April 2017

Hydrogel Microfluidic System to Control Oxygen Gradients for Breast Cancer Cells

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A Major Qualifying Project Report submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science
Submitted on April 27th, 2017

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Keywords:
Microfluidic
Oxygen Gradients
Migration/Proliferation

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Acknowledgements

The team would like to recognize several individuals who supported the team for the duration of the project. Their assistance was vital to success of this project.

First, the team would like to thank our advisor, Professor Dirk Albrecht, for making this project available to us and for his guidance, knowledge, advice, and support throughout the year.

Next, the team would like to thank Professor Adriana Hera for all her guidance in learning the finite element program, COMSOL, and Professor Sakthikumar Ambady for his knowledge in cell culturing and for gifting us our cell line. The team would also like to thank lab managers, Lisa Wall and Elyse Favreau for their assistance in obtaining all needed materials for completing the project.

The team would like to thank the BME Microfabrication Lab and the graduate course BME595T for fabricating the master mold free of charge.

Additionally, the team would like to recognize Professor Raymond Page and Kyra Burnett for their input A-term, and Laura Aurilio for demonstrating how to use the lab equipment and materials at the start of our project.

Lastly, the team would like to thank all of the people affiliated with Worcester Polytechnic Institute, our colleagues, and our family members who played a major role in the completion of this project through their support.

Abstract

Cancer is one of the leading causes of morbidity and mortality worldwide. One challenge is understanding how cancer cells react to heterogenous tumor environments. While there are many current practices for researching cancer, microfluidics provides a new approach; therefore, the goal of the project was to observe human breast cancer cells within a small, microfluidic system. Sharp, predictable oxygen gradients can be modeled and effects such as viability, proliferation, and migration of the cells can be observed. The fabricated devices are capable of producing an oxygen gradient by flowing nitrogen (0% oxygen) and compressed air (21% oxygen) through the PDMS system. Three different gradient lengths were tested, observed, and analyzed: sharp (100μm), intermediate (700 μm), and shallow (5mm). Additionally, the cells were grown in a hydrogel to better mimic the 3D environment of the body. From the data it was concluded that the system with the sharp gradient produced the most migration and proliferation. The cells in this device migrated approximately 5.52 +/-5.98 micrometers toward a more hyperoxic oxygen level in a 48-hour period. The hyperoxic region of the device saw the most migration at approximately 8.5 +/- 7.0 micrometers and the most proliferation as population size increased by approximately sixty percent. All three gradients were able to successfully produce oxygen gradients. The sharp device was best for observing cell migration and proliferation.

Keywords: Microfluidic; oxygen gradient; migration/proliferation

Authorship

Franklin Alvarez and Michelle Henderson contributed to the research and writing of the report. Both team members were responsible for the device design, testing, and imaging. Each person learned how to use the finite element program, COMSOL. Franklin did most of the COMSOL modeling seen in the report. Michelle did the FIJI analysis and the data analysis for the proliferation and migration seen in the devices. Both individuals edited the report. Michelle complied and formatted the report.

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1. Introduction

If you were to ask a room full of people if they knew anyone who has been affected by cancer, many, if not all, would raise their hands. About half of all men and about one-third of all women in the United States will develop cancer during their lifetimes (American Cancer Society, 2016). Cancer is the name given to a collection of related diseases and affects many people throughout the world. It can start anywhere within the body and is characterized as the abnormal growth of cells. These cancer cells grow faster than normal cells and invade surrounding tissue. As one of the leading causes of death in the world, cancer accounted for 8.2 million deaths in 2012 (World Health Organization, 2015). Breast cancer is one of the most common type of cancer. In the United States in 2015, there were about 234,190 new breast cancer cases and about 40,730 deaths (Siegel et al., 2015). Of these cases, about ninety-nine percent were in women and one percent were in males.

Groups all over the world are looking to solve the unknowns of cancer. One challenge is understanding how cancer cells react to heterogenous tumor environments. Microfluidics is an area of study that has become a method for researching cancer. Microfluidics allows precise handling and manipulation of fluids at small volumes, which enables it to be a powerful tool for cell analysis (Ning et al, 2016). This technology is very attractive for researchers as the microfluidic technique uses less samples and reagents, and shortens time of experiments to reduce the overall cost. Cell experiments within a microfluidic device are convenient as performance can be predicted through modeling such as finite element analysis, and different conditions can be observed simultaneously in the device (Albrecht, 2016).

Cells behave and function differently depending on the partial pressure of oxygen. A normal, normoxic environment in the body is between 2% to 9% (30-90 mmHg). Levels lower than these would be considered hypoxic and higher would be hyperoxic. Hypoxic is less than 2% oxygen (5 to 15 mmHg) and hyperoxic is greater than normal, such as 21% (greater than 160 mmHg) (Allen & Bhatia, 2003). Oxygen concentrations are not constant throughout the human body. There are gradients throughout all organs, tissues, and vessels; some are gradual gradients, while others are sharp (Tsai et al, 2003). Tumors typically have exaggerated oxygen gradients because of the high metabolic rate of the cells (Oppegard & Eddington, 2013).

In order to mimic these regions of low oxygen levels in tumor cells, scientists and researchers have created different systems to study cellular behavior in different oxygen levels. Current methods include hypoxia chambers and hypoxia workstations, however these only allow for static oxygen concentration observation (Brennan et al, 2014). Cancer cell growth has been observed within two-dimensional static systems and systems with gradients; however, little research has been done in a three-dimensional environment.

Currently, breast cancer cells have been cultured in different environments within different oxygen levels; however, no sharp oxygen gradients have been created even though cancer tumors have different oxygen concentrations throughout the tumor. The goal of this project is to create a three-dimensional, cost-efficient, reusable microfluidic system that can be used to observe cell response to oxygen gradients, particularly cell growth and migration. Different oxygen gradients will be studied to detect variation in responses; a shallow, intermediate, and sharp gradient will be produced. The information gathered could be used for cancer research to develop new drug delivery methods for patients whose tumors are resistant to chemotherapy as hypoxia limits drug delivery. By observing cancer cells in different oxygen

levels, more can be learned about the cell biology, since it has been shown that gradients of oxygen are crucial metabolic regulators. The designed system will more closely mimic the microenvironment found at cancer sites.

The hydrogel microfluidic system was created throughout the year. The first term consisted of defining the problem and conducting background research. In the second term, materials were gathered, device specifications were created, and conceptual designs were developed and evaluated. By testing several small components of the device with finite element analysis and physical testing, different designs were developed on the DraftSight program. Additionally, oxygen gradients were tested using a fluorescent slide. The third term consisted of verification of the oxygen gradients that were modeled and tested as well as more device testing and analyzing the results for proliferation and migration through the FIJI image analysis program. In the last, final term remaining tests were completed, the report was written, and a presentation was given. Different gradients were produced by flowing nitrogen gas (0% oxygen) and compressed air (21% oxygen) through a microfluidic device. Different devices were produced to create different gradients. These gradients consisted of 0-21% oxygen over a range of 100 micrometers, 700 micrometers, and 5 millimeters (sharp, intermediate, and shallow gradients). The MDA-MB-231/GFP/Blasticidin, human breast cancer cell line was used to observe the growth and migration in the device. PureCol EZ collagen hydrogel was used to simulate the 3D environment of the body. To be successful throughout the year, the teammates were open-minded, communicated effectively, managed their time well, and were well aware of the problem they were investigated.

The report contains literature review, the project strategy, the design process, followed by the final design specifications, verification, validation, and results. Next, the results will be discussed, followed by the conclusions, limitations, and recommendations in the final chapter.

2. Background

2.1 Cancer

Cancer is used to describe a multitude of different diseases that all involve abnormal cell growth which can destroy and affect body tissue. The rapid growth of abnormal cells beyond their usual boundaries arise from a single cell which becomes a precancerous lesion and then a malignant tumor (World Health Organization, 2015). There are over one hundred different types of cancer. Some common types of cancer include breast cancer, prostate cancer, melanoma, colon cancer, lung cancer, and leukemia (National Cancer Institute, 2016).

Cancer is one of the leading causes of morbidity and mortality in the world. In the United States it is the second leading cause of death behind heart disease (Siegel, Miller, & Jemal, 2015). In 2012, there were fourteen million new cases and over eight million deaths worldwide (World Health Organization, 2015). Within the United States, about half of all men and a third of all women will develop cancer within their lifetime; there are 1.5 million new cancer cases each year and more than 15 million people alive in the United States have had some type of cancer (American Cancer Society, 2016). The risk of developing cancer can be reduced by changing one's lifestyle. For example, it is beneficial to not smoke cigarettes, to limit time in the sun, to eat healthy, to limit alcohol consumption, to limit exposure to radiation and chemicals, to be physically active, and to maintain a healthy weight. The risk of developing cancer also increases with age; about 9 out of 10 cancers are diagnosed in people over the age 50. Cancer can also be passed down in families through genes inherited from previous generations (American Cancer Society, 2016). The bottom line is that no one knows the exact cause of most cancer cases besides the fact that it can occur when there is a change in the cells of the body. Scientists and researchers are studying cancer to learn more about how it forms and grows.

Early diagnosis of cancer is essential in order to try and prevent the disease from spreading (World Health Organization, 2015). There are typically three main types of cancer treatment: surgery, chemotherapy, and radiation (American Cancer Society, 2016). Surgery is usually the first treatment to remove cancer from the body. Chemotherapy and radiation are used to shrink the cancer before and/or after the surgery. Chemotherapy is drugs that are used to kill cancer cells through the bloodstream. These are taken through IV or by mouth. Some side effects include a low blood cell count (which leads to a higher risk of infection), bleeding and bruising, anemia, hair loss and nausea. With external (out of the body) or internal (inside tumor) radiation therapy, high energy rays are used to kill or shrink the cancer cells. The side effects include skin irritation and fatigue. Other types of cancer treatment include targeted therapy, stem cell/bone marrow transplants, immunotherapy, and hormone therapy (American Cancer Society, 2016). Others use palliative care, which relieves the symptoms, rather than cures the cancer (World Health Organization, 2015). While these methods are used to treat cancer, it is surprising that the success rate of chemotherapy is only 2.1% over a five-year period (in America) (Morgan et al, 2004). This means that cytotoxic chemotherapy is a very minor contribution of cancer survival, and that the cancer cells are resistant to the chemotherapy treatment. It is imperative to better understand cancer cell biology in order to find an alternate solution for treating cancer.

2.2 Microfluidics as a new means of researching cancer

Microfluidics is the precise handling and manipulation of fluids at small volumes, such as microliters, nanoliters, or picoliters. This allows for a "small scale benefit (Albrecht, 2016)." The minimum unit of a system is addressed as the chambers and channels are controllable, which allows for experiments to be reproducible and tunable (Ning et al, 2016). At the microscale, the process is faster, less energy is used, and less reagent volumes used; this makes them cheaper than alternatives (Albrecht, 2016). Microfluidics has been widely investigated and applied in chemical microanalysis, clinical evaluation, environmental monitoring, and biological fields (Ning et al., 2016). Within the biological field it can be used in drug discovery, biology, diagnostics, and tissue engineering (Riahi et al, 2015). Microfluidic systems can be used to create complex drug carriers with precise size and composition; active and localized preprogrammed quantities of drugs can be delivered. This can lessen painful and hazardous injections (Riahi et al, 2015).

Microfluidic chips first emerged in the 1970s (Ning et al., 2016). The materials for microfluidics were developed in three stages: the initial glass-and-silicon based inorganic system for analyte detection, the polymeric substitution (thermoplastic and thermosetting polymers) for cell manipulation and analysis, and the most recent: integrated microfluidics with hydrogel or smart biomaterials (Ning et al., 2016). For an example of a microfluidic device, see Figure 1 below. Currently, microfluidic systems are being used to create complex drug carriers with precise size and composition. This may be used for active and localized delivery of cancer drugs within the future (Allen & Bhatia, 2003). For example, one study observed how cancer cells migrate from an area with little medium, to an area with complete medium. Through this research, the cell migration rate was calculated, cell deformation was observed, and ultimately provided novel drug targets for metastasis (Zhang & Nagrath, 2013).

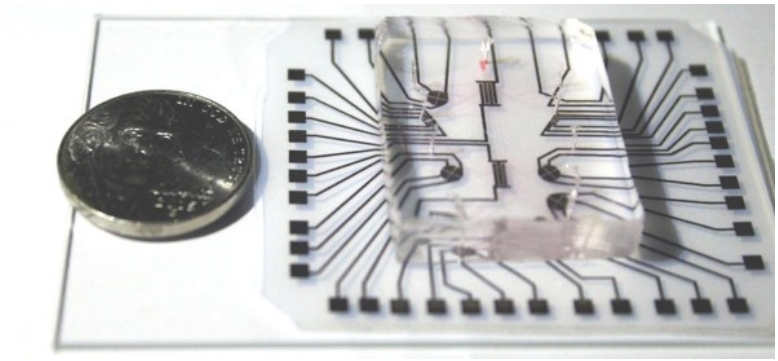


Figure 1: "Lab on a chip"- microfluidic device is about the size of a coin.

Microfluidics is a powerful tool for cell analysis since they can be microanalyzed and dynamically monitored, which gives them a "high-throughput benefit." They also use little reagents and have a low detection limit to better observe and analyze cells. Cell experiments are convenient and integrated when a single device can have parallel function channels; cells can be sorted, cultured, imaged and analyzed within this device (Ning et al., 2016). This allows the user to visualize many environments and a large number of cells at once (Albrecht, 2016). Through these microfluidic systems, microenvironmental signals can be investigated (Cosson & Lutolf, 2014). Cellular microenvironments are very important to study since they determine cell survival, proliferation, and differentiation (Wan, Wang, & Wang, 2015). Experiments have been

used to study cancer cell migration, angiogenesis, and the microenvironment of tumors (Zhang & Nagrath, 2013). Additionally, these microdevices are transparent which makes them noninvasive, highly sensitive, and able to fluorescently imaged (Ning et al., 2016). Time-lapse microscopy of each chamber is possible to thoroughly observe the different conditions. More physiological cell culture conditions not found in in-vitro systems can even be created. These include conditions such as exchange rate of nutrients or mechanical stimulation (Gomez-Sjoeberg et al., 2007).

Lastly, microfluidics have a "quantitative benefit." This means that the performance of the device can be predicted through predictive models such as finite element analysis. By creating models, the surface of the microfluidic device can be modified to be more applicable and specific. The concentrations and stresses can be measured as well, since the device specifications are known (Albrecht, 2016).

By using a smart biomaterial or hydrogel there is better biocompatibility, and cell to cell communication and cell to surface interaction can be observed. These three-dimensional (3D) culture environment better mimics the in vivo environment of the body. It is hard to stimulate cell function in vivo in 2D, therefore 2D was limited to in vitro research (Ning et al., 2016).

While there are many benefits to microfluidics there are also some disadvantages, especially for long term cell culture. For example, these small systems have trouble with medium evaporation, limited space for cell growth, and shear stress of the friction of the fluids against the cell membranes. It is also difficult to perform routine cell culture such as passaging the cells or removing the cells. Hydrogel microfluidics is used to help overcome these challenges . By incorporating hydrogel with PDMS, gradients can be generated within 3D scaffolds and cells can be shielded from flow and shear stresses (Cosson & Lutolf, 2014).

The high sensitivity of microfluidics, high throughput, and enhanced spatio-temporal control allow for researchers to control physics, biology, chemistry, and physiology at the cellular level. Using microfluidics to address the unmet needs in cancer research can expand knowledge of cancer cell biology, management of the disease, and patient care (Zhang & Nagrath, 2013).

2.3 Oxygen Levels and Cell Function

Cells behave and function differently depending on the partial pressure of oxygen. A low oxygen environment is below physiological levels and is known as hypoxic; this is typically less than 2% oxygen. A normoxic environment is normal oxygen levels of 2% to 9%. A hyperoxic environment is above physiological oxygen conditions. Within the atmosphere the oxygen level is 21%; this is a hyperoxic environment to the cells in the body. An environment with no oxygen is anoxic (Brennan et al., 2014). In mmHg, hypoxic is 5 to 15 mmHg of oxygen, normoxic is 30 to 90 mmHg of oxygen, and hyperoxic is greater than 160 mmHg of oxygen (Allen & Bhatia, 2003). These levels have been considered standard for liver cells in mice and humans, breast cancer cells of humans, and colon cancer cells of humans (Allen & Bhatia, 2003) (Lau et al, 2009) (Fukuda, 2003).

Oxygen concentration is not constant throughout the body. In particular, there are steep oxygen gradients seen in blood vessels and in cancer tumors (Tsai et al, 2003). Cancer tumors are generally hypoxic, especially since cancer cells rapidly outgrow their vasculature. If they want to survive, they adapt to these lower oxygen conditions; this creates a poorly perfused hypoxic inner region (Brennan et al., 2015). An example of a tumor spheroid and the gradients that exist can be seen in Figure 2. As can be seen, the nutrient levels and oxygen levels are much

less at the innermost region than the outermost region; this occurs in just 100 micrometers (Chandrasekaran & King, 2012).

