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On the Fabrication and Development of Tissue on a Chip Devices with Included Perfused Vasculatures

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Dedication

This thesis is dedicated to my parents Naveed Ismail and Aliya Rajput, without whose unyielding support and love this thesis would not have been possible.

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The advent of Tissue-on-a-Chip (TiOC) devices has provided a novel way for researchers to approach biological study and drug development. As a platform that enables human *in vivo* conditions to be accurately replicated *ex vivo*, TiOC can accelerate both fundamental biological research to answer basic questions regarding tissue behavior and function (e.g., cell-cell interactions in the tumor microenvironment (TME)) and translational research that includes testing of standard and novel therapeutics. The reported work focuses on development of processes and technologies common to two classes of TiOC. The first device is a single-layer, multi-compartment microfluidic device for investigation of pancreatic ductal adenocarcinoma (PDAC) tumor behavior in a comprehensive TME that includes cancer and non-cancer cell types. The second is a multi-layer, multi-compartment (chamber-over-chamber) device for human gut research. Both systems are designed to support a perfused vascular network in communication with the tissue of interest (tumor or gut epithelial cells). It was found that the devices can be made by utilizing conventional microfabrication methods and can be constructed in such a way that experiments are repeatable and customizable for different cell types and environmental

conditions. One-step soft lithography and a straightforward assembly process were used to mold the single-layer elastomeric (polydimethylsiloxane, PDMS) Tumor-on-a-Chip (TOC). Soft lithography was also used to mold components (two channels and a porous PDMS membrane) of the Gut-on-a-Chip (GOC); however, the small size of the mold pillars, corresponding to pores of the PDMS membrane, necessitated a more sophisticated fabrication approach. Further, the chamber-over-chamber design of the GOC required development of an alignment jig for device assembly. Challenges, pitfalls, and successes are discussed.

Chapter 1: Introduction and Motivations

The past 30 years of pharmaceutical development of treatments and drugs have seen a marked slowdown (Pammolli, Magazzini, & Riccaboni, 2011). While the reasons for this slow down can be attributed to a multitude of different factors (Paul et al., 2010; Scannell, Blanckley, Boldon, & Warrington, 2012), a lack of innovation in technique has had an impact on the pharmaceutical down turn of late. To this end, new microfluidic culture and research vessels known as Organ-on-a-Chip (OOC) or Tissue-on-a-Chip (TiOC) (Esch, Bahinski, & Huh, 2015; Zhang, Korolj, Lai, & Radisic, 2018) have emerged in the disease researching field. These devices seek to make the study of diseases and the effects of drugs on humans better understood by employing a method that helps to augment current development procedures. This thesis will focus on the development, improvement, and application of this type of device, specifically focused on two different pathologies, pancreatic tumor formation and behavior and Necrotizing Enterocolitis (NEC) in the gut of newborns.

Innovation and Effectiveness: The Drug Industry's "Drying Pipeline"

In order to understand the need for the scientific and pharmaceutical industries' foray into exploring new research and development platforms, it is important to grasp the current state of the drug development industry. Rising costs, rates of attrition, increased regulatory scrutiny, and increased pressure to create better drugs (Paul et al., 2010) have all helped to stymie growth in pharmaceutical research and development (R&D). Since the 1950s that the number of approved drugs per billion dollars spent on development has halved every 9 years (Scannell et al., 2012). Additionally, only about 1 in 6 drugs that make it to the clinical trial stage are approved for use in the United States (Dimasi, Feldman, Seckler, & Wilson, 2010). This approval rate has remained generally constant despite the ongoing innovations in science and

technology sectors and the enormous amount of resources and money that are spent creating these drugs. A review of 10 American drug companies' portfolios in the year 2013 suggested that the total capitalized cost to develop a drug is 2.87 billion dollars (DiMasi, Grabowski, & Hansen, 2016). The increase in spending and low rate of approval has led to the coinage of the term "drying pipeline" to suggest that the availability of new drugs and treatment options available to the consumer are decreasing and will continue to decrease in the future (Zhang et al., 2018).

Issues lie not only with drugs in development, but also with drugs that are already in use by patients. Based on data collected between 2013 and 2014 it was estimated that 4 out of every 1000 emergency department cases in the United States were related to adverse effects of drugs taken by patients (Kim & Moon, 2012; Shehab et al., 2016). Specifically, drug induced nephrotoxicity, a state where the kidneys are unable to properly function, filter the blood, and maintain homeostasis, is a leading cause of adverse drug affects. Solutions to the "drying pipeline" of new products and treatments in the pharmaceutical industry will not be discovered by one change or breakthrough. However, the devices that this thesis will present, and those like them, can help researchers by providing a platform to augment and perhaps even one day replace the need for animal testing.

Animal testing is an ingrained step in the drug development process. In order to test the efficacy of target compounds in a complex, multicellular, homeostatic environment, a living host must be used. Despite the biological complexity of animals, the tests that are conducted are often inadequate and/or not reproducible in human clinical trials. An examination of high impact journals showed that only one third of the work done with animal models translated to humans in clinical trials and that only 10% of these studies were approved for use in patients (Stephan, Hill, Xi, Delft, & Moecklinghoff, 2012; van der Worp et al., 2010). How this improvement will be

made is another topic currently up for debate in the industry. The solutions that are being applied and will be applied in the future are entirely dependent on what researchers and the industry decide the shortcomings with the animal model are. These critiques include a lack of effective randomization in animal studies (Pound, Ebrahim, Sandercock, Bracken, & Roberts, 2004) or the use of improper dosages of drugs in animals to produce effects that can be observed by the researcher (Holmes, Creton, & Chapman, 2010). One of the most important issues with translating data from an animal model to a human is that the genetic structure and sequence of an animal and a human are different. This can lead to promising work done in animals, which is not replicable in human trials. Several notable examples of this non-transference of results exist such as in the case of the drugs Encainide and Fleacainide in the 1990s. These drugs showed promise in animal models as cardiac arrhythmia regulators (Harrison, Winkle, Mason, & D, 1980) but failed to demonstrate the same results in human trials (Sommer et al., 1991). In a more extreme circumstance the clinical trials of Fialuridine, a possible treatment for Hepatitis-B, in 1993 led to the death of a third of the patients in the study (Smow, 1995). The development of TiOC devices will help to avoid the mistranslation of results seen in animal studies to human studies. By providing a flexible, controllable, and *in vivo* like environment for testing scientists can be better informed about the effects drugs and other treatments will have on humans.

2D v. 3D Cellular Culture and Study: Arising from the Flat Plates of the Past

On the pathway to developing a new testing platform, an emerging challenge to biological and pharmaceutical research is the medium in which the research is conducted. Traditionally, work done with cells has been done in a petri dish that is either lined with or immersed in a culture medium to feed the cells seeded on the plate. This method has served scientists well in the past, and major discoveries, such as the discovery of penicillin, have been

found on these plates. However, it has become increasingly clear that the better the cell culture environment matches the parallel environment *in vivo*, the better the results will translate to humans (Huh, Hamilton, & Ingber, 2011; Ma et al., 2012; Shamir & Ewald, 2014).

