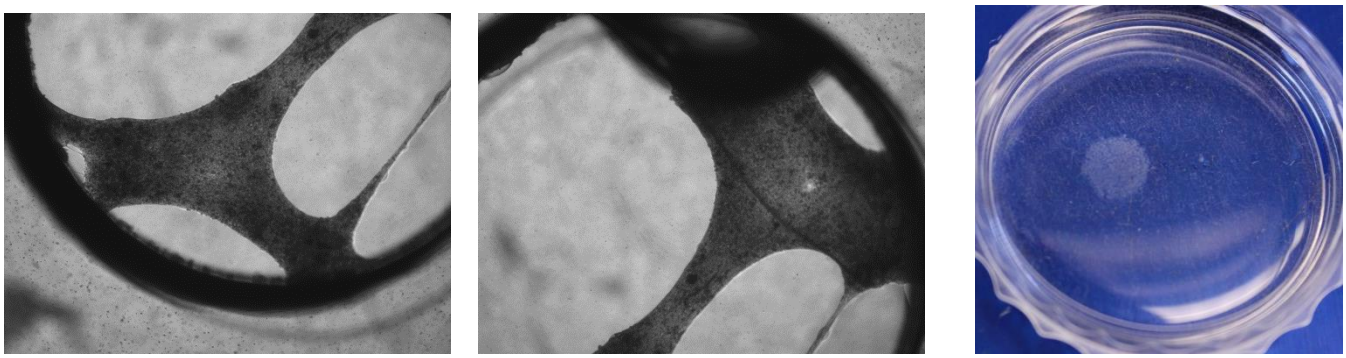


Figure 6 The two images on the left show two chips imaged 24 hours post seeding which have pulled apart. The image on the right is a well formed control sheet made the same day as the images on the left. Images on the left taken at 2X using a Nikon overhead microscope and image compressor.