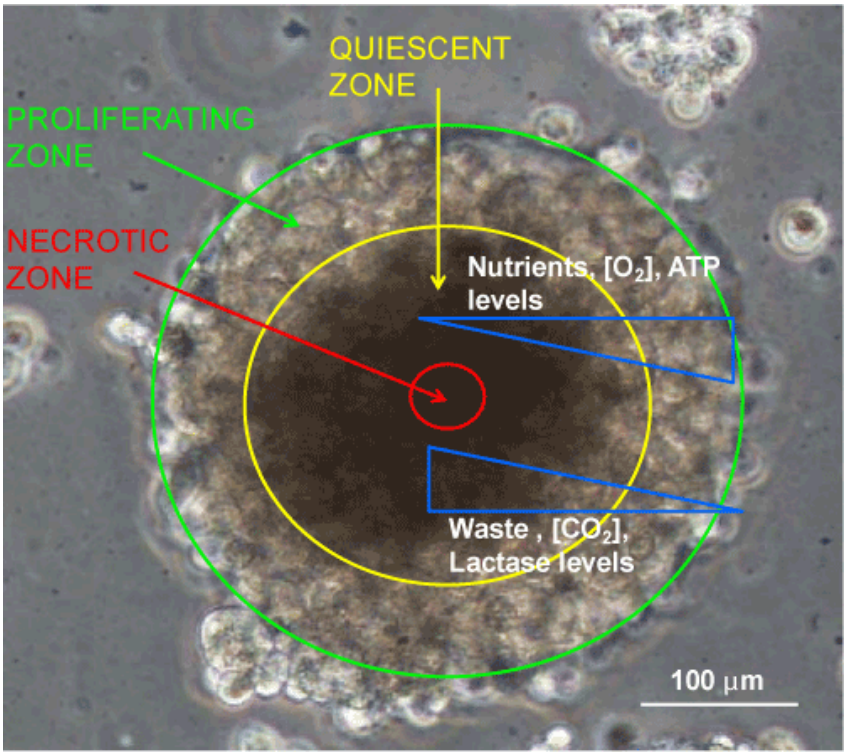


Figure 2: Tumor spheroid nutrient, waste, and gas levels (Chandrasekaran & King, 2012)

It is important to study cancer cells under controlled hypoxic conditions to understand the pathophysiology. Research has shown that hypoxia may enhance aggressive phenotypes, tumor progression, metastasis, and resistance to therapy (Brennan et al., 2015). Hypoxia and oxygen levels are known to alter cancer gene expression by altering transcription of these genes. These alterations are under the activity of the transcriptional hypoxia inducible factor (HIF) (Brennan et al., 2015). By adapting to hypoxia in cells, the HIF-1 is responsible for transcriptional induction of a series of genes that are involved in development, angiogenesis, iron and glucose metabolism, cell proliferation, survival, and migration, and apoptosis (Oppegard & Eddington, 2013). HIF-1 is overexpressed in various cancers and targeting this factor may be a novel approach to cancer therapy (Ke & Costa, 2006).

In order to mimic these regions of low oxygen levels in tumor cells, scientists and researchers have created different systems to study cellular behavior in different oxygen levels. Current methods include hypoxia chambers and hypoxia workstations. Hypoxia chambers are small enough to fit inside a standard incubator, are inexpensive (about $500), and do not require specialized equipment. There is a vessel to place the cell culture plates and dishes. Some drawbacks of this chamber are that they are prone to leaks, cannot replicate anoxic (no oxygen) conditions, equilibrate slowly, have imprecise levels of oxygen, and are not compatible with microscopic analysis. Hypoxic workstations are large, sealed biosafety cabinets that contain the gas of interest. There are oxygen sensors and an incubator in the corner of the cabinet. It even has a small, gas modulated bench top to perform biological assays. Some drawbacks of the workstation includes the cost (over $50,000), and that it cannot be coupled with live cell

microscopy (Brennan et al, 2014). Both of these systems only allow for static oxygen conditions to be applied and observed. Two-dimensional (2D) and three-dimensional (3D) oxygen gradients have been produced, but it is much more common to create them in 2D. For example, one group used human lung cancer cells to observe migration toward different oxygen levels in a 2D microfluidic system (Figure 3). They found that the cells slightly migrated from an area of 9% oxygen, toward an area of 3% oxygen (Chang et al, 2014).

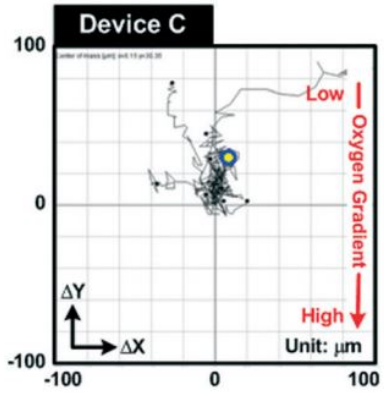


Figure 3: 2D migration of human lung cancer cells (Chang et al, 2014)

Another group researched human breast cancer cell viability in a 3D oxygen gradient (Figure 4). The gradient went from 0-21% oxygen in an agarose gel either 3mm or 6mm. They found that cell viability was not affected in the different concentrations of oxygen (Oppegard & Eddington, 2013). Their setup can be seen in Figure 4 below.

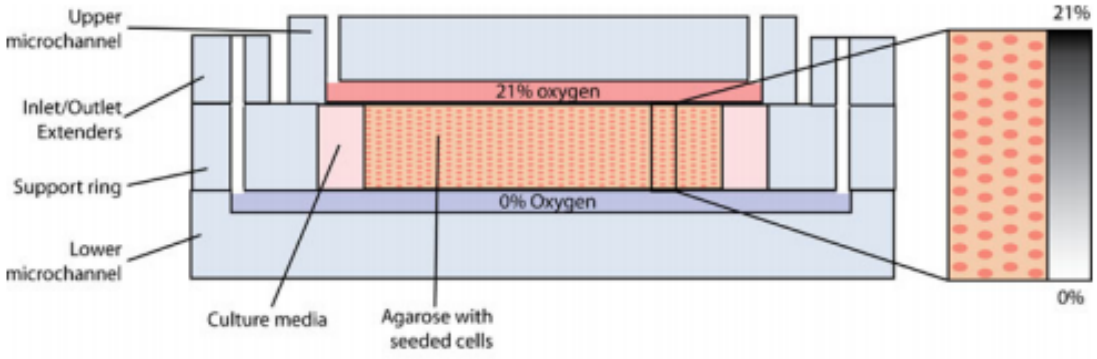


Figure 4: Setup of agarose gradient system (Oppegard & Eddington, 2013)

2.4 Breast Cancer

2.4.1 Breast Cancer Background

Breast cancer occurs when cells in the breast grow out of control. Breast cancer commonly beings in the ducts that carry milk to the nipple. This is known as ductal cancer. In some cases it begins in the glands that make the breast milk, which is known as lobular cancer. When breast cancer spreads, it spreads through the lymph system which includes the lymph nodes, vessels, and fluid (American Cancer Society, 2016).

As previously mentioned, breast cancer is one of the most common cancers in the United States and in the world (Siegel, Miller, & Jemal, 2015). About one in eight women in the United States will develop invasive breast cancer within her life, and approximately 30% of the cancers diagnosed in women are breast cancer (U.S. Breast Cancer Statistics, 2017). In 2015 there were 234,190 new breast cancer cases and 40,730 deaths (Siegel, Miller, & Jemal, 2015). In 2017 there are an estimated 252,710 new cases expected to be diagnosed in women in the US, and an estimated 63,410 new cases. Besides skin cancer, breast cancer is the most commonly diagnosed cancer in women. There is an estimate of 2,470 new cases of breast cancer diagnosed in men (U.S. Breast Cancer Statistics, 2017). This means that the population that breast cancer affects is about 99% women and 1% men (Siegel, Miller, & Jemal, 2015). Additionally, 40,610 women from the US are expected to die from breast cancer in 2017; this is the highest cancer death rate besides lung cancer. Luckily, the death rate of breast cancer has been decreasing since 1989. This decrease is likely due to early screening detection, treatment advances, and more awareness (U.S. Breast Cancer Statistics, 2017).

The five year survival rate for people with breast cancer is 89%, the ten year survival rate is 83%, and the fifteen year survival rate is 78%. The chance of surviving breast cancer depends on when the cancer is found (as is true with most cancers). If the breast cancer is found during stage 1, which means it is only located in the breast, the five year survival rate is 99%; 61% of cases are diagnosed at this point. If it has spread to the lymph nodes, the five year survival rate is 85% and if it has metastasized and spread throughout the body, the five year survival rate is 26% (Cancer.Net, 2016).

2.4.2 Breast Cancer Cell Line

There are many different human breast cancer cell lines available for purchase, in fact, there are forty-five available from the American Type Culture Collection (ATCC). One of the most commonly research human breast cancer lines is the MDA-MB-231/GFP/Blasticidin line. These cells are derived from metastatic breast cancer, mammary gland epithelial cells. The GFP means "green fluorescent protein," which means that the cell line expresses the signal-enhanced GFP reporter in the genome. While under a blue light of a fluorescent microscope, a strong fluorescence is produced (GenTarget Inc., 2016). This is localized in the nucleus and cytoplasm to produce fluorescence throughout the cell (Hoffman, 2015). The cell line also has a Blasticidin resistance. Blasticidin doesn't allow for translation to occur in prokaryotic and eukaryotic cells, which would ultimately cause cell death (ThermoFisher Scientific). This is useful to protect the cells in hopes that they remain viable and can proliferate.

The growing environment for these cells are standard. The media used for culturing is composed of Dulbecco's Modification of Eagle's Medium 1X (DMEM), 10% Fetal Bovine Serum (FBS), L-Glutamine, and Penn-Strep. In a 50 mL solution, there is 44 mL of DMEM, 5 mL of FBS, 0.5 mL of L-Glutamine, and 0.5 mL of Penn-Strep. The DMEM is a basal medium used to support growth of cells and contains high glucose, amino acids, vitamins, and phenol red. It does not contain any proteins or growth factors ("DMEM, high glucose," (n.d.)). This is why FBS is needed. FBS is a growth supplement for cell culture and has a high concentration of embryonic growth factors that promote growth and protect cells from oxidative damage and apoptosis ("Fetal Bovine Serum (FBS)," (n.d.)). The L-Glutamine is an essential amino acid that supports the growth of the cells, especially those that require a lot of energy by synthesizing large amounts of proteins and nucleic acids. It acts as an alternative energy source ("Glutamine in Cell Culture," (n.d.)). The Penicillin-Streptomycin (Penn-Strep) is used to prevent bacterial

contamination. The penicillin interferes with the bacterial cell wall and the streptomycin inhibits protein synthesis to cause death to the bacteria ("Penicillin-Streptomycin," (n.d.)).

The growing conditions of the cells require a pH of 7.3 or 7.4 (Sokol et al, 2016) and normal carbon dioxide levels between 4% to 10%. This level is necessary to keep the pH at the appropriate level ("pH & CO2 Levels," (n.d.)). The temperature is set to 37 degrees Celsius to mimic body temperature conditions. The population doubling time is approximately 38 hours at these conditions.

2.5 Microfluidic System

Microfluidic systems can be composed of a variety of different components. The system used in this experiment consisted of the device, a membrane, a well, slides, hydrogel, cells, media, and the gases- nitrogen and compressed air. All of these components have specific functions to create the best device.

A PDMS microfluidic device will be plasma bonded to a glass slide. Additionally, a thin PDMS membrane will be plasma bonded to the top of the device. The gas channels that run through the device are 100 micrometers deep; the membrane allows for diffusion of the gas to occur into the hydrogel (and cells). Plasma bonding activates the PDMS surfaces, which allows bonding to occur. This ensures that the device and membrane remain stationary. If the membrane were to come off or peel up, the gases within the channels would mix. Also, the membrane keeps the hydrogel, media, and cells from going into the channels of the system. On top of the device will be a well, which holds the hydrogel, cells, and media. The gases will flow into the system through inlets. The device can be seen in the image below (Figure 5). The device (more clearly shown by adding a piece of tape on top before plasma bonding to the slide and membrane) is approximately 50mm long, 25mm wide, and 9mm tall. It has three horizontal channels that are approximately 30 mm long and 5mm wide. There are three inlets where the tubing from the gas tanks will be inserted.

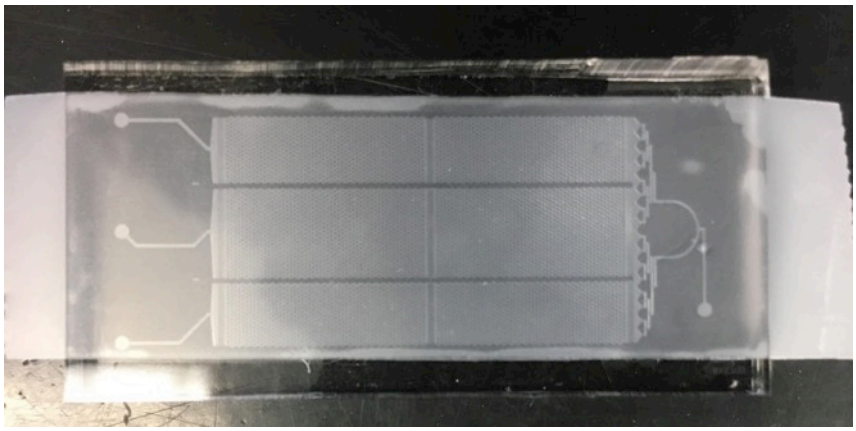


Figure 5: Microfluidic device made of PDMS. There are three channels, three inlets, and one outlet.

2.5.1 Hydrogel

Hydrogels are crosslinked polymers that have a hydrophilic group. Water molecules are attracted to the negative charges in the crosslink and this creates hydrogen bonding between water and the hydrophilic group. Hydrogels have a high water content and some hydrogels are able to absorb up to five hundred times its own weight (GSCE, 2015). Additionally, hydrogels

have easy mass transportation, better cell viability, and better cell proliferation. They are ideal for mimicking the native extracellular matrix of the body (Ning et al, 2016).

The extracellular matrix (ECM) plays a critical role in the development and maintenance of epithelial tissues. The ECM in human breast tissue is a complex mix of protein fibrils that are interwoven within a network of glycosaminoglycan carbohydrate chains. The proteins provide resistance to tensile forces and the carbohydrates provide resistance to compressive forces. Hydrogels are able to more closely mimic this environment (Sokol et al, 2016). Hydrogels are used for tissue engineering applications because they have material properties like permeability, mechanical strength, and biocompatibility that can be engineered to fit the needs of the application. Additionally, the high water content allows them to easily exchange nutrients and wastes with the environment (Nuttelman et al, 2001). The highly flexible nature of the hydrogel is similar to that of natural tissue (Mellati et al, 2014). It is important for cells to be able to attach to the hydrogel as these are strongly influence cell proliferation, migration, differentiation, and extracellular matrix production (Nuttelman et al, 2001). Culturing cells within a hydrogel allows them to grow in a three-dimensional setting and is most similar to the in vivo environment. Three dimensional cell cultures allow for more physiologically relevant modeling ex vivo. All cells, even cancer cells, depend on microenvironmental signals for survival, growth, and metastasis (Kenny et al, 2007). Gene expression changes can be observed and the morphology of the cells can be determined. Breast cancer cells typically have four different morphologies: round, mass, grape-like, or stellate. The MDA-MB-231 cells have morphologies of the stellate type, which are elongated cell body (Kenny et al, 2007). The different types of morphologies can be seen in Figure 6 below.

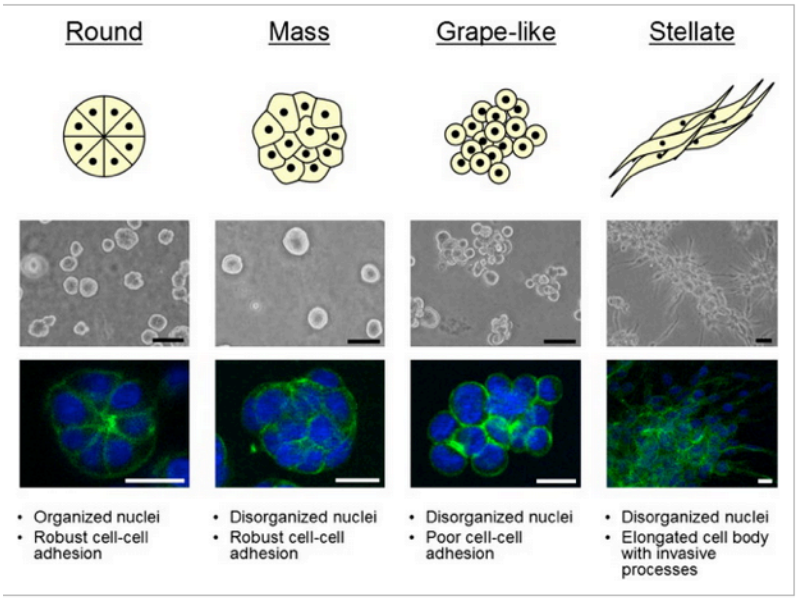


Figure 6: Four different breast cancer cell morphologies seen in 3D cultures (Kenny et al, 2007)

There are different substances that can be used as hydrogels; they can be natural or synthesized. Some synthetic examples include polyvinyl alcohol (PVA), polylactic acid (PLA), and polyethylene glycol (PEG). Natural polymers such as laminin-rich extracellular matrix, collagen, cellulose, and more are used as hydrogels (Ning et al, 2016). Additionally, hydrogels can be loaded with proteins such as collagen and fibronectin as they are present in human breast tissue. Additionally the hydrogel can be loaded with growth factors such as insulin, epidermal

growth factors, and hydrocortisone. These help with growth and differentiation of mammary cells (Sokol et al, 2016). 3D cultures support the growth of complex tissues and is useful for regenerative medicine and for studies tissue development.