Before describing how OOC and TiOC devices harness the power of 3D cell culture, it is pertinent to describe why the 3D method of culturing cells is superior to the 2D method of culturing cells. The most prominent reason why 3D cell culture is preferred to 2D culture is the development of an extracellular matrix (ECM) with which the cells can interact. In the world of cellular development and study, it can be said that function follows form. That is the shape of the cell can help guide its function in a complex biological system. The ECM has been found to have great influence on developmental biology and therefore should be considered a vital part of cellular studies (Lingala & Ghany, 2016). This importance can be demonstrated when looking at how the ECM affects the cellular development cycle.

Studies conducted as early as the 1980s had found that the ECM had a role to play in biological development and cell differentiation. In 1982 Grinnell et al. noticed that the amount and location of fibronectin relative to fibroblasts present in pregnant female rats during pregnancy was correlated to the shape of the fibroblasts at that time (Grinnell, Head, & Hoffpauir, 1982) . Another study examining the Sertoli cell type, which forms tight cell-cell junctions *in vivo*, found that when these cells were cultured on a flat plate they formed a monolayer that did not capture the structure observed in humans. However, when these same cells were cultured in a gel that recapitulated the structure of the basement layer in the adult testis, the Sertoli cells grew in a way that was quite similar to the structure of the cells *in vivo* (Hadley, Byers, Suàrez-Quian, Kleinman, & Dym, 1985). Furthermore, a recent 2013 study showed that the ECM is a vital player in the stem cell differentiation process (Watt & Huck,

2013). This is an important point as OOC/TiOC devices commonly incorporate stem cells in their use and function. Work done with induced pluripotent stem cells (iPSCs) has shown that in combination with the correct transcription factors and environment iPSCs recreate cell types found in humans (Robinton & Daley, 2013). While these are only a few examples of the effect that a cell's environment has on its behavior, there are many more that show how a cell's surroundings will impact its growth and development.

Another reason that the ECM and the three-dimensional cell culture environment are preferred to the traditional flat plate is the ability to better understand how mechanical cues and forces influence biology. Through the study of 3D cellular biology, the interconnected nature of biological/chemical influences and mechanical forces can be elucidated. The most basic example of this phenomenon in human tissue, is the effect of gravity against which muscles, tendons, and bones work (J. H. C. Wang & Thampatty, 2006). These mechanical forces are a major factor that help to influence ECM and cell shape, and by extension influence how well these cells function and/or in what way they function. An example of the way forces affect cells is through the stretching of fibroblasts, a cell type that helps provide structure to the ECM, in the Anterior Cruciate Ligament (ACL) and Medial Cruciate Ligament (MCL). This stretching will result in different levels of mRNA expression which codes for the production of collagen, a structural protein in the body (Hsieh et al., 2000). This result is one of a multitude that demonstrate how the change in mechanical environment can profoundly impact biological function at the cellular level. Another example of this type of behavior that is more relevant to this thesis's work is the effect of shear stress, produced by fluid flow at a wall, on cellular shape and behavior. This mode of stress is often important in the formation and proliferation of blood vessels (Robinton & Daley, 2013).

Vasculature Formation: Connecting the Cellular Environment

Another important aspect of the ECM regarding cellular function is to establish connectivity networks between cells. This thesis will focus on the establishment of vascular networks in OOC/TiOC devices. The vasculature is a series of connected blood vessels that form the circulatory system in humans. There are two main ways to produce such a system in OOC/TiOC devices. The first, angiogenesis, is the process through which new blood vessels are created from existing blood vessels. The second is vasculogenesis, which is the process by which blood vessels are formed *de novo*. The ECM plays a key part in this process in the body. Acting as a scaffold for the appropriate endothelial cells and biological factors, the ECM helps to drive capillary formation in interstitial spaces (Moya, Hsu, Lee, Hughes, & George, 2013; X. Wang et al., 2016)

Based on the action of shear stresses and the presence of an ECM in conjunction with perfusion, it is possible to create a vascular network inside of OOC devices. Previous work, especially that done by Lee and George et al., have shown that it is possible to grow a vascular network in microfluidic devices (Moya et al., 2013; X. Wang et al., 2016).The ability to grow these networks is exciting, particularly in the context of the two tissue devices, Tumor-on-a-Chip (TOC) and Gut-on-a-Chip (GOC), on which this work is focused. The vascular network provides an organic method for drugs, transcription factors, and other biological factors to be transported to cells being studied. This allows clearer examination of these factors effects in an environment that most closely resembles the human *in vivo* environment.

Microfluidic Platform Advantages

The OOC/TiOC platforms both are characterized more broadly as microfluidic devices. Microfluidic devices are those that are fabricated at and deal with fluid flow at the micrometer

length scale. Despite the small sizes of these devices, they offer extreme levels of control and regulation when running experiments and testing hypotheses. The volume of fluid in these devices is as little as 10^{-9} to 10^{-18} liters in the chambers and channels that comprise the microfluidic layout (Sosa-Hernández et al., 2018). While the use of such devices is not limited to biological studies, they are well suited for the biological field.

There are several reasons for this. First, cells can be cultured in a three-dimensional space. As was stated earlier, being able to culture cells in a three-dimensional space adds a level of complexity which elevates any potential results to be closer to the *in vivo* conditions than those of past two-dimensional studies. Further, increased accuracy relates to the length scale at which the studies are carried out. As noted above, the quantities of liquid and biological components that are used in TiOC studies are significantly smaller than those used in Petri dish or flask studies and therefore can lead to data that is more applicable to the human body.

The potential accuracy of microfluidic platforms can only be realized if these platforms provide a method in which data can be collected in a relatively simple and non-destructive manner. The biological microfluidic platforms of today provide a variety of methods for data collection. However, due to the numerous ways that microfluidic devices are used, there are many different ways data can be collected from them. Only a few broad examples of possible assessment techniques will be described here. One example of microfluidic assessments is the ability to evaluate and assay a single cell. Enzyme-linked immunosorbent assay (ELISA) and genetic transcription of a single cells are some of the methods used for evaluation . Another method that is used to evaluate biomicrofluidic devices is use of live cell imaging. The concept of live imaging is one that allows the biological processes that are taking place inside of the

reaction vessel to be monitored over time through the use of microscopic techniques (Duncombe, Tentori, & Herr, 2015).