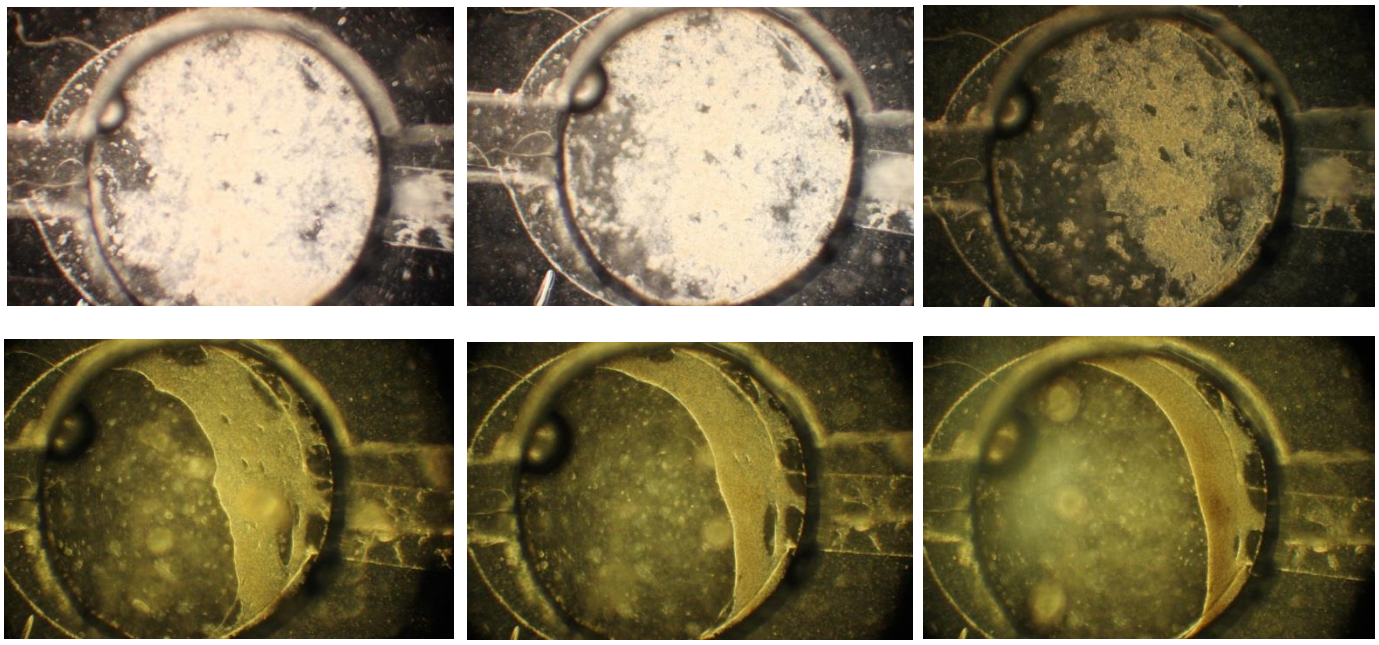


Figure 7 Time course of a single sheet forming. Images are taken at, from top left to bottom right: 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours post seeding. Images are taken at 2X magnification with a digital camera.

It was hypothesized that the pulling on the cells that occurred only in the chips was due to the plasma oxidizing of the devices involved in their bonding. When PDMS is plasma treated it

enables cells to adhere strongly to the treated PDMS, as well as the intended PDMS to PDMS bonds. When the chips are being fabricated, all three pieces are exposed to plasma treatment to ensure one solid device. Unfortunately, this also means the inside of the middle layer's punched hole is treated. To combat this, the end of a 4mm biopsy punch, the same punch used to cut the chamber, was used to plug the hole in the middle layer of the device during treating and in turn preventing the methyl groups from being replaced on the inside surface of the chamber. Therefore, cells would not be as apt to adhere to the chamber well, and only bond with each other.

Around the same time that this change was made, tests using MCF10a began failing, with the control tubes no longer working either. Across the lab, other projects work with MCF10a, and at the same time all MCF10a's stopped working. Different MCF10a stocks were thawed from cryo-freezing, but to no avail. What caused the MCF10a to stop working for everyone is still unknown, but we were forced to change cells types to continue research. Fortunately, MCF10a is not crucial to the project, just that an epithelial cell is used to form a cell sheet, as the end goal is to form these same sheets with human lung cells. It was decided to proceed with canine kidney epithelial tissue, MDCK.

MDCK yielded well-formed cells sheets immediately. Initial test were conducted in test tubes ranging in inside diameter from 10mm down to 5mm, the smallest available to model the 4mm chamber in the PDMS devices. Under all conditions, cell sheets formed, allowing research to continue into the chips moving forward, where sheets also immediately formed. Unlike sheets formed in tubes, these sheets did not cover the entirety of the interface (**Figure 8**). This morphological difference, while unexpected is not detrimental to the integrity of the sheet. After 24 hours of formation in ATPS, the chips were again hooked up to the syringe pump, this time loaded entirely with cell culture medium. After another 24 hours, the medium had changed from its initial purple hue to a faint yellow due to phenol red indicator included in the medium which changes to yellow in acidic conditions. This change in pH is attributed to cells using nutrients and producing waste, indicating that the cells sheets are not only formed, but also still alive.