Some limitations of hydrogels include insolubility, uncontrollable pore distribution, low mechanical durability, and low microstructure reproducibility (Ning et al, 2016).

The hydrogel will sit in a PDMS well on top of the device with the cells and media.

2.5.2 PDMS and Oxygen Diffusion

Polydimethylsiloxane (PDMS) is commonly used in microfluidics because of its optical clarity, biocompatibility, and that is able to be molded down to a submicron resolution (Brennan et al, 2014). Additionally, it mimics some natural interactions of stem cells with the microenvironment (Cosson & Lutolf, 2014).

PDMS is highly permeable to gas, particularly oxygen. The diffusivity of oxygen in PDMS is 3.25×10^{-9} m^2/s (Cox & Dunn, 1986). In water, oxygen has a diffusivity of 1.6×10^{-9} m^2/s at 10 degrees Celsius and a value of 3.33×10^{-9} m^2/s at 40 degrees Celsius (Ferrell & Himmelblau, 1967). There have been experiments for oxygen control that involve gas diffusion through a thin PDMS layer into another area of the device (Brennan et al, 2014). PDMS gas permeable membranes have been used in 3D printed microfluidic devices to allow for oxygen control in cell culture studies (Brennan et al, 2015). Hydrogels are able to easily be incorporated into PDMS structures, and hydrogels can even replace PDMS (Cosson & Lutolf, 2014).

Some of the disadvantages of using PDMS is that liquid evaporates, protein gets absorbed into the media, and non-reacted compounds leach out (Cosson & Lutolf, 2014).

2.5.3 Gas Flow and Oxygen Sensors

To produce a hypoxic environment, 0% oxygen will be needed, therefore pure nitrogen gas will be used to produce this environment. The atmospheric air is 21% oxygen, therefore a tank of compressed air will be used to produce a hyperoxic environment.

There are several ways to measure oxygen, such as luminescent optical sensors or Clark electrodes.

Luminescent optical oxygen sensors can be ruthenium-based or metalloporphyrin-based. These sensors don't require an electrolyte solution compared to the Clark-type electrodes, they don't consume oxygen in the measurement process (non-invasive), and they can measure oxygen in a liquid or gas phase. These sensors have good long term stability and are accurate as they are not influenced by the flow rate of the sample. The sensor of the device contains fluorescent dye and depending on the amount of oxygen molecules present, the luminescence response varies. This luminescent response is then converted into a concentration measurement (Brennan et al, 2014).

On the other hand, clark electrodes require an electrical connection to each position to be measured in a single, low spatial measurement. It has an anode and cathode in contact with an electrolyte solution. The oxygen tension is measured amperometrically, which means that the pressure of the oxygen electrode produces a current at constant polarizing voltage that is proportional to the partial pressure of oxygen (Brennan et al, 2014).

For a more simplified approach, a fluorescent slide can be used to produce a qualitative spatial map. This would not be able to quantify the oxygen levels unless calibrated to the microscope and changes in fluorescence. Different levels of oxygen can be observed by blowing

gas on the slide. A lower level of oxygen would be brighter than a higher level of oxygen; more fluorescence means less oxygen.

2.5.4 Other Devices

Aside from the 2D and 3D systems discussed in section 2.3, several other microfluidic devices have been created by different groups. One group created a microfluidic hydrogel chip and cultured mouse embryonic stem cells on the surface. Through this device they were able to spatiotemporally control the delivery of biomolecules through this system (Cosson & Lutolf, 2014). Another group created a PDMS microfluidic chip where specified conditions in a 96 culture chambers were full automated. These conditions were customized and included seeding density, composition of cell culture, and feeding schedule. Through this system, they were able to observe quantitative measurements of the influence of transient stimulation schedules of proliferation, differentiation and motility in human mesenchymal stem cells (Gomez-Sjoeberg et al, 2007). Another group used a 3D hydrogel scaffold with extracellular proteins and carbohydrates to expand primary human breast epithelial cells. After two weeks the cells had self-organized to form mature mammary tissue (Sokol et al, 2016). This proved that a 3D hydrogel scaffold was able to support the growth of complex tissue.

2.6 Team Plan

There exists a need for a device that can observe cancer cells in a microenvironment similar to what is seen *in vivo*. While groups have created 2D and shallow 3D gradients, not much research has been done on observing breast cancer cells in a 3D environment within a steep oxygen concentration. The viability, proliferation, and migration of the cells will be observed.

The plan is to create a microfluidic system that uses a hydrogel and creates different oxygen gradients (21% to 0% oxygen) in order to represent the two environments: hyperoxic and hypoxic. This will be created by flowing nitrogen air (0% oxygen) and compressed air (21% oxygen) through the system. A normoxic environment will be created between the channels of the device. Three different gradients will be investigated, a shallow 5mm gradient, an intermediate 700μm, and a sharp 100μm gradient. These will be generated by changing the location of the inlet or by changing the chamber gap widths. A 5mm gradient will be created by inserting the nitrogen gas and compressed air into opposite inlets. This means that there will be gas flow into the top chamber, then the middle chamber will have no direct gas flow, but will be the gradient of 5mm, and there will be gas flow into the bottom chamber. The 700μm and 100μm gradients will be created by using different chamber gap width designs (through DraftSight) and inserting the gases into adjacent inlets to each other.

By observing the breast cancer cell line in this environment, cell communication and cell migration can be observed. This would not be seen in static oxygen environments. A basic image of what will be done is seen in Figure 7. There are three channels, one that is a hyperoxic environment, one that is a hypoxic environment, and the gradient between will be determined by the distance between these channels (x in Figure 7). Within this region will be a normoxic range. On top of the channels will be a membrane, and the hydrogel and cells will sit on top of the membrane.

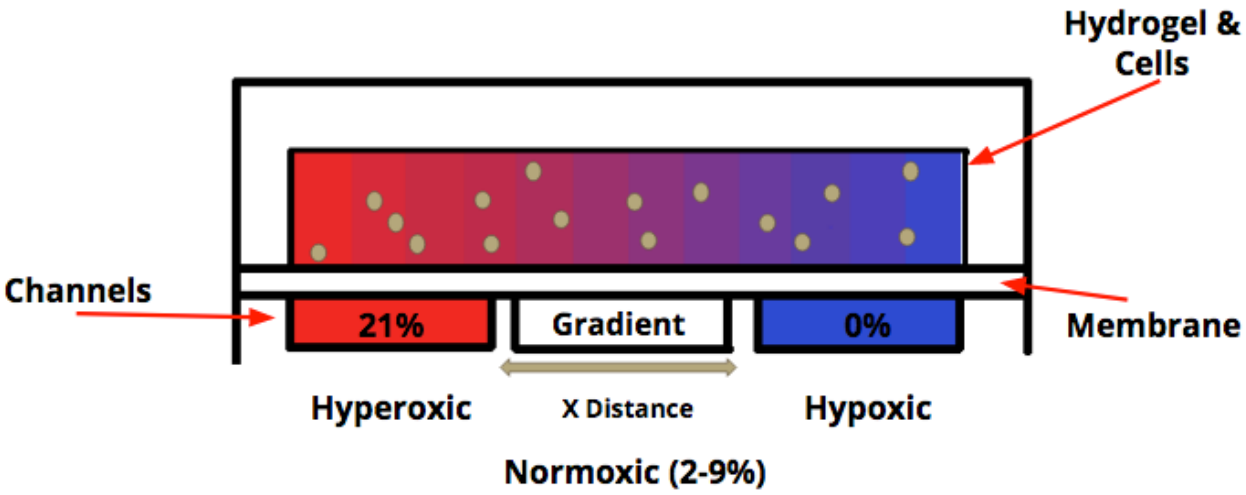


Figure 7: Team plan on creating the microfluidic device with a specified gradient

3. Project Strategy

3.1 Initial Client Statement

The initial client statement was given to the team by the advisor of the project, Dirk Albrecht, before the school year started. It was to "design, characterize, and build a microfluidic system that releases a specified oxygen level to the hydrogel-embedded cancer cells."

3.2 Design Requirements (Technical)

3.2.1 Objectives

In order to complete the project the following objectives were created:

1. Design and fabricate a new microfluidic device to create the desired gradient to best observe migration and proliferation of cells in a 3D environment.
2. Quantify and observe gradients of oxygen within the device.
3. Test viability, proliferation, and migration of cancer cells in three environments simultaneously. These environments include the hypoxic (less than 2% oxygen), the hyperoxic (21% oxygen), and the gradient (which would include a normoxic region).
4. Create a cost efficient, reusable device that is useful in a research setting in order to further understand cell biology.
5. Create a versatile device that allows other cell lines to be cultured within it, and can test the effects of other gases.

3.2.2 Functions & Requirements

A functional device must be produced that satisfies the objectives. It is required that the oxygen diffuses through the membrane of the device and through the hydrogel. This will produce appropriate, accurate oxygen gradients, which will be modeled and verified through finite element analysis and physical testing. The device must be biocompatible.

The three-dimensional environment will be created using a hydrogel. It is important that the cells be able to grow and migrate through the gel, and that all required nutrients from the media can reach the cells. It is important that the cells remain viable and don't die though out the length of the experiment.

There should be no leaking of the contents out of the device. This means that the membrane must be properly plasma bonded to the device. The well should fit tightly on the device, but it will be removable for easy cleaning.

It is important that the gases be flowed at the same speed so that the appropriate gradient is produced. Using a microfluidic device will use less energy and lower reagent volumes; it is also more reproducible. This means that less gas, cells, media, and hydrogel are used. Additionally, the different oxygen environments can be observed at once (hyperoxic, gradient, and hypoxic).

The device must be easily sterilized prior to conducting an experiment, and then after the experiment concludes. Sterilization through UV light is effective, easy, and safe. This allows the device to be reusable.

The team must keep the cost of materials under the budget of $500; the device and materials need to make it must be affordable. The fabrication should be easy for someone new to learn.

Lastly, it is required that the device be useful in observing cancer cells to see the viability, migration, and proliferation. The device must be clear to allow the microscope to focus on it and allow for cell observation.

3.2.3 Specifications

The microfluidic device should fit within a standard size petri dish (60mm in diameter) or within a larger size petri dish (140mm in diameter). The device will be approximately 50mm x 25mm x 9mm and the chambers are approximately 30mm x 5mm x 0.1mm. Different gradients can be produced in the device by changing the location of the inlets or by creating a new device. Since there are three inlets within the device, to create a sharp gradient, the gases would be placed next to each other. To create a shallow gradient, these inlets would be placed on opposite sides. A new device can be made to increase or decrease the gap width between channels. Figure 6 is one of the models in DraftSight.

In order to seed the appropriate amount of cells into the device, a cell count will be done on the passaged cells. Once they are resuspended, a calculation will be done. If 200,000 cells are required and there were 1,000,000 cells per mL then:

$$\frac{1{,}000{,}000}{1{,}000\text{uL}} = \frac{200{,}000}{X}$$

In this case, X would be 200uL.

3.2.4 Constraints

There are some constraints within the project. New programs must be learned in order to predict the gas flow patterns through the device and to design a new one. A finite element analysis simulation is essential in order to observe these model gradients in steady state. Additionally, the team must learn the DraftSight program in order to design a new microfluidic device. Additionally, the team must be comfortable with creating devices using a master, with using a fluorescent microscope, and using an image analysis program to analyze the data.

The team has a budget of $500 dollars to create and test this device. To use the cell culturing materials in the lab is a fee of $100 dollars per person. Additionally, supplies such as gas tanks, cell lines, tubing, PDMS, connectors, and the master fabrication price must all be taken into account. Oxygen sensors, for example, are very expensive. The budget prevents us from purchasing one of these precise sensors.

In order to ensure that experiments are successful, extreme care must be taken. Devices must be properly sterilized, cell cultures must remain uncontaminated, wells must be placed properly, and the appropriate number of cells and hydrogel must be added. Additionally, the images taken to observe the proliferation and migration must be carefully selected and aligned each time the device is imaged.

Lastly, it is important that the hydrogel be completely gelled with the cells before taking images, otherwise they can move, especially when media is added.

3.3 Standards

Standards provide requirements, specifications, guidelines, and characteristics to ensure that materials, products, processes and services are fit for their purpose. This means that the device must perform, meet safety requirements, show consistency, and be compatible with technology.

Some standards include:

- ISO Standards- Requirements , specifications, and guidelines ensure that the product is fit for its purpose.

  For CAD/COMSOL:
  - ISO 11442:2006- Basic rules for managing technical documents
  - ISO 16792:2015- Digital product definition data practices

  For sterility:
  - ISO 11737-2:2009- This is the sterilization of medical devices. The device must be sterilized in order to successfully grow the cancer cells and ensure no cross contamination.

  Petri dish:
  - ISO 24998:2008- Single-use Petri dishes for microbiological procedures

  For safety:
  - ISO 10993-1-Evaluation and testing within a risk management process. Using the microfluidic system will be safe for users and pose no potential toxic effects.

  Standardized methodology for cell counting:
  - ISO/WD 20391-1- General guidance for cell counting methods
  - ISO/WD 20391-2- Experimental design and statistical analysis for cell counting methods

  Gas flow:
  - ISO 9300:2005- Measurement of gas flow by means of critical flow Venturi nozzles
- IEEE standards- The microfluidic system may incorporate electronic components (housed in an incubator) and therefore it must be safe for use and pose no risk of electrocution. The device used does not contain any electrical components and the incubator standard was not found.

The standards will be incorporated into the design through the materials that are selected to be used (hydrogel, oxygen sensor, media, etc). Research will be done to ensure that all materials are safe. When the microfluidic design is made, wires, tubing, cells, etc. will be observed to make sure that there is no potential for injury to the user. Ethical standards will also be incorporated into the design. This will ensure that it is safe for use.

The device incorporated the following standards:

- ISO 16792:2015- Digital product definition data practices
- ISO/WD 20391-1- General guidance for cell counting methods
- ISO/WD 20391-2- Experimental design and statistical analysis for cell counting methods
- ISO 24998:2008- Single-use Petri dishes for microbiological procedures
- ISO 11737-2:2009- Sterility
- ISO 10993-1-Evaluation and testing within a risk management process

The standards that would be recommended before commercialization are:

- ISO 11442:2006- Basic rules for managing technical documents
- ISO 9300:2005- Measurement of gas flow by means of critical flow Venturi nozzles

3.4 Revised Client Statement

After researching what was currently on the market, and what had been done in more detail, a revised client statement was created.