The devices are able to be imaged because most of them are made, at least in part, out of polydimethyloxysilane (PDMS), a transparent elastomer that houses the cells being cultured in the devices. The use of PDMS to create microfluidic devices was first reported in 1998 by Whiteside et al. (Duffy, McDonald, Schueller, & Whitesides, 1998). Since that time, the field of biomicrofluidics has largely been dominated by the use of PDMS to create devices. One reason for this dominance in the microfluidic field is the fact that PDMS is considered biologically compatible (Belanger MC, 2001). In other words, cells will not react with or be harmed by the PDMS. In addition, PDMS does not significantly change the biochemistry of the environment around the PDMS. Understandably, this is a desirable trait in TiOC devices as one seeks to recapitulate the *in vivo* environment and not alter it in any way. Secondly, PDMS is transparent. As stated above, transparency to light allows for imaging of cells within the device. Finally, PDMS is able to be molded with sub-micrometer resolution (Hua et al., 2004). This is helpful to researchers as it allows for greater flexibility in potential device layouts.

Despite the advantages that PDMS gives in the creation of TiOC and OOC devices, there are some drawbacks to using this material. One potential drawback in using PDMS is the gas permeability of the material. While researchers like to have the material permeable to oxygen (Leclerc, Sakai, & Fujii, 2003), there are other gases that can pass through the material that are undesirable. An example of this is water vapor absorption (Velve-Casquillas, La Berre, Piel, & Tran, 2010). In addition to this, PDMS has been shown to absorb hydrophobic materials and drugs, diverting them from cells and the biological environment (Paguirian & Beebe, 2008).

Differences between the GOC and TOC Platforms

While the TOC and GOC devices that are examined in this thesis are similar in how they are fabricated, as well as in the advantages and disadvantages that they have as it pertains to biological study, there are key differences in the structure and function of these two platforms. The most noticeable difference between the two platforms is the structure of the final assembled device. The TOC device is a planar design and contains all of the pancreatic cancer cells, endothelial cells, vasculature, and other biological factors in one layer. While this leads to a relatively simpler assembly process than the GOC device it also leads to a more complicated layout design of the chip. As seen below in Figure 1, the TOC device has many more chambers, ports, and perfusion lines than the GOC device (see Figure 2).

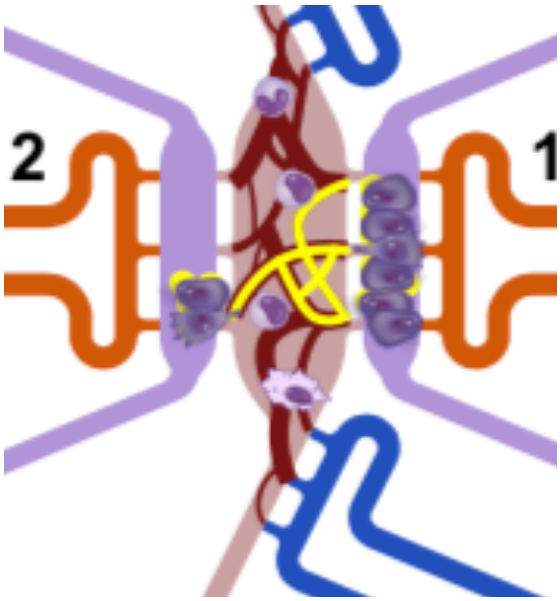

Figure 1. Graphical Representation of the TOC device layout. Only the central chamber of the device has been pictured here. Inlet and outlet pads have been cropped from the image for simplicity.

Part of this complexity is because the device needs separate chambers for certain materials, such as tumor cells and endothelial cells, while providing a method for intra-device communication between the separate chambers through the imbedded vasculature. This leads to

a need for more ports, connection areas, and chambers to be included in the device. All of this means that the fabrication of molds of this device is more generally more complex and challenging than the GOC platform mold creation.

The complexity of the TOC mold design does not mean that the GOC platform is devoid of challenging features. In fact, the simple design of the GOC top and bottom molds leads to complexity in the assembly of the device. As the device has a top and a bottom channel it is not a planar device and so must use a method other than geometry to separate the chambers of the device. A porous membrane is incorporated into the device to allow the vasculature and other components of the device to remain separated. The membrane that divides the top and bottom channels was difficult to manufacture and insert into the device while ensuring that the membrane was correctly aligned with respect to both channels to not allow leakage between the chambers.

While the inclusion of the membrane increases the complexity of the GOC device it can be seen in Figure 2 that the GOC top and bottom channels are relatively simple compared to the TOC device and thus are relatively easier to manufacture.

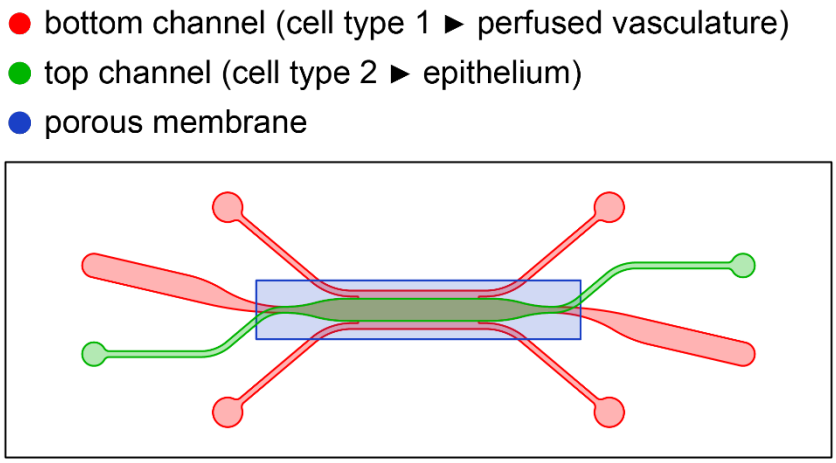

Figure 2. Graphical Representation of the multi-layered GOC device.

Chapter 2: Materials and Methods

Having laid the scientific foundation of the OOC/TiOC devices and the motivations behind trying to fabricate these devices, it is pertinent to describe how the devices used in this body of work were created.

Photolithography & Soft Lithography with SU-8

The molds for both the Tumor-on-a-Chip (TOC) and the Gut-on-a-Chip (GOC) devices were made in the class 100 cleanroom at Washington University in St. Louis. Traditionally, cleanroom procedures were used to create components for the microelectronics industry. However, as techniques have advanced, the ease of fabrication and range of applications for devices made in cleanrooms have broadened to include the biomicrofluidic realm. The basis for the fabrication of the OOC/TiOC master molds lies in lithography. Photolithography is the process by which a pattern of the desired features gets transferred into another material (often a rigid mask material) (Madou, 2002). Photolithography is a complex, expensive, and time-consuming process and thus soft lithography has emerged as a viable alternative for the fabrication of less-expensive microfluidic devices (Kim et. al 2008). Soft lithography is the process through which an elastomeric stamp of the desired device is created by molding and then detaching the device from a negative (Xia & Whitesides, 1998).