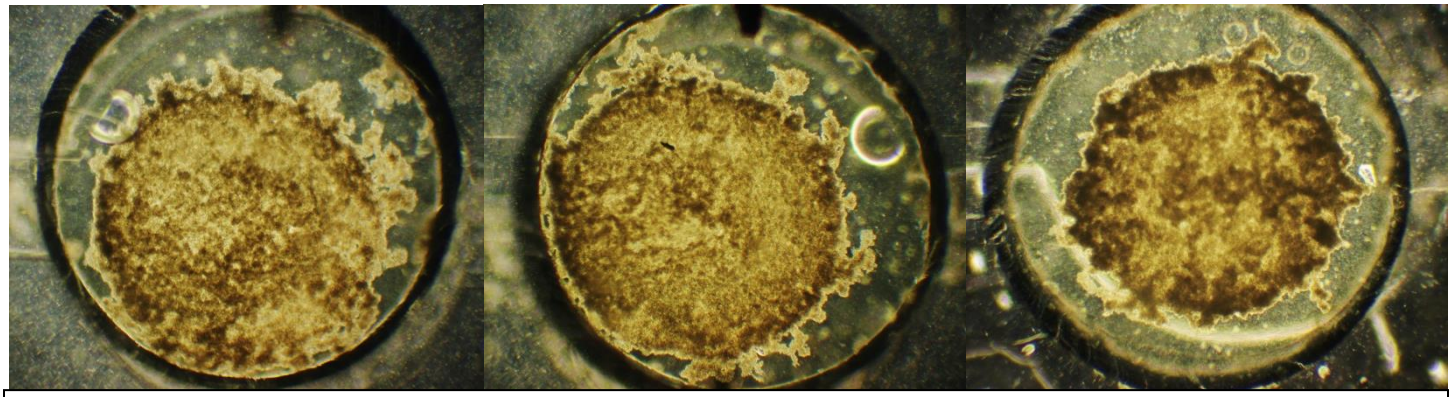


Figure 8 separate cells sheets formed in PDMS chips, imaged 24 hours post seeding. Note that although nearly perfectly round, the sheets do not cover the entire interface. Images taken at 2X magnification with a digital camera.

To determine what leads to the morphological difference between sheets formed in tubes versus sheets formed in chips, sheets were left to form in an Olympus inverted microscope, equipped with a motorized stage and live cell imaging chamber to regulate temperature, CO2 levels and humidity. The conditions in the live cell imaging chamber allow for sheets to form while be imaged once every minute for 24 hours to create a time lapse movie of sheet formation. This movie showed that, similarly to previous MCF10a sheets, the sheet initially formed across the entire interface. Unlike previous tests through, within 30 minutes to an hour of seeding, the sheet began to contract away from the chamber wall, uniformly. This is likely due to cells being less apt to adhere to the non-plasma treated chamber wall, and instead only experiencing pulling forces from other cells and tighter cellular junctions formed. Within 4 hours, all contraction had stopped and the sheet reached its final morphology.

TEER Results

TEER reading on MDCK monolayers on trans well membranes has previously been conducted yielding resistance measurements of 50-150 Ω*cm^2 [3]. Therefore, we expected readings in the same range, though not necessarily identical due to the differences induced by growing the sheet unsupported in ATPS versus on a membrane. Initial data showed an unexpected frequency-dependent decay in measurements of resistance with respect to frequency (**Figure 9**).