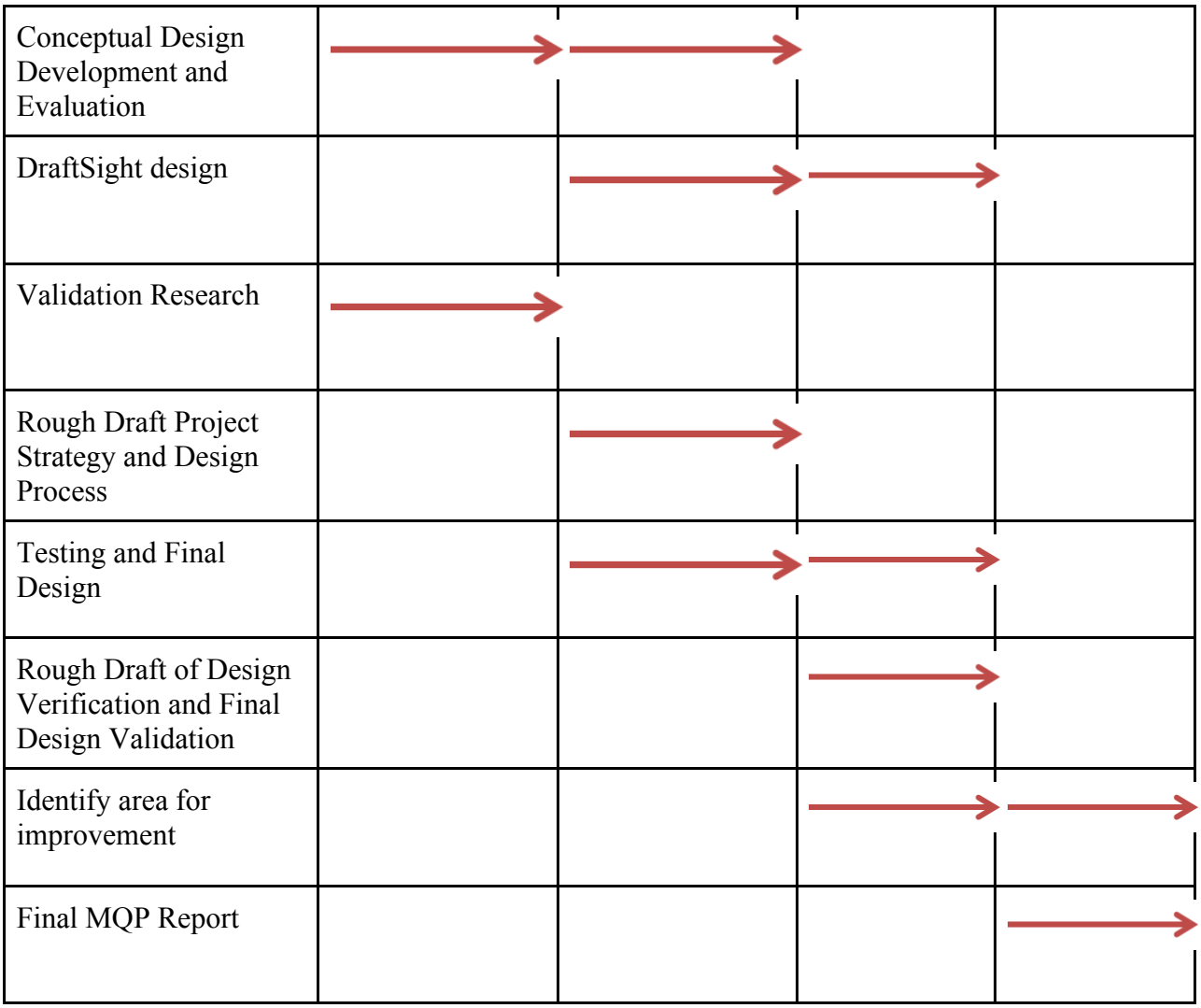
"Since there exists a need for a device that can observe the cancer cells within a steep oxygen environment similar to what is seen *in vivo*, the goal was to create a microfluidic system that enables human breast cancer cells (MDA-MB-231) to grow within a three dimensional environment by using a hydrogel. Within the system there will be a hyperoxic channel (21% oxygen), created by flowing compressed air, and a hypoxic channel (0% oxygen), created by flowing nitrogen gas. The area between these channels will be an oxygen gradient. The steepness of the gradient will be adjusted depending on the length between the channels. Observing the cell viability, proliferation, and migration in these distinct environments and gradients would better the understanding of cancer cell biology and how these human breast cancer cells react to oxygen microenvironments."

3.5 Management Approach

In order to accomplish the project objectives, it was necessary to breakdown the work to be done. A basic approach chart can be seen in Table 1 below.

Table 1: Rough Work Breakdown by term for the entire year

	A-Term	B-Term	C-Term	D-Term
Research Background	→	→		
Define Problem/Objectives/functions	→			
Rough Draft Chapter 1 and 2	→			
Project Specifications	→	→		



As can be seen in Table 1, A-term consisted of a lot of background research, and coming up with preliminary designs.

In B-term, materials were gathered and additional research was done. Preliminary testing was done such as verifying that the oxygen gradient could be visualized with a fluorescent slide, that the cells could grow within the hydrogel, that migration could be observed, and that the well height was high enough to hold the hydrogel, cells, and media. The team also began culturing the MDA-MB-231 cells and made different microfluidic devices during this term. Experience was gained in cell culturing, device making, and plasma bonding, which proved useful for the future work.

C-term consisted of confirming oxygen gradients in the device through finite element analysis and by observing the gradient within the device through the fluorescent slide. It was decided that three different gradients would be investigated: shallow, intermediate, and sharp. Migration and proliferation experiments were run in both sharp and shallow gradients.

In D-term, the intermediate gradient was tested and final conclusions were made. A presentation was given and the report writing was finalized.

The team was able to effectively manage their time even with just two team members. All tasks were completed on time.

4. Design Process

4.1 Needs Analysis

Table 2: Analysis of the needs for the device. The table is split up into requirements and how important each requirement is (weight).

Requirement	**Weight**
Can produce steep oxygen gradient	4
Can produce 3 different oxygen environments (hypoxic, hyperoxic, gradient (with normoxic))	5
Hydrogel is oxygen permeable	5
Hydrogel allows for successful cell culture (3D environment)	5
Hydrogel contains additional growth factors	3
Can further knowledge on cell biology of cultures	3
Cells grown in a temperature of 37 degrees Celsius	5
Low cost (Less than $500 budget)	2
Reusable	2
Able to culture other cell lines	2
Able to test the effects of different gases	1

We rated our requirements by their importance in order to achieve our goal. They are weighted from 1 (not necessary) to 5 (must have).

5 (Must Have)

The final product must have three different oxygen environments (hypoxic, hyperoxic, and a gradient). The gradient will be changed based on how wide the chamber widths are. Diffusion of oxygen through the membrane will be diffused into the hydrogel that is permeable by oxygen. The hydrogel should also be able to successfully culture cells in a 37°C environment; it can vary since some hydrogels are specific for certain cell lines.

4 (Important)

The second goal is to provide a microfluidic system that can produce a steep oxygen gradient. The term “steep gradient” is subjective. Research articles have produced gradients of 3mm or 6mm in 3D, but none have been found smaller than this. The steep gradient would be 100 micrometers. This is much smaller than the previous research done and more applicable to what can be seen *in vivo*.

3 (Good to Have)

By creating this device, it will be useful in a research setting and hopefully further the knowledge on cell biology of cultures. The hydrogel that is selected for use within the system could be injected with additional growth factors to further the growth of the cells.

2 (Not Needed, but Would Be Nice to Have)

An additional goal is to provide a low cost and reusable device can be used to culture any cell line and provide more research on how oxygen levels affect the chosen cell line. As far as the cost goes, oxygen sensors are expensive and alone would be over the budget. Regardless, they are important for this system and necessary in order to ensure accurate oxygen environments.

1 (Not Necessary)

The last goal is to test the effects of different gases on cell lines. Since oxygen is already being used, the supply to a different gas can easily be changed, but it is not a priority.

Currently there has been little research done on observing cell growth within a steep oxygen gradient hydrogel microfluidic system. Cancer cell growth has been observed within different oxygen concentrations, particularly in a static or 2D setting. By observing the cells over steep gradients, cell response to different environments can be observed. The cells may migrate; this wouldn't be seen in a static system. Additionally, proliferation and viability within the different environments can be simultaneously be observed.

4.2 Concept Map & Conceptual Designs

The basic concept map in the figure below shows the fundamentals of the device. This device will have an oxygen gas gradient that ranges from steep to shallow. Within this device cancer cells will be cultured in a three-dimensional environment.

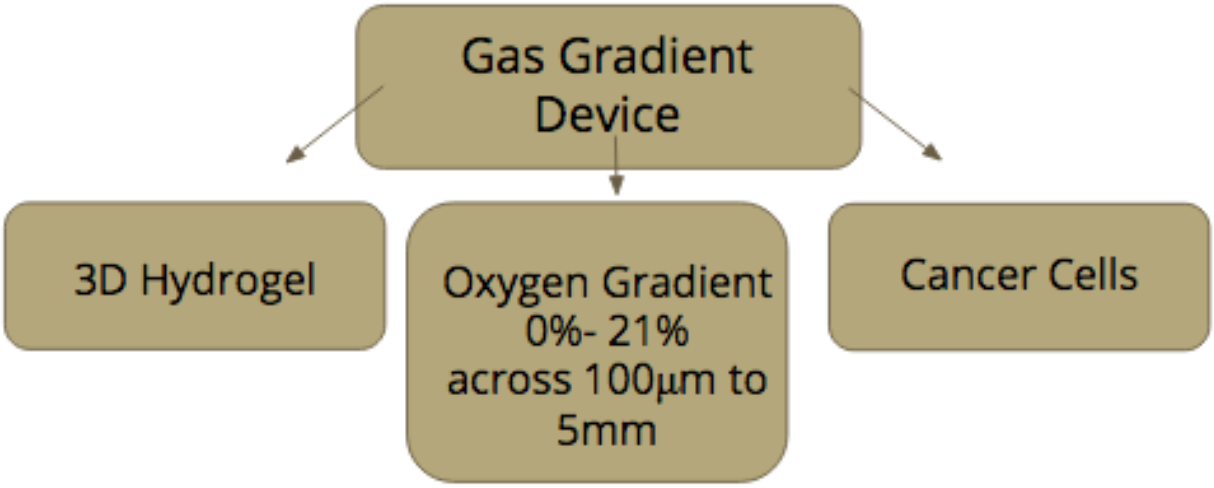


Figure 8: Basic Concept Map

A more advanced concept map can be seen below (Figure 9).

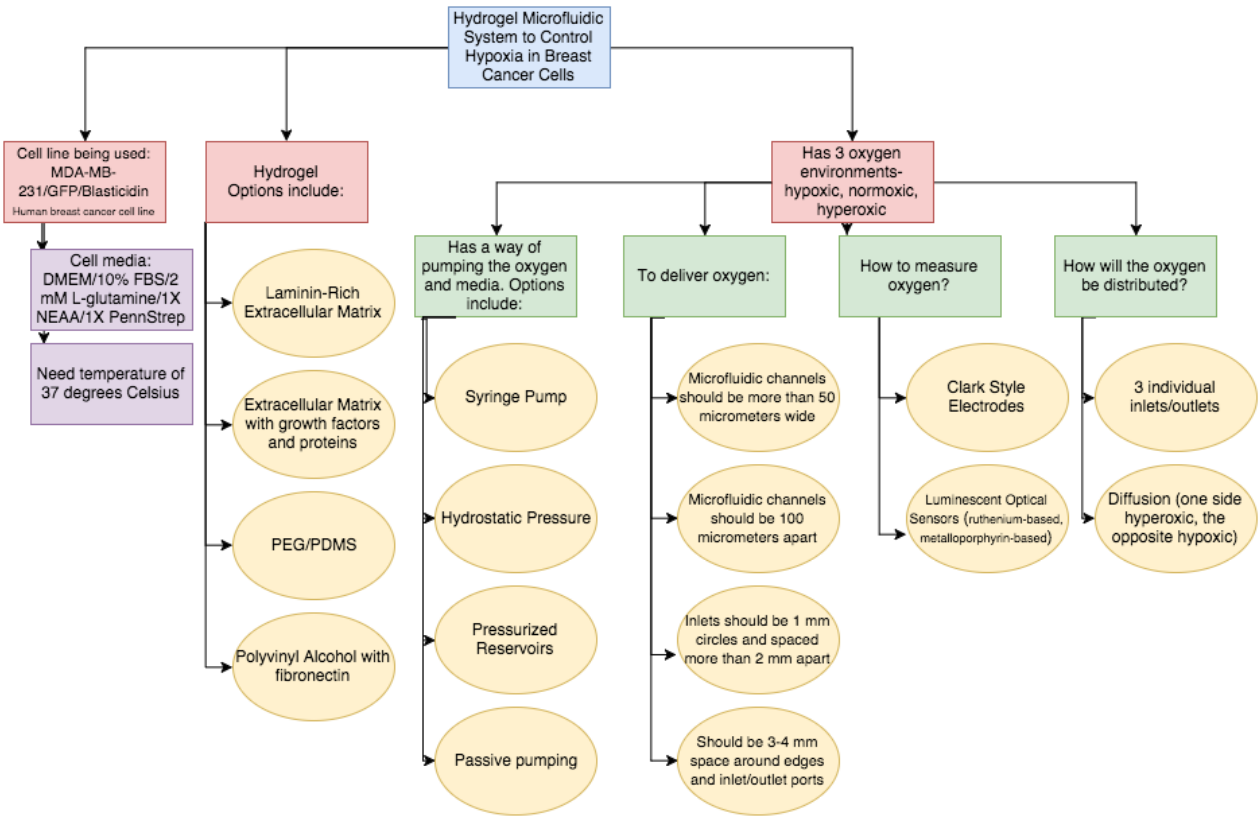


Figure 9: Advanced Concept Map

The concept map above includes several important design attributes. Within the system, the MDA-MB-231/GFP/Blasticidin, human breast cancer cell line will be grown. The cell media used will be DMEM/10% FBS/2 mM L-glutamine1X PennStrep and the cells need to grow in a temperature of 37 degrees Celsius. There are several hydrogel options as can be seen above. Some options include laminin-rich ECM, ECM with growth factors and proteins, a PEG/PDMS gel, polyvinyl alcohol with fibronectin, or collagen. Laminin rich and loaded ECM hydrogels best resemble natural *in vivo* since they contain natural growth factors and proteins. These help with growth and differentiation. PEG/PDMS can be molded down to the sub-micron resolution and is optically clear. Polyvinyl alcohol is an option but fibronectin is needed to promote cell adhesion. Lastly, using collagen is a more natural approach. PureCol EZ can be used to provide a firm gel or in a thin layer. The system also has to be able to create three different oxygen environments (hypoxic, normoxic, and hyperoxic), by flowing nitrogen gas and compressed air. There are different ways of delivering the oxygen and media. Syringe pumps are an easy way to deliver media; the pump is set to the rate at which to expel the media from a syringe so the flow rate is directly controlled. Other methods would use a difference in pressure such as hydrostatic pressure or pressurized reservoirs. These are easy to regulate but the flow rate can change. Passive pumping is easy, but it is sensitive to evaporation and flow rates are variable. The oxygen will be delivered by the gas tanks. The design of the device will include channels that are more than 50 micrometers wide, and more than 100 micrometers apart. The inlets will be circles approximately 1 millimeter in diameter. Additionally, there needs to be space around the edges and ports, so that they are not too close to the edge; this minimizes diffusion out of the device. Oxygen can be measured quantitatively with clark style electrodes or luminescent optical sensors. Oxygen can be distributed through individual inlets, by diffusion, or by both.

4.3 Alternative Designs

4.3.1 Alternative Design #1

One alternative design was given to the team at the beginning of the project. There are three different compartments for each of the different oxygen environments. There would be at least three inputs and three outputs, each containing a different oxygen level (Figure 10). There is a thin PDMS membrane that separates the channels from the extracellular matrix hydrogel and cells. This would allow for diffusion of the gases to occur into the hydrogel. The device is made of PDMS.

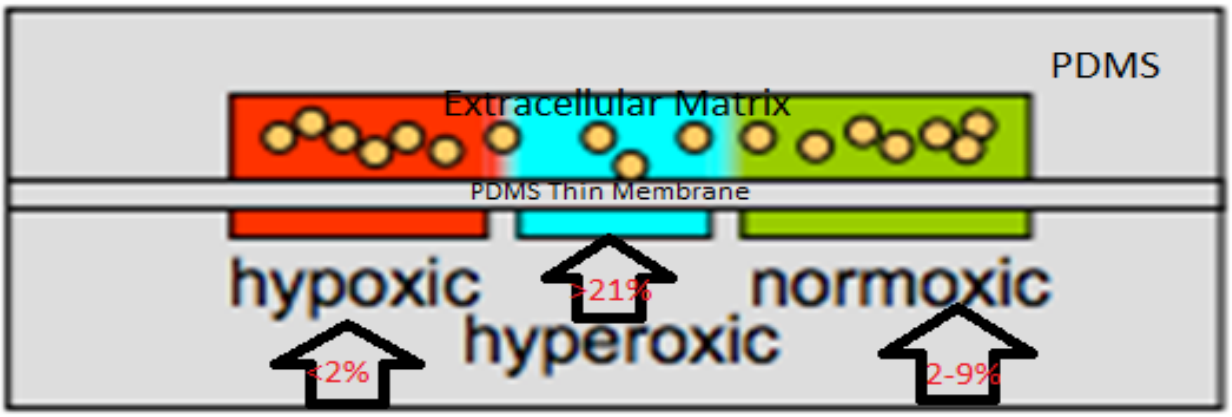


Figure 10: Alternative Design

4.3.2 Individual Tests for Final Design

Different individual tests were done which led the team to create the best design.

4.3.2.1 Well Test & Media Replenishing

Three different well heights were made using SYLGARD 184 Silicone Elastomer Kit. One well had a height of 5.6mm which was made of 20g of PDMS, the next well had a height of 7.4mm which was made of 30g of PDMS and the final well had a height of 12.3mm which was made of 70g of PDMS. To test the wells, each well was placed on top of the microfluidic device and 1mL of water was added in increments until the well either leaked or spilled. This amount was chosen because the hydrogel, cells, and media within the well equated to approximately 2mLs of liquid. If the well could hold more than this amount without leaking, it proved that the well was capable of holding the components needed for each experiment.

Result: From this well test, it was concluded that 12.3mm height well would be ideal for our final design because it was able to hold the necessary volume for each experiment. The smaller well heights couldn't properly hold all components and anything larger would affect the petri dish covering.

The team discussed replenishing the complete media within the 12.3mm well in case of media evaporation or the capillary effect. The capillary effect was common in wells lower in height; the large amount of liquid within the well tended to touch the top glass cover slide. This

caused the liquid to be drawn up and out of the device. A syringe pump was used and set to different speeds like 0.25mL/hr and 0.1mL/hr. A syringe pump filled with 10% FBS complete media was attached to tubing that went into the incubator. A special inlet was made within the well to provide a stable access point for the media to drip onto the hydrogel

Result: From tests using the syringe pump it was determined that a syringe pump was not needed because evaporation was not occurring that fast. It was much easier to replenish the media by hand if necessary. Usually it was not necessary as one to two milliliters of media was plenty for 200,000 cells for 48 hours. Additionally, with the higher well height, the capillary effect did not cause any media to be drawn up and out of the device.