While the process of soft lithography has allowed for the rapid creation of OOC/TiOC devices, a photolithography step is needed first to create the negative master mold that was used in the soft lithography process. A common way to fabricate the master mold is to use SU-8 2000 series negative photoresists as is standard practice in the OOC field. Most of the procedures that were used to create the SU-8 master molds used in this thesis follow standard soft lithography techniques.

The first step in the photolithography process was to print a photomask that was used to expose photoresist on a 4-inch diameter silicon wafer. This process involved using a Heidelberg DWL 66+ laser writer to print a copy of the SU-8 features onto a chromium backed glass mask coated in AZP-1500 photoresist. The exposure parameters for the mask were 0% focus, 70% exposure, 47mW laser power, 25% filter. This resist was a positive resist, meaning once exposed by the laser it washed away in the developer. After exposing the design, the mask was developed in AZ-400K developer for 5 minutes and then rinsed in deionized water to remove the resist and reveal the design from the top of the mask. At this point any light passing through the mask would be blocked by the chromium backing of the mask so the chrome was also etched away. This process occurred by immersing the mask in a chromium etchant for 90 seconds. After this wet etching process, the back side of the mask was now clear to any light passing through the glass. However, to fully ensure that all the features on the mask were free of any residual photoresist the mask went through a final cleaning process. There are two ways that this final cleaning process can be accomplished. The first is a two-step process in which the mask is first washed in acetone and then placed in an oxygen plasma cleaner at 100% power for 15 minutes. The second way that this mask can be cleaned is by immersing it in a piranha solution for 5 minutes. Piranha is a solution that is made up of a 3:1 ratio of sulfuric acid and hydrogen peroxide. Both methods were used to make the devices used in this thesis.

Having created a mask with the OOC design on it, the SU-8 features were created using standard soft lithography techniques. First, silicon was chosen as the substrate to build the negative mold on. The choice of silicon as the substrate is largely influenced by the microelectronics fabrication basis of all cleanroom processes (Madou, 2011). To ensure that photoresist will adhere well to the silicon wafer, the wafer was dehydrated on a hot plate at

150ºC for 15-30 minutes. Afterwards, the wafer was cooled at room temperature and then SU-8 series 2000 resist was spun over the surface of the wafer. Next, the wafer was soft baked on a hotplate, the time and temperature of the bake was based on the height of the SU-8 on the wafer; these specifics can be found in the Microchem SU-8 2000 series datasheet. For example, 100 micrometers was a common height chosen for TOC devices. A device this tall was baked for 5 minutes at 65 ºC and then for 16 minutes at 95ºC. Next, the wafer was exposed at 100% power for 8 seconds in the mask aligner to transfer the pattern from the photomask to the wafer. Then, a post exposure bake was done to drive free radicals throughout the resist and help crosslink the polymers. Again, the time and temperature of the bake was based on the height of the photoresist. A 100 micrometer layer of SU-8 was baked for 4 minutes at 65 ºC and for 9 minutes at 95ºC. Following this post-exposure bake the wafer was washed in liquid SU-8 developer to strip away unexposed resist and reveal the SU-8 master mold. The wafer was then rinsed in isopropyl alcohol (IPA) and then deionized water; if this rinse was found to turn a milky white color, the wafer was underdeveloped and was developed longer. The wafer was dried with compressed nitrogen.

The final step in the soft lithography process was the creation of the PDMS stamp from the SU-8 negative. However, uncured PDMS, when poured on silicon, will bond irreversibly when cured. To combat this, the surface energy of silicon must be lowered. The method of choice to accomplish this was to coat the surface of silicon with a solution of trichloro (1H,1H,2H,2H-perfluorooctyl)silane (POTS). The POTS treatment has been shown in the literature to help PDMS structures release from SU-8/silicon master molds (Zhang, Wu, Wang, Xiao, & Wen, 2010). The treatment was done by placing the wafer and a glass slide with a small amount of POTS pipetted onto it (~50 μL) into a vacuum chamber. The chamber was then placed

under vacuum and held for 12-24 hours. Afterwards, the wafer and mold were rinsed with IPA and placed in an oven at 150ºC for 20 minutes. Following this treatment, degassed PDMS in a 10:1 wt% ratio of base to crosslinker was poured over the SU-8 master mold and left to cure in an oven at 60ºC for 4 hours.

Silicon Etching Master Mold Creation

Another method that was explored to create the master mold was to etch them out of the silicon wafer itself. This process is considerably more intensive and time consuming than soft lithography. However, generally the resolution that can be achieved with this process is better than that of the soft lithography method (with respect to the fabrication of the TiOC devices used in this study). As with the soft lithography process, the first step was to dehydrate a silicon wafer for 30 minutes at 100ºC on a hotplate. After allowing the wafer to cool to room temperature for 5 minutes, AZP-4620 photoresist at a target thickness of 7 micrometers was spun onto the wafer. The wafer was then soft baked at 95ºC for 90 seconds. Following this bake the TOC pattern was exposed to ultraviolet (UV) light using a Heidelberg DWL 66+ Laser Writer. The parameters for the exposure was 0% focus, 100% intensity, 70mW of laser power, and 100% filter. It is important to note that as AZP-4620 is a positive photoresist the area on the wafer that is exposed will be developed away exposing the bare silicon underneath. As such, the laser writer does not expose the design itself, but rather exposes everything on the wafer excluding the device. It was also found that due to the thickness of the resist, good fidelity of the pattern of the device was achieved after exposing the device twice in the laser writer system.

After the exposure process, the wafer was developed in AZ-400K developer, rinsed with deionized water, and dried using compressed nitrogen. In order to set the resist and ensure that the pattern of the device did not change during the etching process, the wafer was then hard

baked at 100ºC for at least 30 minutes. Care must be taken at this point to not allow the wafer to heat too much or too quickly. AZP-4620 is a quite viscous resist and if heated too much, even after the exposure and development process, it can reflow and destroy the shape of the features.

At this point, the wafer was ready to be etched using a deep reactive ion etching (DRIE) or Bosch process. This process is a dry etching process where a plasma consisting of gaseous SF_6, used for etching and C_4F_8, used for passivation, was used to anisotropically etch into silicon by alternating etching and polymerization/passivation steps to create features with nearly vertical sidewalls (Madou, 2002). Using the pattern in the photoresist as a mask, the area around the desired features was etched down to a depth of 100-110 micrometers. Following this, the wafer was rinsed in acetone and placed in an oxygen plasma cleaner for 15 minutes. At the end of this process the mold for the TOC/GOC device has been formed in the silicon.