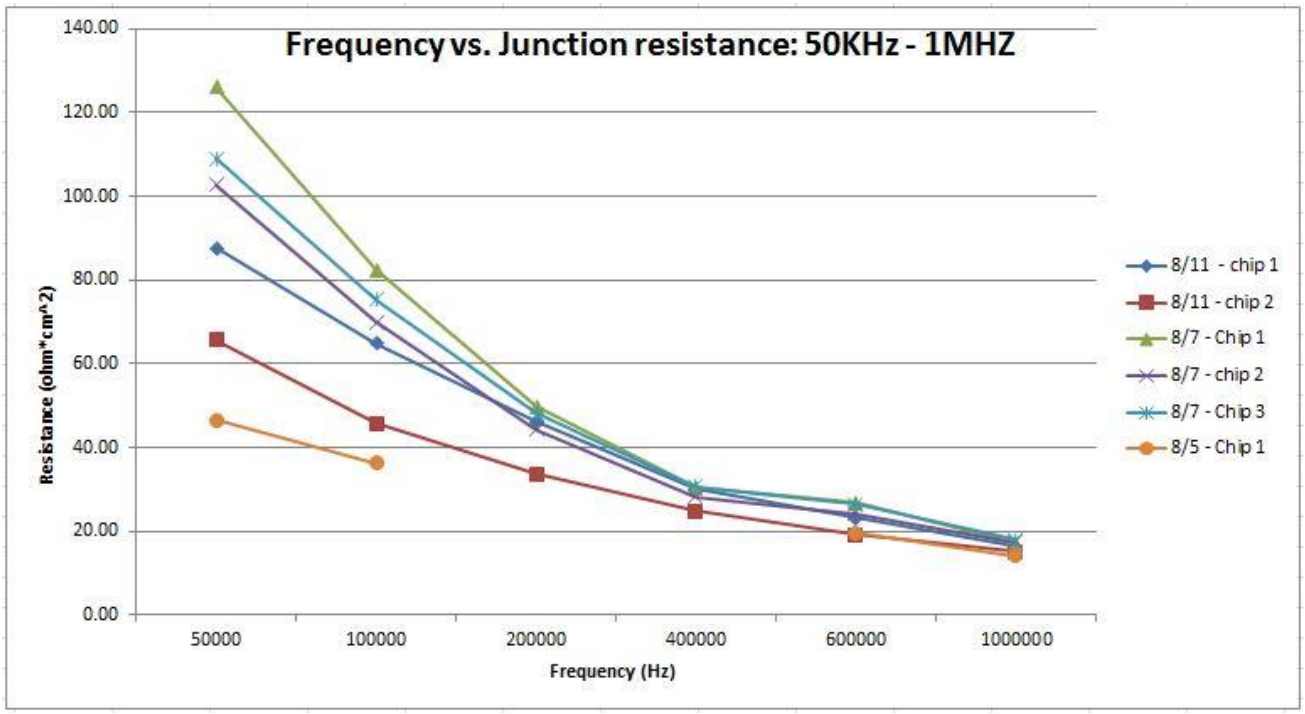


Figure 9 plots cellular junction resistance measurements at a range of frequencies from 50KHz through 1MHz for 6 different cell sheets. Junction resistance is a calculated value of a single electrical component, so no slope should be present in the data.

Each measurement, regardless of frequency, is a measure of the resistance through the same sheet, and therefore measurements should be constant, not decaying as we see in **Figure 9**. After going over our calculations finding junction resistance, it was determined that there must be an additional circuit element which we are overlooking. Initially, resistance of a chip loaded with only medium (negligible resistance) was measured to ensure that our setup was functioning properly. These measurements were used to model the ATPS solution surrounding the cell sheet. This was overlooking that ATPS has macromolecules suspended within it, as well as an interface that could have an effect on the flow of current. To analyze these affects, chips were loaded with various concentrations of ATPS as well as PEG and DEX only to see if these caused any unexpected resistivity.

We expected to see an impedance element from the macromolecules of PEG and DEX suspended within the medium, though we expected the interface to have no electrical effect. We found that PEG and DEX solutions do impose an added impedance effect, but both of these in the chip at once as an ATPS raises this impedance significantly, as can be seen in **Figure 10.**