4.3.2.2 Hydrogel Experiments

Preliminary testing of hydrogel began with PureCol EZ bovine collagen. The purpose of the experiments was to observe cell proliferation and migration in the hydrogel. Images were taken at 0 hours, 24 hours, 48 hours, and 72 hours using the ZEN Program with the Zeiss Axiovert 40 CFL Fluorescent microscope. Using FIJI, images were compared at each time point to observe cell proliferation and migration.

Results:

Experiment #1: After 24 hours, cell growth was observed on the hydrogel. After 48 hours, a sterile scalpel was used to add a scratch to the hydrogel to observe migration across the scratch. A pipette tip would not be able to cut the hydrogel due to its jello-like substance. A cross was drawn on the bottom of the petri dish to be used as a reference point when taking the images under the microscope. 2mL of fresh media was added after aspirating the two-day-old media from the hydrogel well. After 72 hours, contamination was observed. A cloudy substance had formed on top of the hydrogel preventing clear images of the cells seeded within the hydrogel. The assumption was made that the contamination came from the scalpel cute done the day before and the experiment ended at this time.

Experiment #2: The same area of the hydrogel was observed at each time point when taking images. After comparing the 0 hours, 24 hours, 48 hours and 72 hours it was concluded that there was proliferation in the hydrogel (Figures 11 & 12), and therefore justified the choice for using PureCol EZ as the hydrogel. The Figures get brighter and more cells are seen after each 24 hours, which indicates an increase in proliferation. A scratch test was done in the middle of the hydrogel. Upon observing particular cells, it was seen that the scratch had cells within it, therefore proving that there was migration.

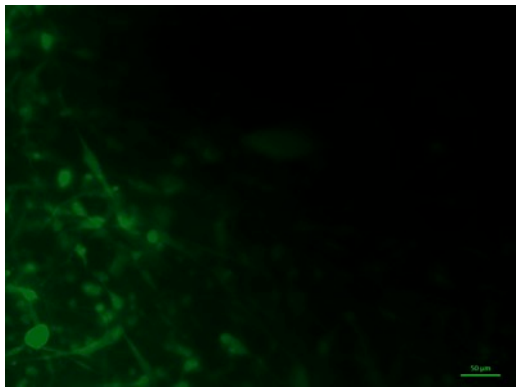


Figure 11: Cells after 48 hours with fluorescence (20X magnification) for experiment 2

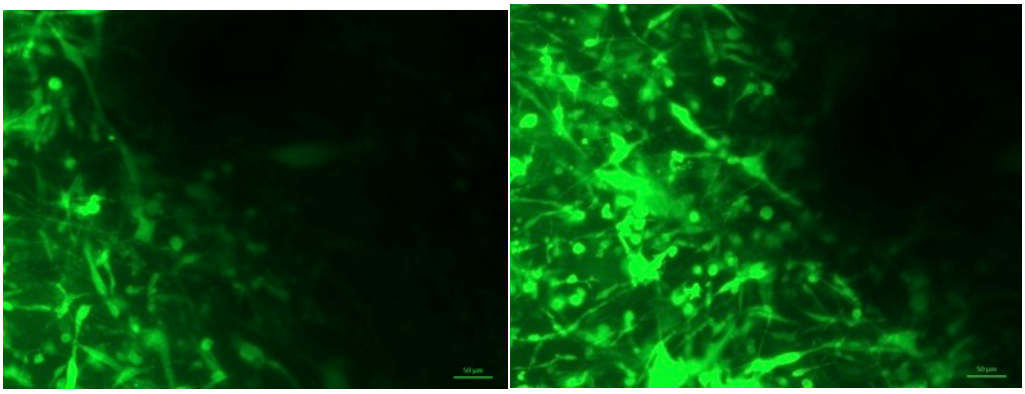


Figure 12: Cells after 72 hours (left) compared to after 96 hours (right) for experiment 2

4.3.2.3 Hydrogel Amount Test

Another hydrogel experiment was done in a 24 well plate to determine the necessary amount of PureCol EZ to use for cell proliferation and migration in our final design. The amounts tested were 250µl, 500µl, and 1mL.

Images were taken at 0 hours, 24 hours and 48 hours using the ZEN program with the Zeiss Axiovert 40 CFL Fluorescent microscope. Using FIJI images were compared at each time point to observe cell proliferation and migration.

Result: There was cell proliferation and migration in all three well amounts. At 0 hours, all cells were between the hydrogel and by 48 hours the cells had migrated closer to the bottom of the well. This could be caused by the fact that the bottom of the wells are treated for cell culturing.

The team experimented with adding 250µl, 500µl, 750µl, and 1mL of PureCol EZ hydrogel to the device.

Result: 1mL of hydrogel was chosen. A hydrogel amount of 250µl or 500µL tended to peel up when the gases began to flow since it was so thin. An amount of 1mL was perfect as it remained stationary and all layers could be seen in the device. This ensured that all cells were seen. An image the 1mL hydrogel without the well can be seen in Figure 13. Additionally, Figure 14 shows a hydrogel that peeled up because it was too thin.

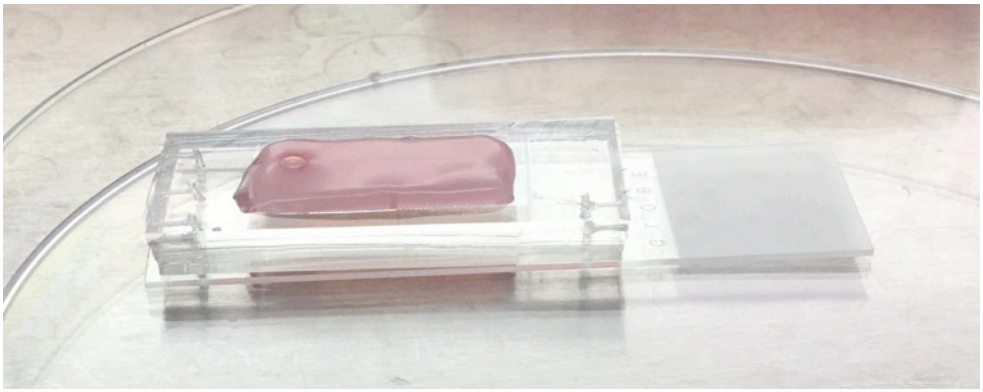


Figure 13: Successful 1mL hydrogel on device with no well

Figure 14: Thin hydrogel that peeled up and folded over (seen near top of well)

4.3.2.4 Cells to Seed

The MDA-MB-231 cell line was chosen because it is a commonly researched human breast cancer cell line and it expresses the GFP reporter, which will allow migration and proliferation to be easily observed under the microscope. For information on cell passaging, and the number of cells that were passaged, see Appendix A.

Experiments were conducted to determine the proper amount of cells to seed into the hydrogel to allow proliferation and migration in our final design. Experiments were seeded with 2.5 million cells, 2 million cells, 1 million cells, or 200,000 cells.

Results: From the experiments, it was concluded that 200,000 cells was the ideal number of cells to seed because it allowed more space to observe proliferation and migration compared to the larger number of cells seeded. Having too many cells was difficult to observe migration since not a lot of empty space was available to clearly see the migration of cells.

4.3.2.5 COMSOL Simulations

COMSOL is a finite element analysis program. The entire device was simulated and modeled in 2D in COMSOL, however, only the hydrogel, membrane, and chambers are depicted in the following simulations, as these are the components that affected the gradient. The following parameters were used to create the model:

- Hydrogel Dimensions: 27mm x 1.7mm(Thickness)
- Membrane Dimensions: 25mm x 0.063mm(Thickness)
- No flux boundary all around the system
- Diffusivity of Oxygen in PDMS: $3.25*10^{-9}[m^2/s]$
- Diffusivity of Oxygen in Water: 1.97×10^{-9} $[m^2/s]$ (Ferrell & Himmelblau, 1967)
    - Simulates diffusion of oxygen in the hydrogel
- 3 Chambers: 5mm x 0.1mm each
- 100um gap between each chamber
- 21% oxygen in one chamber
- 0% oxygen in one chamber

The entire device was modeled in COMSOL and meshed; however, for clarity purposes only the chambers, hydrogel, and membrane are represented in the section, since adding the bottom of the device did not affect the slope of the gradient. The boundaries were considered no flux because of the large width between the edges, particularly the area from the chambers to the

bottom of the device, and the chambers to the sides of the device. A complete image of the device model can be seen in Appendix C.

Using COMSOL three different oxygen gradients were modeled of the three different designs. The shallow gradient (5mm gap) had a broader oxygen gradient compared to the intermediate gradient (700µm) and sharp gradient (100µm). These can be seen in the Figure below. For the oxygen gradient models, the parameters stayed the same. The shallow gradient has the 21% oxygen and 0% oxygen inlets on opposite sides creating a 5mm oxygen gradient in the model. In the intermediate gradient, the gap between each chamber was changed from 100µm to 700µm to and the 21% oxygen and 0% oxygen inlets were next to each other to produce the oxygen gradient shown in the model. For the sharp gradient, the 21% oxygen and 0% oxygen inlets were next to each other over a 100µm gap to produce the oxygen gradient shown in the model. The maximum slope of the oxygen gradient for the shallow gap is 0.31 O_2%/100µm (over approximately 6mm of the gradient) (3.1 O_2%/mm), the maximum slope for the intermediate gradient is 1.4 O_2%/100µm (in 700µm of gradient) (14% O_2%/mm), and the maximum slope for the sharp gradient is 2.5 O_2%/100µm (over approximately 200µm of the gradient) (25 O_2%/mm).

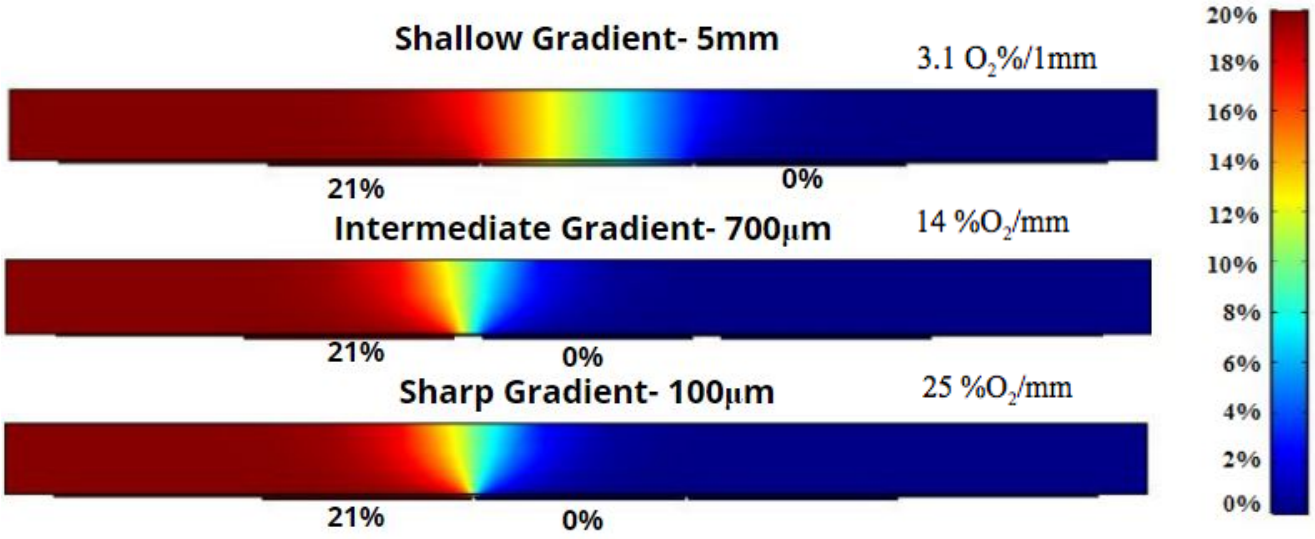


Figure 15: COMSOL Model Comparing Oxygen Gradients

COMSOL was used to simulate other important factors.

Thickness of Membrane: The thickness of the PDMS membrane is an important factor for our experiment (Figure 16). If the membrane is thick it would take longer for the gases to diffuse through and reach the seeded cells. A 63.5µm, 250µm, 500µm and a 1mm thick membrane were compared and are shown in the figure below. For the membrane thickness modeling, the parameters stayed the same except the membrane thickness was changed to 63.5µm 250µm, 500µm, and 1mm. The difference in gradient can be seen when comparing the average oxygen gradient of the 63.5µm membrane to the 1mm membrane. The maximum slope of the oxygen gradient for the 63.5µm membrane is 25.00 O_2%/mm. The maximum slope of the oxygen gradient for the 250µm, 500,µm, and 1mm were taken by looking at the steepest part of the average slope. This created lower values than what would be seen if it was zoomed in, because it is a sharp gradient. The average maximum slope of the 250µm membrane is 3.33 O_2%/mm. The average maximum slope of the oxygen gradient of a 250µm membrane is 7.00 O_2%/mm and the

average maximum slop of the oxygen gradient of a 500µm membrane is 4.8 O_2%/mm. It was decided to use a 63.5µm thick membrane because the oxygen gradient was the sharpest compared to the oxygen gradients in the other models; a thicker membrane has a broader gradient.

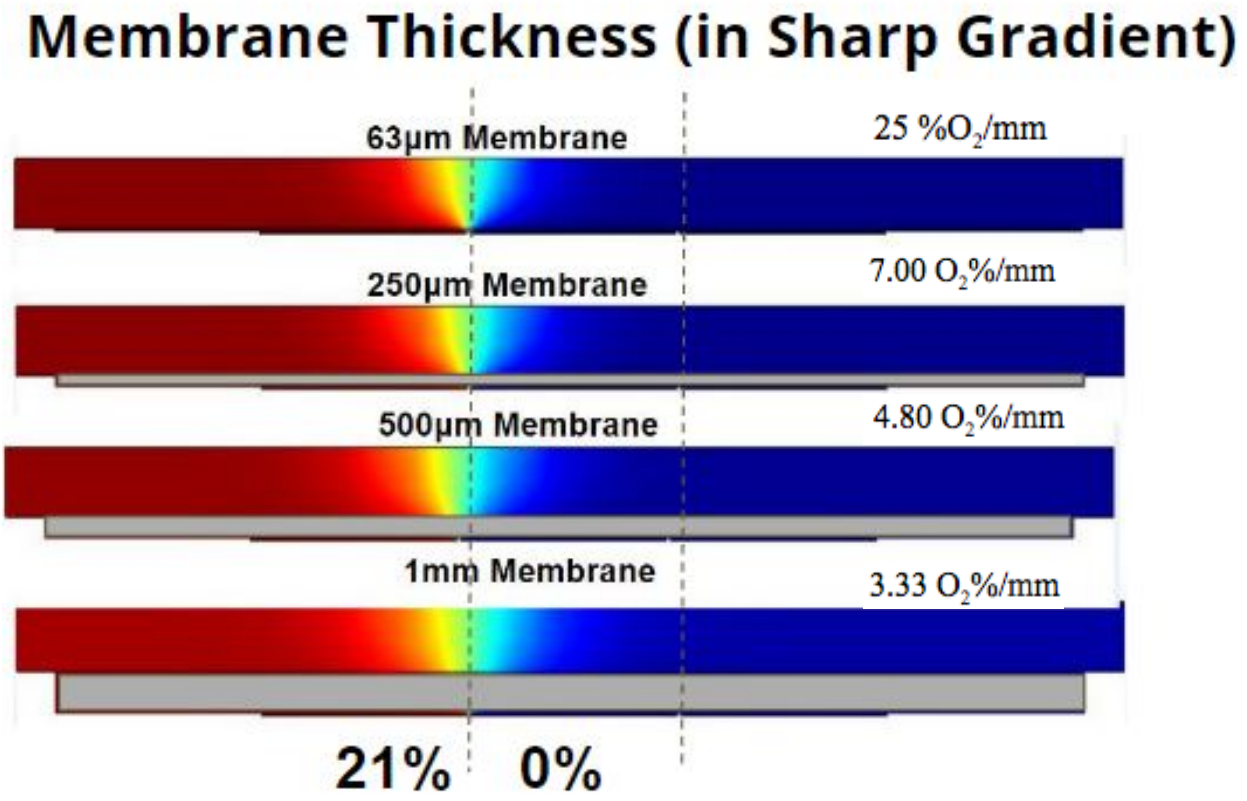


Figure 16: COMSOL Model Comparing Membrane thickness

Slide Covering: Another important factor was whether to use a slide cover or not (Figure 17)-. For the device covering model, the parameters stayed the same except that a 21% oxygen concentration was allowed on the top boundary (open top). For the closed top, the top boundary was set to no flux, preventing 21% oxygen to enter the system (oxygen gradient). From the open top model, it can be observed that there is a much smaller oxygen gradient visible because the oxygen coming from the top interferes with the gradient within the hydrogel. The atmospheric air affects a lot of the hydrogel and there is a sharp gradient that moves vertically throughout the gel. The team wanted to observe the cell affects of an oxygen gradient across a horizontal gradient. The vertical gradient that the open top produced could not have been observed under the inverted microscope. In the closed top model, which did not allow oxygen through the top, it can be observed that the oxygen gradient stays consistent throughout the device. The maximum slope of the oxygen gradient of the closed lid is 3.38 O_2%/mm and the maximum slope of the oxygen gradient of the open lid is 6.5 O_2%/mm. From the COMSOL model it was concluded that having a slide cover would keep the oxygen gradient consistent throughout the entire device and the experiment duration. If the well was left open, the atmospheric oxygen would interfere with

the experiment thus affecting the results. For this reason the team decided to use a slide cover to maintain a consistent oxygen gradient as shown in the closed top model.