As the etched mold also was to be used as a soft lithographic stamp with PDMS, the surface energy of the wafer must be lowered so that the PDMS does not permanently adhere to the mold. However, instead of the POTS treatment that was employed with the SU-8 molds, a new method was used to help ensure liftoff of cured PDMS from the mold. This method involved the conformal deposition of Parylene C, a poly(p-xylylene) dimer, onto the wafer, creating a barrier between the silicon and the PDMS layers. However, as the PDMS eventually had to be cut off of the mold using a scalpel, steps had to be taken to improve and promote adhesion between the silicon and parylene layers. This was done by first treating the wafer with Silane-174. Improving the adhesion between these two layers by treating the wafer with this silane solution before depositing the parylene helped to ensure that the parylene layer would stay bonded to the silicon and not peel away if cut.

Using a method adapted by Hsu et al. (Hsu, Rieth, Kammer, Orthner, & Solzbacher, 2008), 1 mL of Silane-174 was added to 100 mL of water and 100 mL of IPA. This mixture was stirred using a magnetic stir bar on a hot plate for at least 2.5 hours before the wafer was added. The wafer was first soaked for 10 minutes in IPA before being rinsed and soaked in a deionized water bath for 10 minutes. At this point, the wafer was removed from the water bath and added to the Silane mixture. The wafer was allowed to sit in this mixture for 30 minutes before being removed and air dried for 30 minutes. Next, the wafer was soaked in IPA for 5 minutes before being placed into a 115ºC oven for 30 minutes. After this process, the wafer was put into the Parylene deposition system. While the inner workings of this device are not detailed here, it should be noted that the deposition is conformal so the only parameter that needs to be controlled during the deposition process is the amount of Parylene added to the system prior to deposition. Through optimization, it was found that approximately 1 micrometer of parylene was deposited on the surface for every 1 gram of dimer added to the system.

Device Assembly: GOC

Having a functional master mold of the top and bottom channels, whether made in SU-8 or silicon, brought the fabrication process to its final step, molding. To create the PDMS stamp of the top and bottom channels, PDMS was mixed in the standard 10:1 ratio and then degassed in a vacuum chamber. After the uncured PDMS was found to be free of trapped air, it was poured onto the awaiting master mold that has been placed into the bottom of a 6-inch plastic petri dish. After pouring the PDMS onto the mold it was once again degassed to ensure no air was trapped in the eventual devices and then the dish was placed into a level oven at 65ºC for 4 hours. After removing the mold from the oven, it was allowed to cool at room temperature for 30 minutes. Using a scalpel and tweezers, the devices were cut out of the mold and pulled up from the

surface of the silicon revealing the channel cast in PDMS. Using a biopsy punch, the inlet and outlet holes were created in the device.

As mentioned in Chapter 1, the assembly of the TOC and GOC platforms differs due to the complexity and amount of ports in each device. As a result, the ways that the two devices were assembled are also different. The GOC device, due to the need for a top and bottom channel, requires the additional fabrication step of creating a PDMS membrane that separates the two channels. The method of creating this membrane is largely adapted from a process established by Huh et al (Huh et al., 2013). The first step in this process was to create a pillar array, using cleanroom techniques, in silicon. These techniques were largely the same ones that were used to fabricate the master molds of the devices in silicon. The design of the pillars can be seen in Figure 3. There were some differences in the two procedures, however. The first of these differences is that instead of AZP-4620 positive photoresist, SC-1827 positive photoresist was used. The other major difference between the two processes is that the wafer was only exposed in the Heidelberg system once instead of twice as was done when making the TOC molds. The parameters for the laser writing were: 0% focus, 100% intensity, 47mW of laser power, and 50% filter. Following the etching of silicon to create the pillar array, the wafer was placed through the same silane and parylene treatment mentioned before to ensure PDMS did not stick to the surface of the wafer.

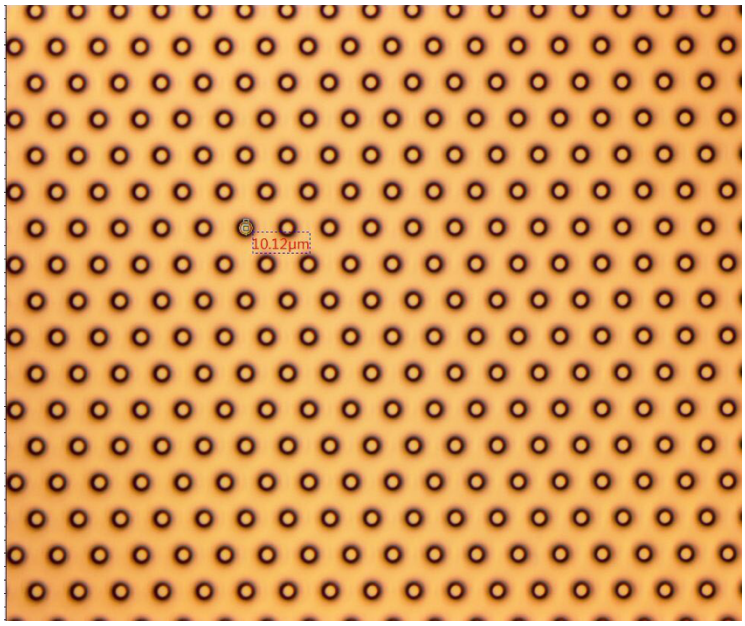

Figure 3. Layout of the Pillar array used to create the PDMS membrane. The pillars are hexagonally packed. The diameter of the pillars is 10 micrometers and the center to center distance is 25 micrometers. The height of the pillars is 10 micrometers.

Next, 15:1 wt% PDMS was spin coated onto the surface of the wafer to a height of approximately 10 micrometers. On top of the wafer was placed a 1-inch thick PDMS slab that had been POTS treated along with a weight that went on top of the slab. The weight pushed the pillars up into the slab ensuring that the membrane that was formed was porous. Following a bake overnight in the oven at 65ºC, the weight was removed from the slab, and the slab was cut out with a scalpel and removed. At this point the membrane should have been a thin layer that sat on top of the PDMS carrier slab. To move the membrane from the slab to the device, the carrier slab and the top channel were cleaned with scotch tape and then placed into a plasma cleaner for 60 seconds. Once removed from the plasma cleaner, the top channel stamp was quickly, but carefully, aligned over the membrane pores and the two layers were adhered together. The assembled top channel-membrane was placed into an 85ºC oven overnight. After removing from the oven and allowing to cool to room temperature, the top channel should have been irreversibly bonded to the PDMS membrane. The devices were cut out from the carrier PDMS slab and

pulled up to reveal a membrane over the central chamber of the GOC device. The device was completed by bonding the bottom channel to the membrane and top channel using the same plasma process as before. After this step the device was assembled and ready to use.