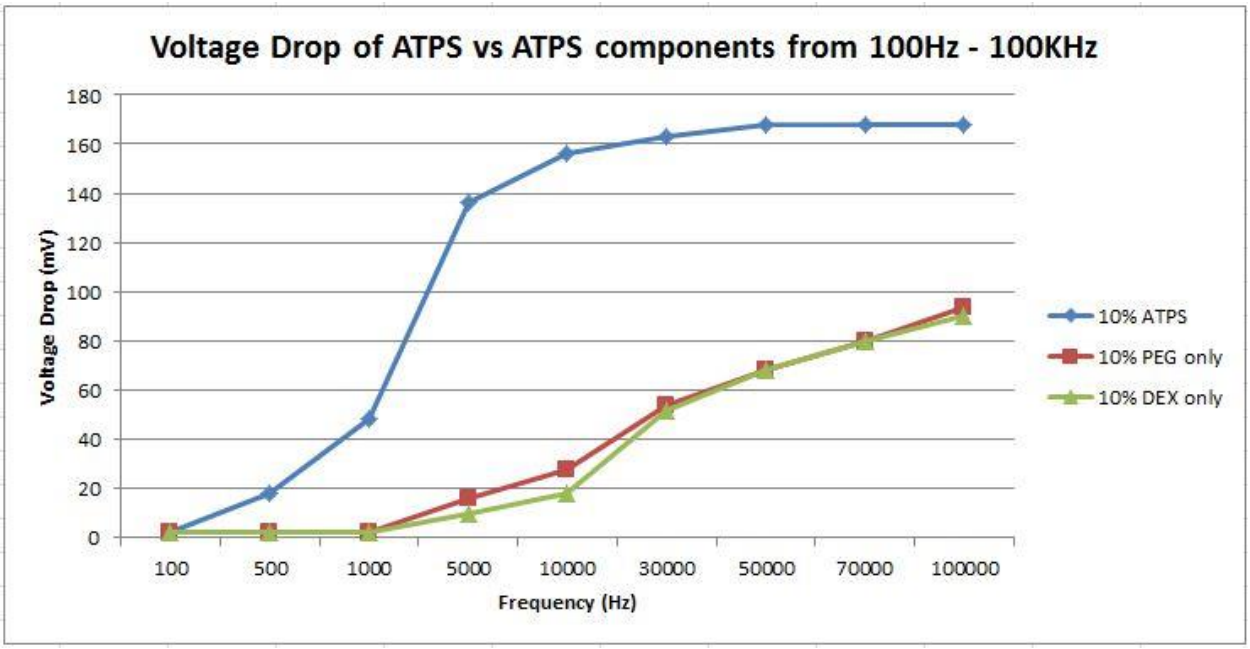


Figure 10 When the chip was filled entirely with 10% PEG and 10%DEX, voltage drops were nearly identical, but when the same two solutions were used in ATPS, the resulting voltage drop was significantly higher with a different shaped curve. Because voltage drop is directly related to impedance and resistance, it can be concluded that the interface of an ATPS adds an additional resistive element.

Though work has not been done within the lab to characterize this added element due to ATPS, it will be an interesting topic of study as the project continues. Zdenek Samec worked with similar liquid-liquid interfaces in trying to characterize their properties [4]. His research suggests that such a system can be characterized as two resistive elements from the macromolecules in liquid suspension in series with a capacitive element at the interface. It would seem as though our system would fit this model, though further work will be done to fine tune our model based off of this work.

Discussion

The recent findings regarding TEER as an additional electrical component are very exciting for future research. With this information, we can integrate the interface as an added circuit component and hopefully get accurate measurements of junction resistance, and in turn be able to quantitatively describe the quality of the cell sheets. In the near future much of the work on this project will be done to fine tune the TEER circuit analogy such that consistent data can be collect without unexpected trends. Also, the interface of such an ATPS as an electrical circuit component isn't a topic with much previous work, which could lead to further applications characterizing a relationship between interfacial tension and resistivity.

Within the lab, we will soon be moving away from MDCK for cell sheet formation to work with the end goal of lung epithelium. Lung sheets will be used in a much larger project as an *in vitro* model for the lung in the human on a chip project which aims to model the entirety of the human metabolism on a microfluidic device for the purposes of drastically improving personalized medicine. Previously in the lab, projects have been conducted to model the adipose and kidney aspects of this future device. MDCK was used primarily as a model cell type to conduct many experiments and fine tune protocol for forming and characterizing cell sheets. Not only is lung more expensive to work with, but once work begins with a stock, it can only be passaged a finite amount of times before it is no longer workable. Because of this, it is important to have all aspects of cell sheet formation fine-tuned, so that, in theory, lung sheets can be made effectively when we begin work on them.

Conclusion

Over the course of the summer, we were able to successfully design a microfluidic system capable of growing a cell sheet. We were able to fine tune the protocol for growth and begin tests to characterize the sheets. Most characterization work was devoted to developing a circuit for TEER testing and analyzing these results. While TEER testing is not complete, it lead to a new discovery regarding the interface as an electrical circuit component, which will be the focus of much research in the future. We were also able to begin testing on the long term survival of the

sheets, which was initially promising. More work will be required though, to design a way to catch the cell sheet when ATPS is removed, because when replaced with medium, the cell sheet falls to the bottom of the chamber as there is no longer an interface to support it. This should be able to be solved by altering the design of the middle chamber. In the future, our primary goal will be to grow the cell sheets within a chip using lung tissue. Once this is accomplished, we will move on to characterizing these sheets through imaging and TEER measurement techniques developed over the summer.

References

1. Rajni Hatti-Kaul. "Aqueous Two Phase Systems: A general overview." Molecular Biotechnology. **2001, Vol. 19.**
2. Kathryn Hatherell. "Development of a three-dimensional, all-human in vitro model of the blood–brain barrier using mono-, co-, and tri-cultivation Transwell models." Journal of Neuroscience Models. **2011, Vol. 199.**
3. Nicholas J Douville. "Fabrication of Two-Layered Channel System with Embedded Electrodes to Measure Resistance across Epithelial and Endothelial Barriers." Anal Chem. **2010 March 15; 82(6).**
4. Zdenek Samec. "Electrical Double Layer at the Interface between Two Immiscible Electrolyte Solutions." Chemical Reviews. **1988, Vol. 18, No.4.**