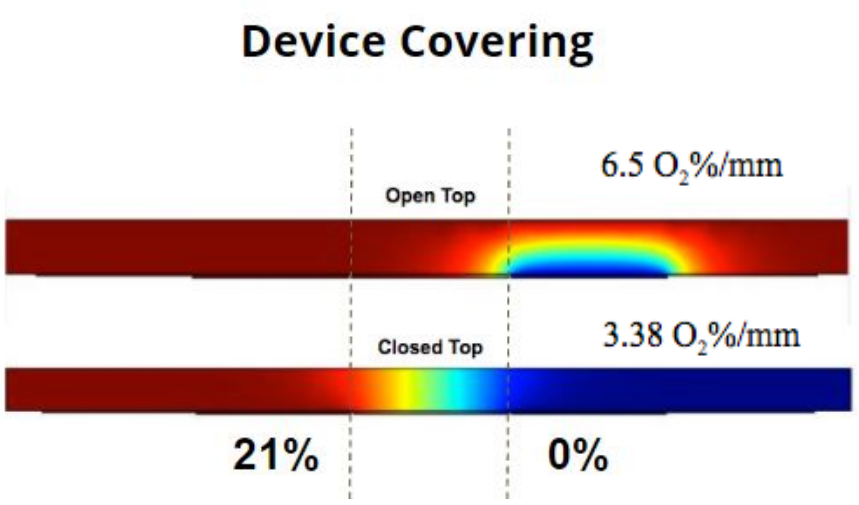


Figure 17: COMSOL Model of Device Covering

There were several other COMSOL simulations done. One of them was investigating the hydrogel height. This had a minimal effect on the gradient. For the hydrogel height model, the parameters stayed the same except the hydrogel height was changed to 0.5mm, 1mm, 2mm and 3mm. As can be seen in the models below (Figure 18), the oxygen gradient stays consistent through each model. The max slope of the oxygen gradient of a 0.5mm hydrogel height is 3.5 O_2%/mm, the max slope of the oxygen gradient of a 1mm hydrogel height is 3.25 O_2%/mm, the max slope of the oxygen gradient of a 2mm hydrogel height is 3.0 O_2%/mm and the max slope of the oxygen gradient of a 3mm hydrogel height is 2.75 O_2%/mm. Concluding that the hydrogel height does not affect the oxygen gradient. This concludes that the hydrogel height does not affect the oxygen gradient. Additional less important factors can be seen in Appendix C, along with the graphs of the oxygen concentration throughout the device. These include the width of the chambers (3mm or 5mm), and a two-chamber device as opposed to a three-chamber device.

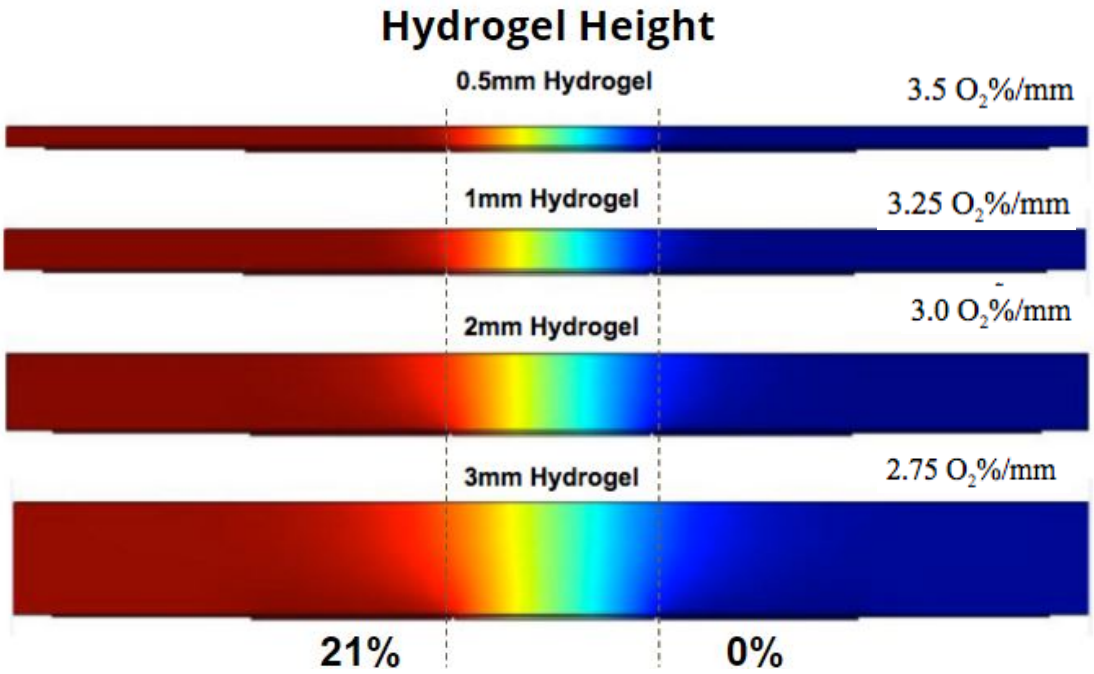


Figure 18: COMSOL Model Comparing Hydrogel Height

4.4 Final Design Selections

The team has decided to use a 47mm X 28mm X 5mm (~50mm x 25mm x 5mm) device made from the silicone master provided. The device is made out of polydimethylsiloxane (PDMS) and it is plasma bonded to a glass slide. Within the device are 1mm holes punched from the top and side. There are three inlets and one outlet hole punched. Only two inlets will be used; one will flow nitrogen gas (0% oxygen) in order to produce a hypoxic environment and the other will flow compressed air (21% oxygen) to produce a hyperoxic environment.

There is 63.5µm thick PDMS membrane plasma bonded to the top of the device that covers the top holes and system since only the side holes will be used to flow the gases as discussed in 4.3.2.5. This membrane will allow for the gases to diffuse through and produce the appropriate oxygen conditions. On top of the membrane will be a well with a height of 12.3mm made from 70g of silicone base and 7g of silicon curing agent in a white weigh boat, as the well is sturdy and able to contain the necessary volume of cells, hydrogel, and media without leaking or spilling as discussed in 4.3.2.1. The well will be thoroughly cleaned with ethanol and kimwipes before it is placed on the device. This component will not be plasma bonded to the device to make the device reusable and easier to clean. To see the cleaning protocol go to Appendix B. PDMS naturally adheres and creates a seal with clean PDMS. The user will be able to easily remove the well after the experiment is complete and clean the components individually.

Within the well, there will be MDA-MB-231/GFP/Blasticidin human breast cancer cells seeded in PureCol EZ and media for cell growth. There will be approximately 200,000 cells (75µL-85µL) seeded in 1mL of PureCol EZ and 1mL of complete media for each experiment as discussed in 4.3.2.1, 4.3.2.2, 4.3.2.3, and 4.3.2.4. A slide cover will be placed on top of the well to prevent any atmospheric air from interfering with the experiment, providing an airtight seal.

The device will be housed within a large petri dish (140mm x 20mm). The petri dish will be contained within an incubator set at 37°C, to allow proper growth conditions of the breast cancer cells. The tubing will be placed in the incubator in such a way that it does not affect the seal of the incubator to ensure that no heat or carbon dioxide is wasted. Gases will flow into the inlets through a 6.35mm tubing that is attached to a compressed air tank and a nitrogen gas tank. The pressure of the tanks will be kept at 14 kPa (2psi), which is sufficient to produce the gradient, but also ensure that the membrane isn't stressed. Too much flow will cause evaporation. The tubing at each tank is secured by a zip tie. At the end of the tubing, a syringe tip is secured to the tubing by a luer adapter. The syringe tip is attached to a smaller diameter tubing (0.85mm), which fits inside a pin that is placed within the inlets of the device. This allows a tight fit to ensure that there is no leakage while the experiment is running.

An image of the breast cancer cells in the device will be taken each day after they are seeded into PureCol EZ to observe migration and proliferation. In particular, reference points will be chosen on the device to clearly see any migration or proliferation (Figure 19). The team expects to observe cell migration to a certain oxygen environment, particularly the hyperoxic region since the most oxygen is within that area. This area may also have the most proliferation. The length of each experiment will be 48 hours. The final setup can be seen in Figure 20. The estimated prices of all the needed materials are in Appendix D. Additionally, the Standard Operating Procedure for setting up and running the experiment can be found in Appendix E.

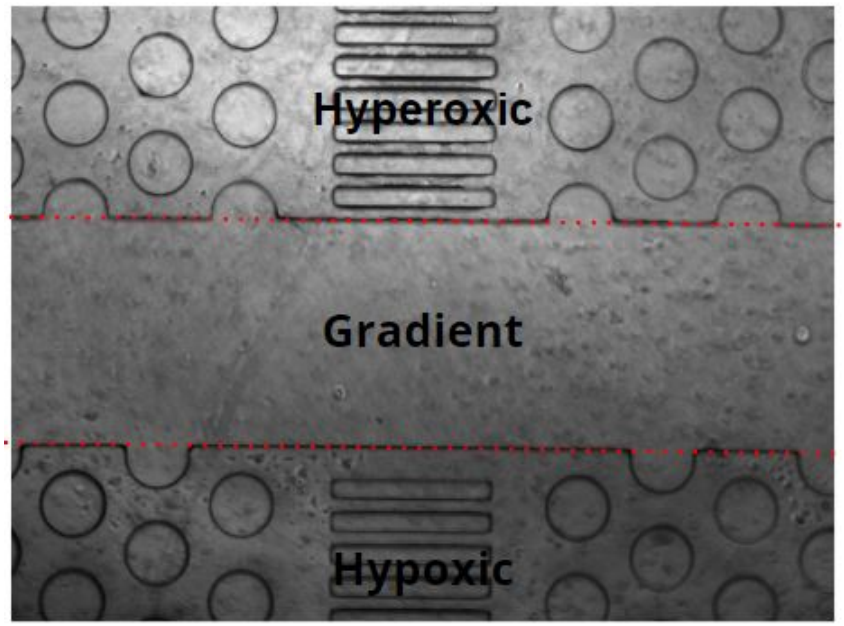


Figure 19: Example of a reference point used to observe cell proliferation and migration.

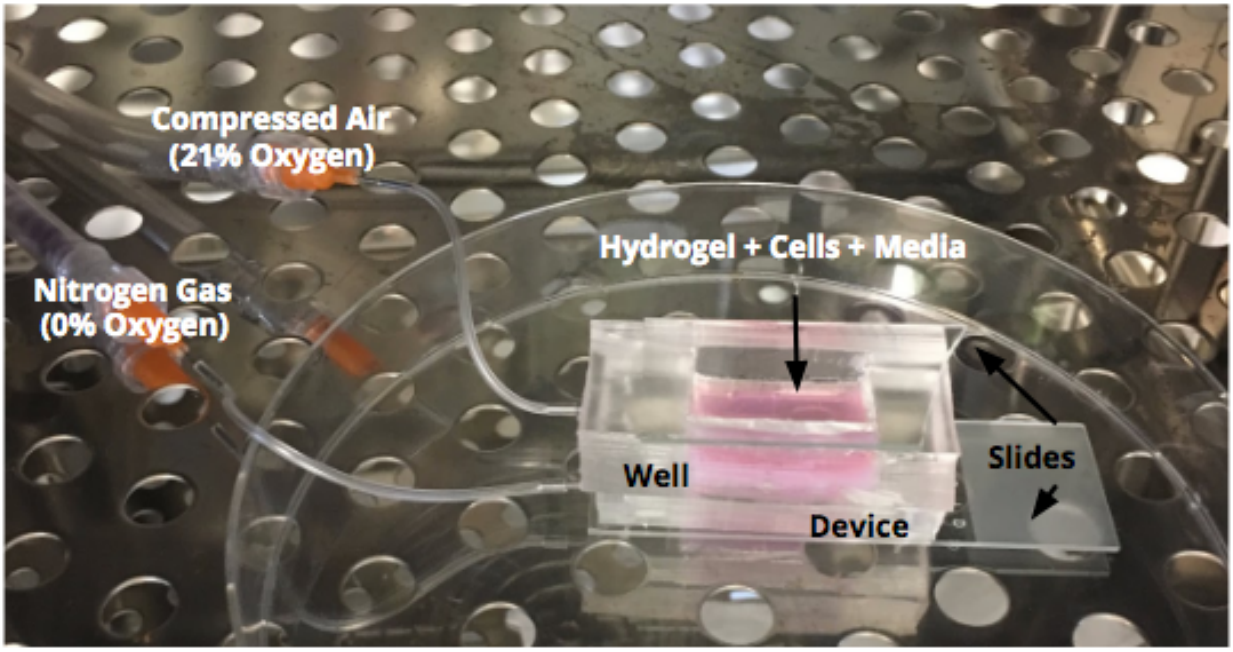


Figure 20: Final setup of device in incubator

5. Design Verification

The tests done in Chapter 4 allowed for a final design to be selected. This chapter presents the core project findings for the oxygen gradients produced and for the viability, proliferation, and migration of the human breast cancer cells within the three different oxygen gradients (5mm, 700μm, and 100μm).

5.1 Oxygen Gradient Confirmation

A qualitative spatial map was used to visualize oxygen levels. By using the ZEN program with the Zeiss Axiovert 40 CFL Fluorescent microscope, gas flow was observed on a fluorescent slide and within the device. The brightfield phase was used and the light from the microscope was closed to only observe fluorescence. Additionally, instead of using auto-exposure, it was set to 100. The FIJI program was used for the image calculations. These images were unmodified and were saved as ome.tiff. This made sure that there were no pixel modifications or adjustments by the program.

5.1.1 Oxygen Gradient Confirmation on Florescent Slide

Carbon dioxide gas (0% oxygen) was blown onto an OceanOptics Ruthenium-Coated fluorescent slide (part name FOXY-SGS-M, price $275.00) under green light. Figure 21 shows a visual on how the syringe tip was attached to the tubing and blown onto the slide under the microscope. The syringe tip was taped down to ensure that no movement occurred.

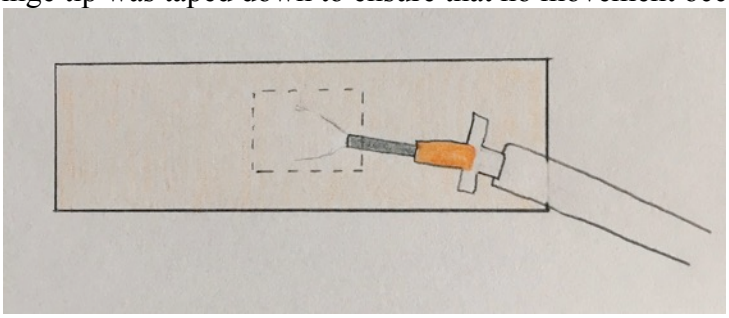


Figure 21: Conceptual image of carbon dioxide being blown onto fluorescent slide

The slide before the gas was turned on and during the gas flow can be seen in Figures 22 and 23.

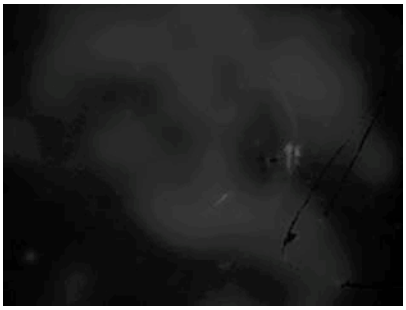


Figure 22: Before carbon dioxide flow on slide (left)/ Figure 23: During carbon dioxide flow on slide (right)

The images are rather dark. Using FIJI, the location during the time when the gas was flowing was divided by the location when the gas was turned off; the image with carbon dioxide flow was divided by the initial image (atmospheric air hitting the slide, approximately 21% oxygen). The result was showed as a 32-bit (float) result. The images can be seen in Figure 24. The black and white image was what was originally outputted since the images themselves were in black and white. To add more contrast, the look-up table (LUT) 'Fire' was added (Figure 24). The flow of gas can clearly be distinguished. A value of one meant there was no change; this is equivalent to atmospheric air (21%). A value greater than one meant that the image got brighter. The more saturated regions had a value of approximately two; there was a large amount of change here and meant that the oxygen content was approximately 0%. The lower left-hand corner had a value of approximately 1.4, so while it doesn't look especially bright (especially when comparing to the brightness around the tip) it still changed. This method allows the team to qualitatively visualize spatial oxygen levels at the micron-scale, which makes it suitable to measure the sharpest gradient the team intends to make (100μm).