Device Assembly: TOC

The assembly of the TOC device was much simpler than the GOC device. The device only had one layer that was fabricated out of PDMS. The assembly procedure, involving oxygen plasma cleaning, was identical to that which was mentioned above for the GOC device fabrication. Following the creation of the PDMS stamps and the punching of the inlet and outlet holes, the device was bonded to a PDMS slab bottom using the same plasma cleaning method used for the GOC device. A difference in the fabrication steps listed here than those for the GOC device is that instead of tubing being added to the device for continuous flow of liquid, pipette tips were placed in the holes as the TOC device uses passive gravity fed flow to fill the device.

Chapter 3: Results and Discussion

The evaluation of the performance and overall success of the devices made in this work is focused primarily on the manufacturing processes that are used to create them. While a goal of the field of biomicrofluidics is to evaluate capabilities to model the *in vivo* environment in a controllable and reproducible manner, the engineering of the devices being used to recapitulate this environment must first be evaluated and known to be satisfactory before biological conclusions can be reached. Therefore, the results presented here will be examined through a craftsman's lens. How well did the molds created live up to the expectations set out for them, does the fabrication process allow for repeatable and tunable creation of devices, and will the assembly process allow for the creation of complete and working devices are all questions that will be answered in this chapter.

SU-8 Mold Evaluation

The process used to create molds out of SU-8 encountered several problems that are difficult to overcome and, in some cases, cannot overcome entirely. The first of these problems is the formation of an edge-bead that develops on the edge of the silicon wafer when photoresist is deposited on the surface of the wafer. The edge-bead is a wall of photoresist that forms on the edge of a wafer when thick viscous photoresists are spun onto wafers. As the target height for the channels was 100-120 micrometers tall (already quite high for photolithography purposes) the edge-bead created on the wafer reached a significant height. The edge bead impacts the fabrication process by not allowing the middle of the wafer, where the features are exposed, to come into conformal contact with the mask. As a result, the light that passed through the mask to reach the photoresist had to travel through an air gap between the mask and the wafer, diffracting around the edges of the mask and spreading out. This has the effect of causing the pattern that is

exposed into the photoresist to be slightly expanded beyond what was initially designed, and causes rounding of features and a loss of resolution. The rounding of these features can be seen below in Figure 4.

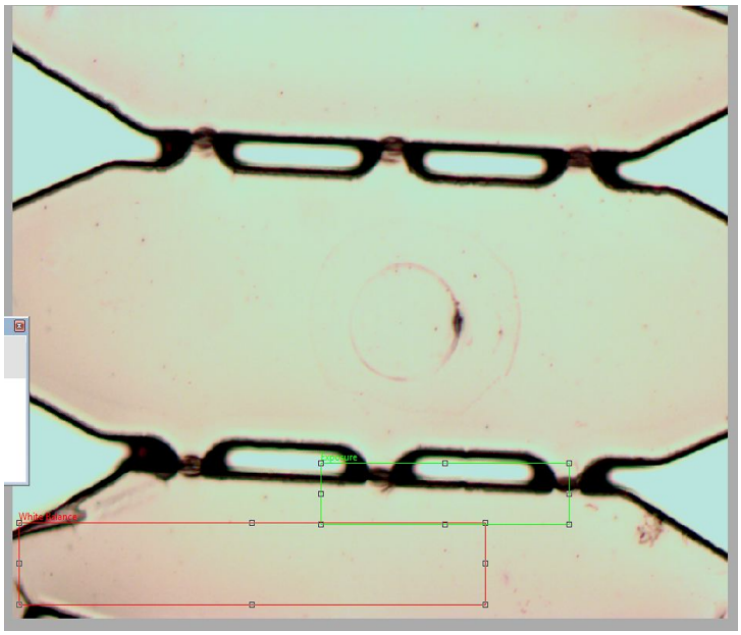

Figure 4. Figure of a TOC device that has been poorly exposed and inconsistently developed. The dark edges in the corners of the device indicate feature rounding and inconsistent exposure and development of the device.

The rounding phenomenon had a profound effect on the TOC mold as this device had small, 30-micrometer wide ports that were critical to the design of the device. Rounding and expansion of these features severely damages the ability of the TOC platform to successfully compartmentalize cells in specific chambers and also allows leakage between sections of the device.

Another reason for the feature rounding, and also the poor development, that can be seen in Figure 4 relates to the exposure system that was utilized. A Suss MJB3 model mask aligner was used to expose the SU-8 2000 series resist that was used to create these devices. SU-8 develops best when exposed by light that has passed through a long pass filter to remove UV

wavelengths below 350 nanometers. However, the available mask aligner does not have this filter, and while it uses a mercury short-arc lamp that exposes from 350-450 nanometers, experimentally it is found to not expose completely in the corners and channels of the devices. It should be noted that as of the time of submission of this thesis, a new Kloe UV-KUB 3 LED-based light source mask aligner, which exposes at a single wavelength of 365 nanometers, is available and has preliminarily proven to preserve the fidelity of features with much improved resolution over the MJB3. As can be seen in Figure 5, the new mask aligner seems to eliminate most feature rounding and the underdevelopment that plagued the devices made with the MJB3 system.

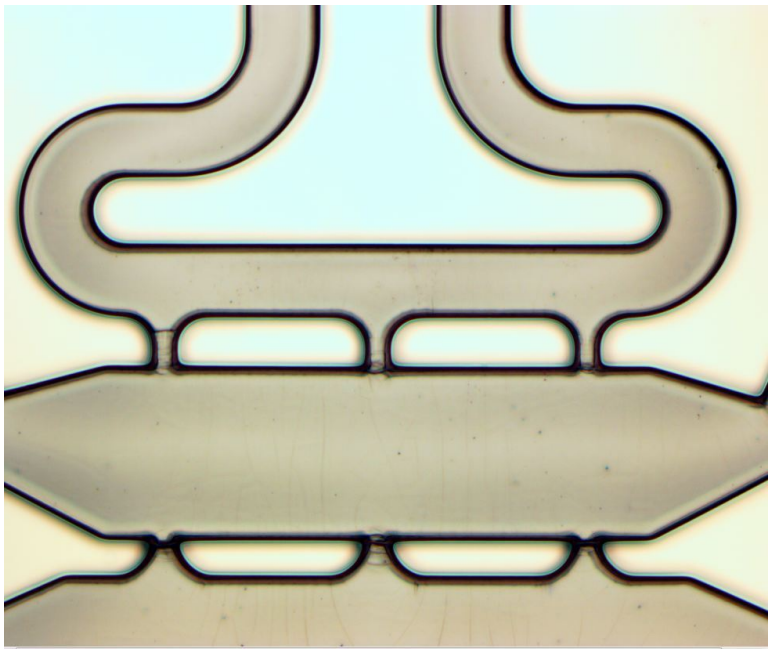

Figure 5. Image of TOC device exposed by the new LED based mask aligner. Features are crisp and well developed

Silicon Etching Mold Evaluation

While the LED based mask aligner has allowed for the creation of devices with good resolution and preservation of designed features, another method that was attempted to make OOC molds was direct etching of the master mold into silicon. DRIE silicon etching by the

Bosch process as described in Chapter 2 enables micron to submicron resolution, depending on the etch parameters and gas flow rates (Jo et al., 2005). Even with the single-wavelength exposure system, the edge bead present in the SU-8 process can lead to issues with feature expansion. Silicon etching was attempted as a way to fabricate molds with better resolution and aspect ratios than the SU-8-based process.

The molds that were created with the silicon etching process are found to have excellent aspect ratio and feature resolution as can be seen in Figure 6. As discussed in the fabrication portion of this work, the Bosch etch process with its alternating etching and polymerization steps allows for the etch of deep channels or features into the surface of silicon wafers anisotropically, creating nearly vertical sidewalls in the devices. However, due to the configuration of the of the available DRIE machine, the final etched device has sidewalls with a 5-6 degree undercut.

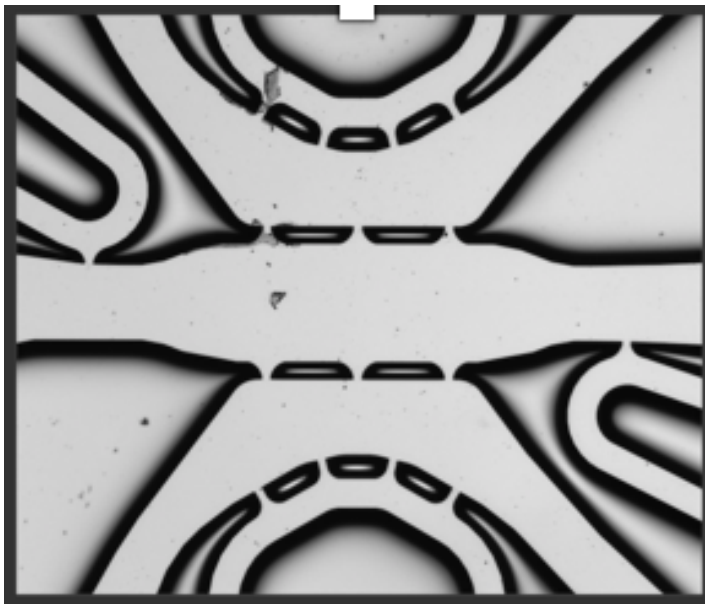

Figure 6. Image of a TOC device created through Silicon Etching.

The undercut impacts the final PDMS device in two ways: the release of cast PDMS from the etched mold is challenging, and the chambers and channels that are created from the mold have tapered side walls. The conventional method to release PDMS from molds to create TiOC

devices has been the surface treatment of the mold with POTS. It was found that the POTS treatment is insufficient in helping the PDMS release from etched silicon molds. Even after the treatment of the surface with POTS, PDMS will routinely stick to the edge of features on the mold and not lift off cleanly. One possible explanation for the adhesion of PDMS to POTS treated silicon etched molds is the undercut that is created during the etching process. Either the POTS is unable to conformally coat the corners of the under-etched mold, or the under etch is large enough that the PDMS, despite the POTS treatment, latches on to the corners of the features and is unable to lift off from the wafer.

To overcome this challenge a new method of surface treating the etched silicon wafer is employed. Parylene-C is a dimer that is biocompatible, non-toxic, and readily forms anti-adhesion layers between PDMS and other materials. Also, as the layer of Parylene that was deposited was very thin, ~1 micrometer, the vapor-phase deposition of the dimer onto the silicon surface was conformal, a desirable trait that helped to preserve the shape and design of the mold as shown in Figure 7.

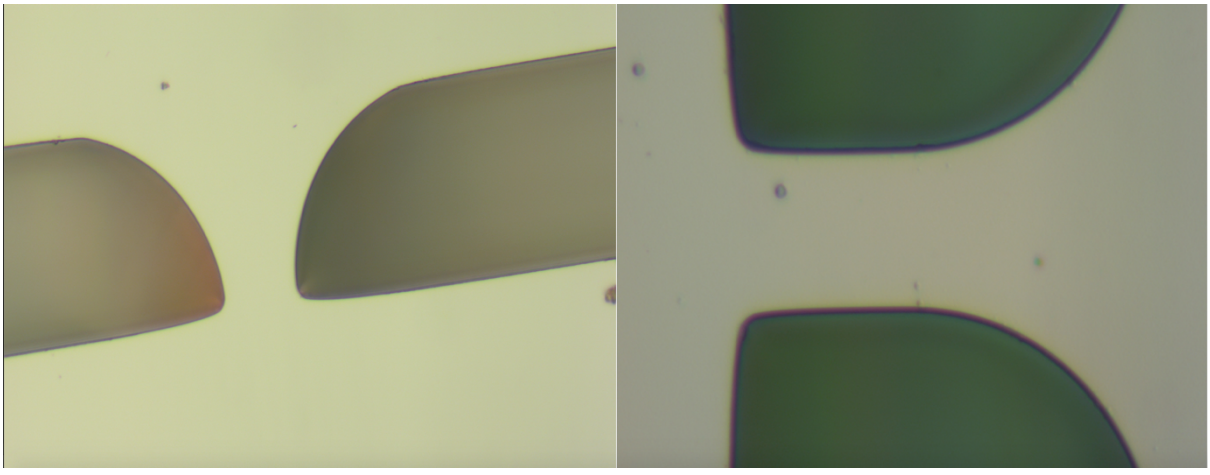

Figure 7. On the left of the photo features of a TOC device can be seen before parylene is deposited on the surface of the mold. On the right of the image the same features can be seen after the parylene deposition.

The etched silicon molds post-Parylene deposition allow for the release of PDMS from their surface and thus, is ready to function in their role as negative stamps for the final PDMS devices that were created. Upon molding and cutting the PDMS devices from the Parylene-coated surfaces, a new challenge is revealed. Because the parylene is deposited in a very thin layer, the entire layer will tear and begin to peel up from the surface of the wafer when cut by a scalpel. This presents the dilemma of having to clean the surface of the wafer to remove all Parylene debris and then redeposit Parylene between every PDMS casting. This is undesirable for a number of reasons. First, repeated Parylene deposition can become a costly and time-consuming process. The primary benefit of the soft lithographic method of creating TiOC devices is the relative speed and accuracy with which the devices can be made. Having to stop and coat the mold with Parylene multiple times during the life cycle of a mold is impractical. A second problem with having to deposit parylene on the mold before between every PDMS casting is that multiple depositions of the dimer could cause a buildup in of parylene in smaller sections of the mold or in places where Parylene from previous depositions cannot effectively be removed. As a result, the mold shape can change and the sharpness of the features could become rounded.