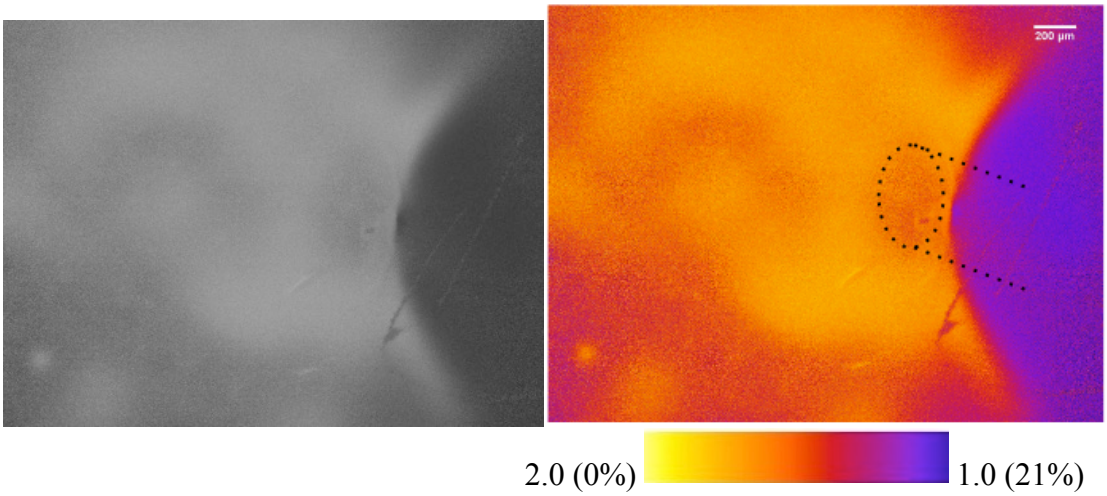


Figure 24: Black and white result of division (left). LUT Fire result of division (right).

Figure 24 also shows a tracing of the needle to help visualize the flow of gas. The brighter, more yellow region has a low level of oxygen. The purple region behind the needle represents the atmospheric air hitting the slide. This test confirmed that the slide could effectively show oxygen change.

5.1.2 Oxygen Gradient Confirmation Within the Devices

Since it was confirmed that oxygen gradients could be visualized with the fluorescent slide, it was used to observe gradients within the device. Nitrogen was flowed into one inlet, and compressed air was flowed into the adjacent inlet. The chamber gap between both of these regions was focused on under the microscope. Both gases were flowed at the same rate. The device was placed upside down on the fluorescent slide so that the membrane was in contact with the slide. This can be seen in Figure 25. The pins and tubing were stabilized to ensure that no movement of the pins, tubing, or slide occurred, as this would produce inaccurate results.

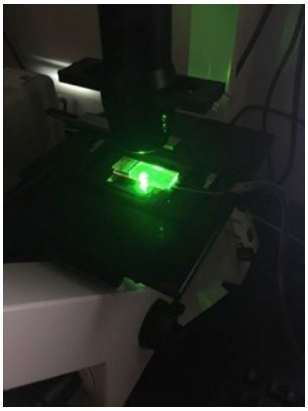


Figure 25: Device on fluorescent slide under fluorescent microscope

Black and white images were produced; images were taken before the gases were turned on and when the gases were flowing. FIJI was used to create the division images and the LUT 'Fire' was added. The images produced were similar to what was expected.

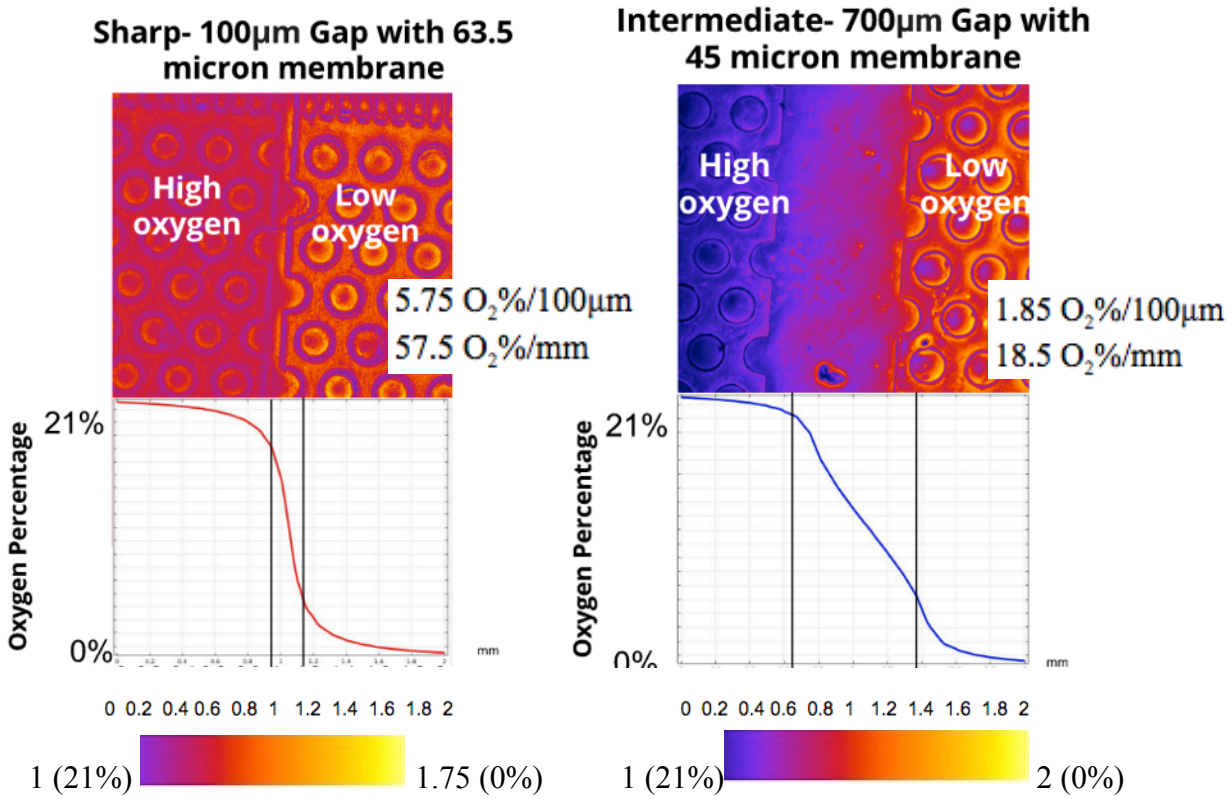


Figure 26: Oxygen gradients in sharp and intermediate gap devices

As can be seen in Figure 26, there is a visible difference between the hypoxic region (low oxygen) and the hyperoxic region (high oxygen). The low oxygen is again visualized by a brighter color (yellow), while the high oxygen is visualized by a darker color (purple).

The sharp 100µm gradient can be visualized between the two chambers. The plot below is to scale; it shows the oxygen percentage from 0-21% along the field of view. The field of view is 2mm in width, and therefore so is the plot. The slope of the sharp gradient plot is steep, which corresponds to the sharp color change within the chamber gap of the device. The scale for 21%

oxygen would be a dark pink, since that is what is seen within the hyperoxic chamber. The range goes from 1, which is approximately 21% oxygen, to approximately 1.75, which is 0% oxygen.

The intermediate 700µm gradient can be visualized more easily than the sharp gradient. The slope of the plot is less steep than what is seen in the 100µm. The gradient change within the gap can be seen and corresponds to the plot. It goes from a bright yellow on the hypoxic chamber side (value of 2), to a dark purple in the hyperoxic chamber (value of 1).

The maximum slope of the oxygen gradient for the sharp gradient is 5.75 O_2%/100µm (over approximately 200µm of the gradient) (57.5 O_2%/mm (in the gradient)), the maximum slope for the intermediate gradient is 1.85 O_2%/100µm (in 700µm of gradient) (18.5 O_2%/mm (in the gradient)), and the maximum slope for the shallow gap is 0.31 O_2%/100µm (over approximately 6mm of the gradient) (3.1% O_2%/mm). There is a large difference in gradients. The oxygen gradient with the units O_2%/mm was done if the gradient was extrapolated to a millimeter.

Oxygen Gradient 200µm Above the Membrane

These tests confirmed that an oxygen gradient can be visualized on the membrane of the device. Since the cells will not sit directly on the membrane as they will be within the hydrogel, plots were made to visualize the gradients at this elevated area above the membrane. This was simulated at 200µm from the membrane. These can be seen in Figure 27. The location of the cells above the membrane can be seen in the COMSOL Figure below (Figure 28).

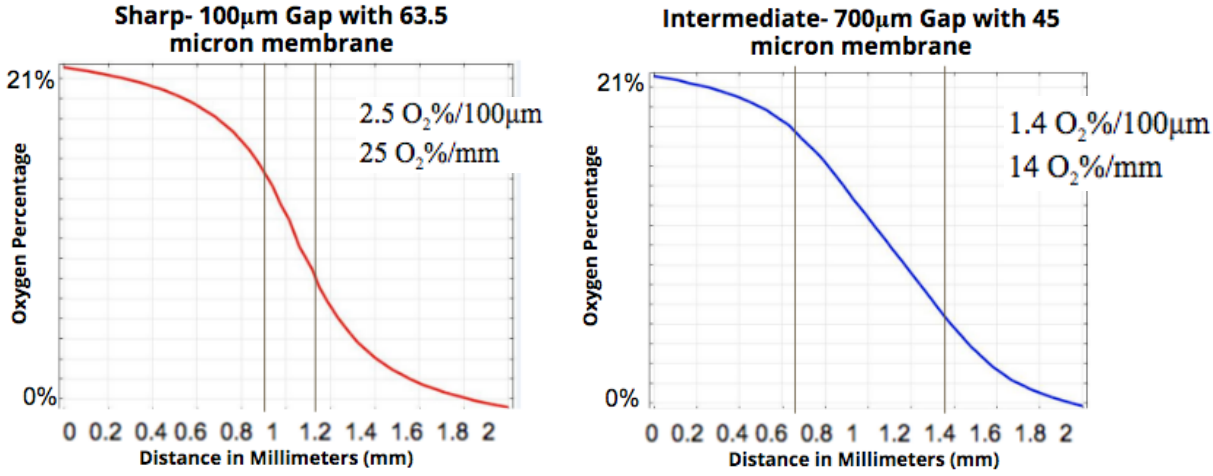


Figure 27: Oxygen gradients within the hydrogel where the cells are seeded

21% O_2 0% O_2

Figure 28: COMSOL model of cells in gel. The red axes show 200 micrometers above the membrane to simulate where the cells are in the hydrogel (1.97 x 10^{-9} m^2/s oxygen diffusivity in hydrogel, and 3.25 x 10^{-9} m^2/s oxygen diffusivity in PDMS)

As can be seen in the Figures, the oxygen gradient was not as drastic for each of the gaps widths as was seen in Figure 26, but there is still an obvious difference in slopes. By observing the figures, the max slope for the sharp gradient is approximately 2.5 O_2%/100µm (25 O_2%/mm

in the gradient). The intermediate gradient has a slope of about 1.4% O_2%/100μm (14 O_2%/mm in the gradient). There is quite a difference in the sharp gradient at the membrane compared to 200μm above the membrane. This is why the cells were seeded as close to the bottom as possible.

It must be noted that there is no visual gradient for the 5mm shallow gradient. This is because the 5mm gradient could not be visualized under the 5X lens of the microscope. It must be assumed that the COMSOL models were correct, especially since they were correct for the sharp and intermediate gradients, and that the gradient was in fact produced.

5.2 Proliferation & Viability

To observe the proliferation and viability FIJI was used. A fluorescent image focused on the cells at the specific reference point was taken. These images were saved as ome-tiffs; they were 16-bit images. Using FIJI, the background was subtracted by adjusting the threshold. The threshold was adjusted so that only the cells were visible. This was a value of approximately 5,000-7,000, depending on the original brightness of the image. The bottom condition was turned up to 100 percent threshold so that the entire image was red (a value of 65,520). The top condition was then lowered until only the cells were visible. Only about 5-10% of the image was visible then. This depended on how many cells were visible within the screen. The cells in clumps were separated through the watershed process. Then the cells were counted through the 'analyze particles' analysis tool. The defaults were kept to view the results. The size was kept from 0-infinity, the circularity went from 0-1, and clear results and summarize were checked within the window. The cell number was then displayed under 'count.' Other components such as average size, and percent area were displayed. A before image and after image analysis can be seen in Figure 29.

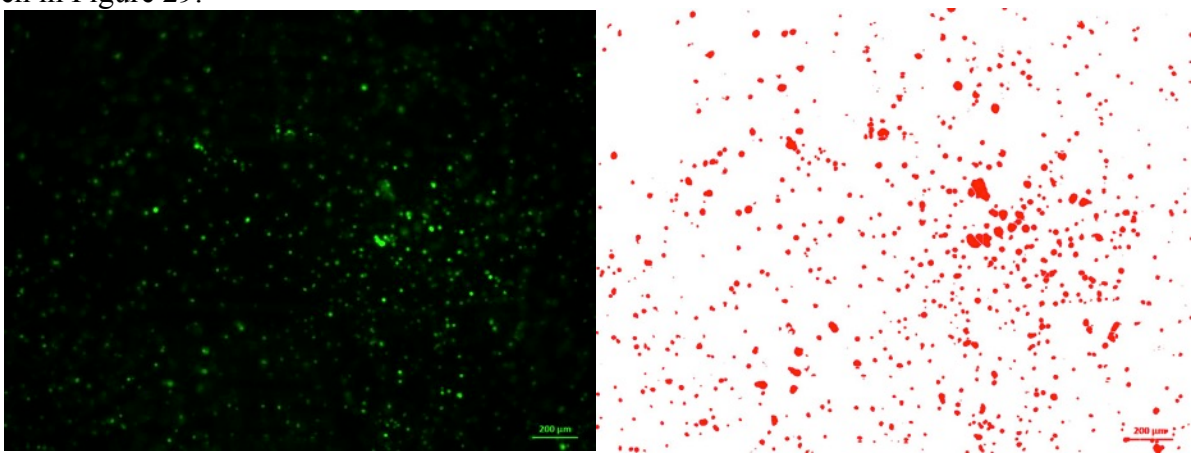


Figure 29: Fluorescent image of cells before FIJI analysis (left) and after (right)

A cell count was done for each experiment at each timepoint (0, 24, and 48 hours). These were each compared and added to a table. Originally the entire proliferation was compared to the next entire image at each timepoint, but to make it more specific the analyzed image was broken into the appropriate regions. The original reference image for each timepoint was used to observe where the particular regions were. This allowed for an accurate cell count of the hyperoxic, hypoxic, and gradient region.

The data for the sharp experiments can be seen in Tables 3 and 4. The cell counts for each environment (hyperoxic, gradient, and hypoxic) can be seen. The percentage increase was found by taking the cell count at the time point, subtracting the initial cell count, and dividing by the initial cell count. The answer would be multiplied to find the percentage. For example:

%Increase = ((24 hour count) - ((0 hour count))/(0 hour count)) x 100
%Increase = ((529-253)/253) = 109.1%

Table 3: Sharp Experiment #1 Cell Proliferation

Time	0 hours	24 hours	48 hours	% Increase After 24 hours	% Increase After 48 hours
Hyperoxic	253	529	676	**109%**	**167%**
Gradient	49	57	58	**16.3%**	**18.4%**
Hypoxic	151	151	134	**0.00%**	**-12.7%**
Total Cells	453	737	868		

Table 4: Sharp Experiment #2 Cell Proliferation

Time	0 hours	24 hours	48 hours	% Increase After 24 hours	% Increase After 48 hours
Hyperoxic	569	602	678	**5.80%**	**19.2%**
Gradient	158	168	179	**6.33%**	**13.3%**
Hypoxic	189	204	212	**7.94%**	**12.2%**
Total	916	974	1,069		

To find the averages percentages (seen in Table 5), each cell count in the environment at both experiments was averaged first, and then the percentage increases were found. The standard deviations of the two sharp experiments were calculated as well.

Table 5: Sharp Experiment Average Proliferation

	0 hours	24 hours	48 hours	% Increase After 24 hours	St.Dev.	% Increase After 48 hours	St.Dev.
Hyperoxic	411	565.5	677	**37.6%**	**+/- 73%**	**64.7%**	**+/- 104%**
Gradient	104	113	119	**8.65%**	**+/- 7.1%**	**14.4%**	**+/- 3.59%**
Hypoxic	170	178	173	**4.71%**	**+/- 5.6%**	**1.76%**	**+/- 17.8%**

As can be seen in the average percentages after 48 hours in Table 5, the hyperoxic region had the most proliferation at approximately 64.72%. The gradient saw the next highest proliferation with an increase of approximately 14.40%. There was not much proliferation in the hypoxic region.

The data for the intermediate experiments can be seen in Table 6. The cell counts for each environment (hyperoxic, gradient, and hypoxic) can be seen along with the percent increase.