To combat this issue, the adhesion of Parylene to the silicon surface needs to be improved. Through an examination of the literature, it was found that Silane-174 increases the adhesive properties of Parylene to different surfaces including silicon. After the silane treatment and subsequent Parylene deposition, PDMS has been found to lift off from the mold without sticking, while keeping the shape of the features on the mold. This process is also found to work for the silicon pillar arrays that were created for the casting and creation of the PDMS membrane of the GOC device.

Assessment of Device Assembly

As mentioned in Chapters 1 and 2 the assembly of the TOC device, after the casting process of the PDMS channels, is straightforward and requires no specific alignment or inclusion of a membrane. As a result, no relevant or interesting information can be gleaned from discussing the assembly of the TOC device.

In contrast to the TOC device, the assembly of the top channel, bottom channel, and membrane required for the GOC device proved challenging. There are a number of factors that contributed to the difficulty of assembling this device. Two factors are the size of the pores that made up the membrane and the thickness of the membrane itself. Both dimensions are on the order of ~10 micrometers. Having to cast, remove, and then insert a PDMS layer of this size by hand is difficult and inconsistent. That is why the PDMS slab is used to remove the membrane from the wafer to a more robust carrier that could be handled and worked with during transfer of the membrane to the channel devices. The use of a PDMS carrier to transfer the membrane from the wafer to the channels presented its own difficulties, however. Unwanted adhesion between the PDMS membrane and the PDMS carrier was a problem. This adhesion made the transfer process between the slab and the channel nearly impossible. While the slab was treated with POTS to avoid adhesion, the treatment seemed to make no measurable difference in the adhesion between the PDMS layers.

The method used to combat this dilemma was to apply the knowledge that Parylene-C can be used to form an anti-adhesion layers between PDMS and other substrates, including other layers of PDMS, and to treat the PDMS transfer slab with Silane-174 and parylene. Following the procedure set out by Huh et al. (Huh et al., 2013) to attempt to fabricate the membrane, but substituting in the Parylene-coated slab for the POTS-treated slab, leads to a thin layer of PDMS

being left on the wafer. This thin layer of PDMS can ideally now be directly bonded to the channels and eliminates the need to use a slab to transfer the membrane to the channels. The results of this new type of membrane fabrication are still being evaluated, and the effectiveness of pore development has yet to be measured.

Another issue that revealed itself in the GOC device fabrication process was the need to align the top and bottom channels in the bonding process. For the device to effectively function, both the top and bottom central chambers must be aligned with the membrane. Any misalignment could cause leakage between chambers, which would limit the ability of the device to function properly. In an effort to make the alignment of the channels less random and prone to human error, an alignment jig was created to help semi-automate the channel alignment process.

The alignment jig, as pictured below in Figure 8, consists of a stage with a plastic platform that can translate and rotate in the x-y plane. Above that stage is a glass stage that could move up and down. PDMS device alignment is done by temporarily sticking the top channel to the top glass stage by pressing the PDMS channel to the glass. The channel stuck to the glass by surface tension and capillary action. Using a microscope pointed down onto the stages, the bottom channel is aligned under the top channel by translating and rotating the bottom stage. Once the channels are aligned the top channel is brought down and pressed together with the bottom channel. This method has demonstrated to work when bonding top and bottom channels together. However, it has yet to be seen how the inclusion of the PDMS membrane will affect this process due to the continued difficulties related to its fabrication and insertion into the GOC device.

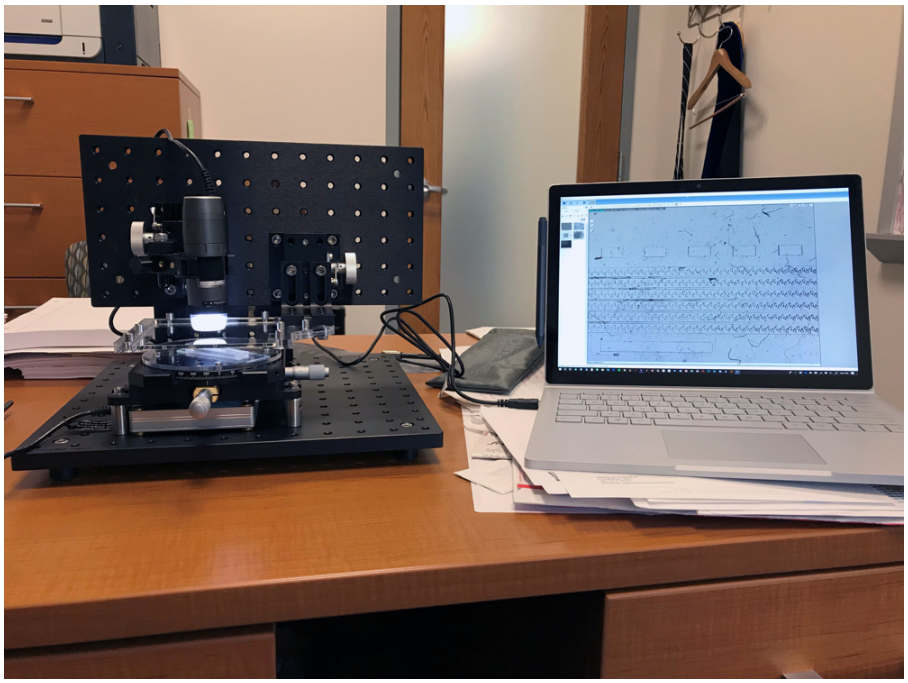

Figure 8. Picture of the alignment set up devised to help assemble the GOC channels in a uniform fashion.

Chapter 4: Conclusions and Future Work

The TiOC platform has enormous potential to become a leading method for biological and physiological studies by researchers studying human tissue behaviors. The ability to capture the *in vivo* condition *ex vivo* in a small, compact, reproducible, and controllable platform is exciting. The prospect of having a platform that can help circumvent animal models is also desirable as these types of studies have proven to be expensive, and their results often prove inadequate when applied to humans. Despite these future possibilities for the TiOC platform, several challenges remain to its implementation. The difficulties of device fabrication and assembly are one that persists in the reported work and throughout the entire field. Capturing the biology and environments within the human body using micrometer-sized chambers is difficult and requires constant refinement of manufacturing techniques. However, the work presented in this thesis should provide a foundation from which the manufacture of the TOC and GOC devices can be built and improved to provide better results to researchers in the future.

One useful direction of future work is to translate the designs of the devices to complex with a 96-well plate format. This configuration will allow multiple experiments to be run in parallel and enable researchers to visualize in real time how certain changes in the microenvironment affect the behavior of whatever disease is being modeled. Also, in the future, it will be interesting to note how the microvascular network can be grown and combined with other TiOC devices to investigate how different systems of the body interact with one another. In conclusion, the TiOC field is full of future possibilities and areas for potential growth, all of which will help drive medicine and research practices to be more refined and customized to the needs of individual patients.

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