Table 6: Intermediate Experiment Cell Proliferation

	0 hours	24 hours	48 hours	% Increase After 24 hours	% Increase After 48 hours
Hyperoxic	174	227	302	**30.5%**	**73.6%**
Gradient	358	401	415	**12.0%**	**15.9%**
Hypoxic	318	332	343	**4.40%**	**7.86%**
Total	563	960	1,060		

The hyperoxic region saw the most proliferation at approximately 73.56%, followed by the gradient which saw 15.92% increase in cell number. The hypoxic saw the least proliferation at approximately 7.86%.

The entire gradient of the shallow 5mm gradient could not be visualized. The top gradient was imaged. This is the area between the air chamber and the middle chamber (top of the gradient). For the two experiments, the top reference point cell numbers were averaged and can be seen in Table 7.

Table 7: Top Gradient of Shallow 5mm with Averages

	0 hours	24 hours	48 hours
Experiment #1	901	878	906
Experiment #2	221	154	162
Average	561	516	534

The completely hypoxic and hyperoxic regions of a the device were imaged and the cell count was analyzed. This data can be seen in Table 8 below in combination with the top gradient data from Table 7. The cell percent increases were calculated.

Table 8: Shallow Gradient Cell Proliferation

	0 hours	24 hours	48 hours	% Increase After 24 hours	% Increase After 48 hours
Hyperoxic	310	289	330	**-6.77%**	**6.45%**
Top Gradient	561	516	534	**-8.02%**	**-4.81%**
Hypoxic	356	301	319	**-15.4%**	**-10.4%**

As can be seen from the table, there was not much proliferation in any of the shallow region. The only percent increase in cell number was in the hyperoxic region. The numbers stayed relatively consistent, so little to no proliferation occurred.

The data from Tables 3-8 above was normalized. The time point of 0 hours was considered to have a cell count of 100%. The percent increases/decreases seen in the Tables was added/subtracted from 100%. To visualize the increase or decrease in cell number, the data was plotted for each of the three gradients.

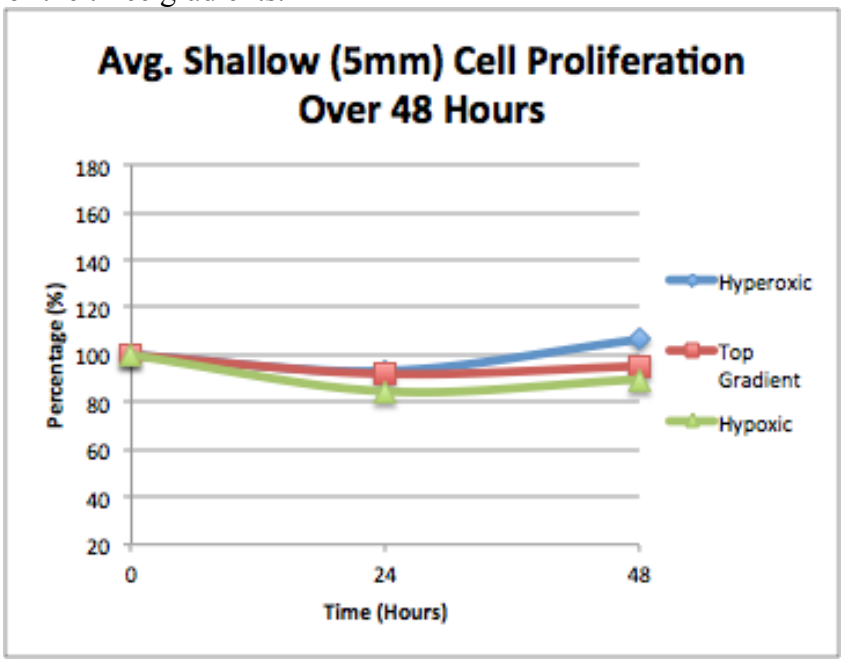


Figure 30: Average Shallow (5mm) Gradient Cell Proliferation Over 48 Hours

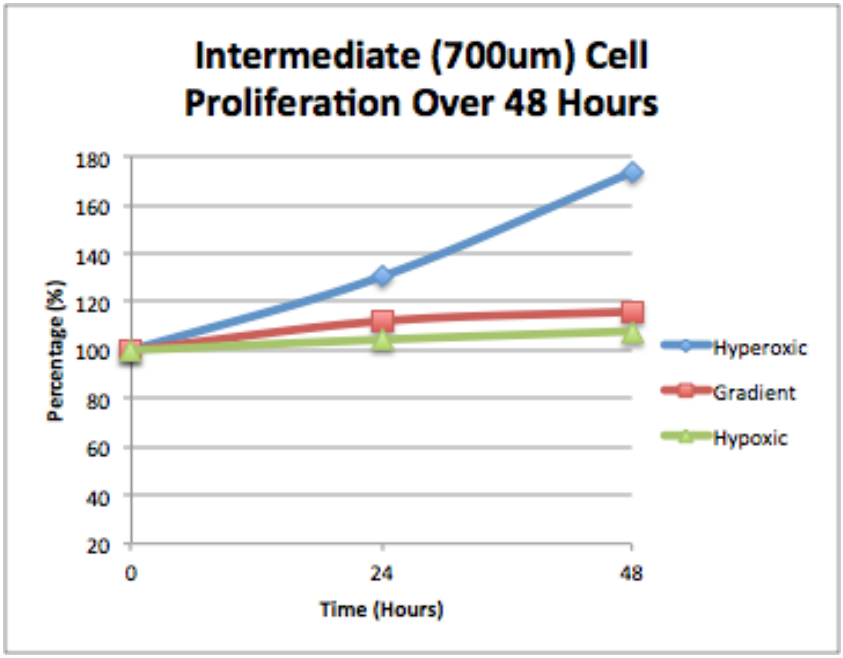


Figure 31: Intermediate (700μm) Gradient Cell Proliferation Over 48 Hours

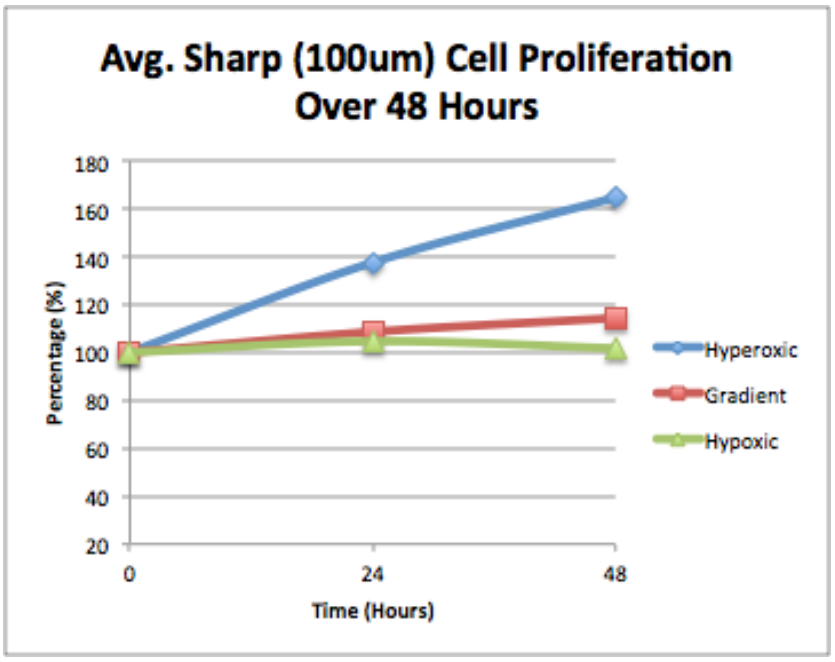


Figure 32: Average Sharp (100μm) Gradient Cell Proliferation Over 48 Hours

For all three gradients, the most proliferation was observed in the hyperoxic regions. Cell count increased by approximately 60% in the sharp and intermediate gradients. There was a moderate amount of proliferation in the gradients for the sharp and intermediate gaps (approximately 20%). The least amount of proliferation was seen in the hypoxic regions.

5.3 Migration

To visualize the migration, images at 0 hours and 48 hours were overlaid and aligned. However, it was deemed more accurate to quantify migration distance by using the individual overlaid image (of the fluorescent image of focused cells and the image focused on the reference point) at 0 hours and at 48 hours, and measuring the distance that the cells migrated. This was done by drawing the line measure tool in FIJI to the same location. This process can be observed within the first two columns in the Figure below (Figure 33). Approximately fifteen random cells were observed in each of the three regions (hyperoxic, gradient, hypoxic) of one device at 0 hours and 48 hours. Images were zoomed in to get a more accurate measurement (Figure 34). The scale was set such that 0.53 pixels was a micrometer. This was found by using the 200 micrometer scale in the image. On the 0 hour image, a line was drawn from the middle of the cell to a reference line. The same was done for the 48 hour image- the same cell was found and a line was drawn to the same reference line. The migration calculations were done for the sharp and intermediate gap devices. It could not be done for the shallow gradient, because the entire gradient wasn't visible within the microscope field of view.

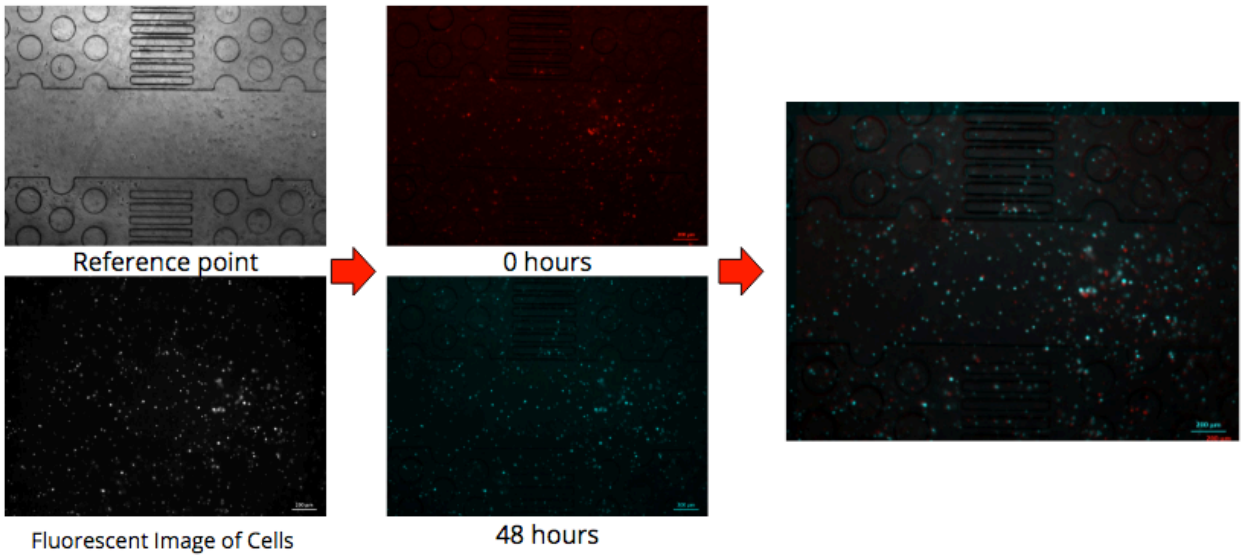


Figure 33: Overlay process. Reference point and focused fluorescent cells are overlaid into an image, such as that seen at 0 hours or 48 hours. These two images are then overlaid and aligned to observe migration. (This method was not used to calculate the migration).

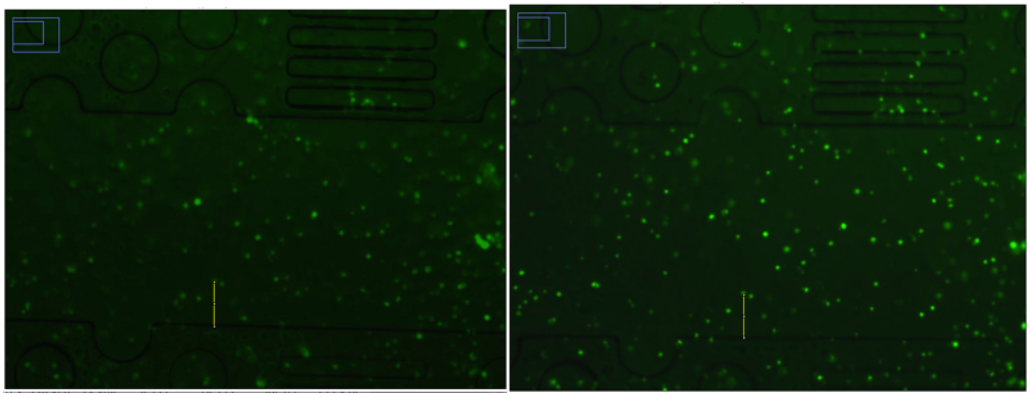


Figure 34: Migration Measurement in FIJI. FIJI analysis using the measure tool to observe how far cells migrated by comparing cell distance to the same location on the reference point.

The migration of approximately fifteen random different cells was measured (Tables 9 and 10). The length of the cell was measured to a reference point (length 0 hours). The same cell was measured to the same reference point (length 48 hours). These measurements are the cell in relation to the reference point. The length moved was found by subtracting these numbers. It was recorded which way the cell moved; up was indicated by a positive number and down was indicated by a negative number. This up and down movement is in referral to the gradient the cell moved toward. A positive number is toward the hyperoxic region and a negative number is toward the hypoxic region (Figure 35).

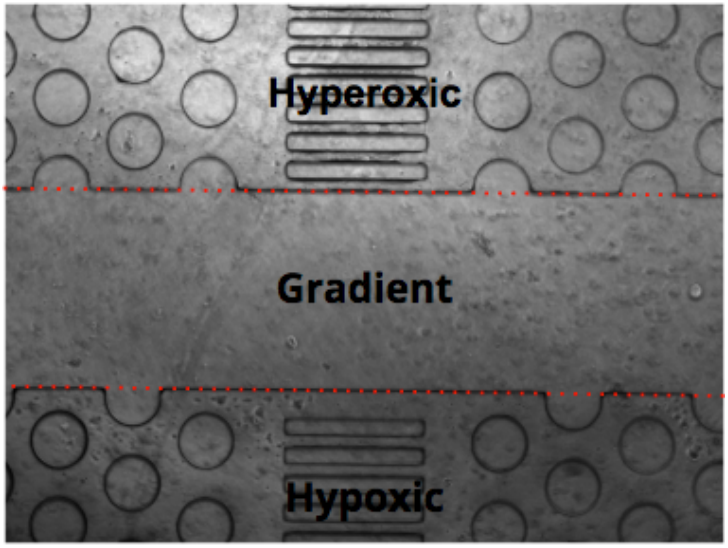


Figure 35: Image to show how positive is up to the hyperoxic environment and negative is down to the hypoxic environment.

5.3.1 Sharp 100µm Gradient Migration

The data gathered on the migration of the cells in the sharp environment can be seen below. The length moved for each region is bolded. A positive number means movement to a higher oxygen gradient, and a negative number means movement to a lower oxygen gradient.

Table 9: Sharp Gradient Migration

Hyperoxic			**Gradient**			**Hypoxic**		
Length 0 hours	Length 48 hours	**Length moved (µm)**	Length 0 hours	Length 48 hours	**Length moved (µm)**	Length 0 hours	Length 48 hours	**Length moved (µm)**
204.6	203.0	**-1.6**	20.12	25.15	**-5.03**	35.21	35.22	**0.01**
39.75	47.79	**8.04**	42.76	37.73	**5.03**	50.37	47.79	**2.58**
129.0	138.3	**9.3**	57.91	52.80	**5.11**	70.43	60.43	**10**
213.3	213.8	**0.5**	27.70	42.76	**15.06**	30.10	27.67	**2.43**
322.6	334.6	**12**	15.09	10.06	**5.03**	196.3	193.7	**2.6**
324.8	334.6	**9.8**	57.91	60.03	**-2.12**	103.1	105.6	**-2.5**
34.80	42.76	**7.96**	72.99	75.51	**-2.52**	334.7	332.0	**2.7**
74.44	80.50	**6.06**	32.70	35.22	**2.52**	337.1	327.1	**10**
47.12	64.26	**17.14**	10.37	7.955	**2.415**	788.5	785.9	**2.6**
704.7	701.9	**-2.8**	65.40	65.50	**0.1**	708.1	704.6	**3.5**
168.5	178.6	**10.1**	75.46	83.01	**7.55**	72.95	65.40	**7.55**

35.30	40.70	**5.4**	62.90	52.89	**10.01**	511.0	510.9	**0.1**
377.3	402.5	**25.2**	95.59	85.53	**10.06**	226.5	223.0	**3.5**
691.8	706.9	**15.1**	37.73	47.79	**10.06**	359.7	359.7	**0**
178.6	186.1	**7.5**				470.4	465.4	**5**
135.8	140.8	**5**				650.2	651.8	**-1.6**
Average		**8.418**			**4.520**			**3.029**

The most migration was observed within the hyperoxic region of the sharp device. Cells moved an average of 8.418 +/- 7.01µm. They moved higher into the hyperoxic region toward higher oxygen levels. The cells within the gradient moved an average of 4.52 +/- 5.72µm. These cells moved toward higher oxygen levels toward the hyperoxic region. A few cells moved down toward the more hypoxic region. The cells in the hypoxic region moved an average of 3.029 +/- 3.67µm. These cells moved the least. The data in the table was created into plots for the three different environments of the sharp gradient. These plots can be seen in Figures 36-38 below.

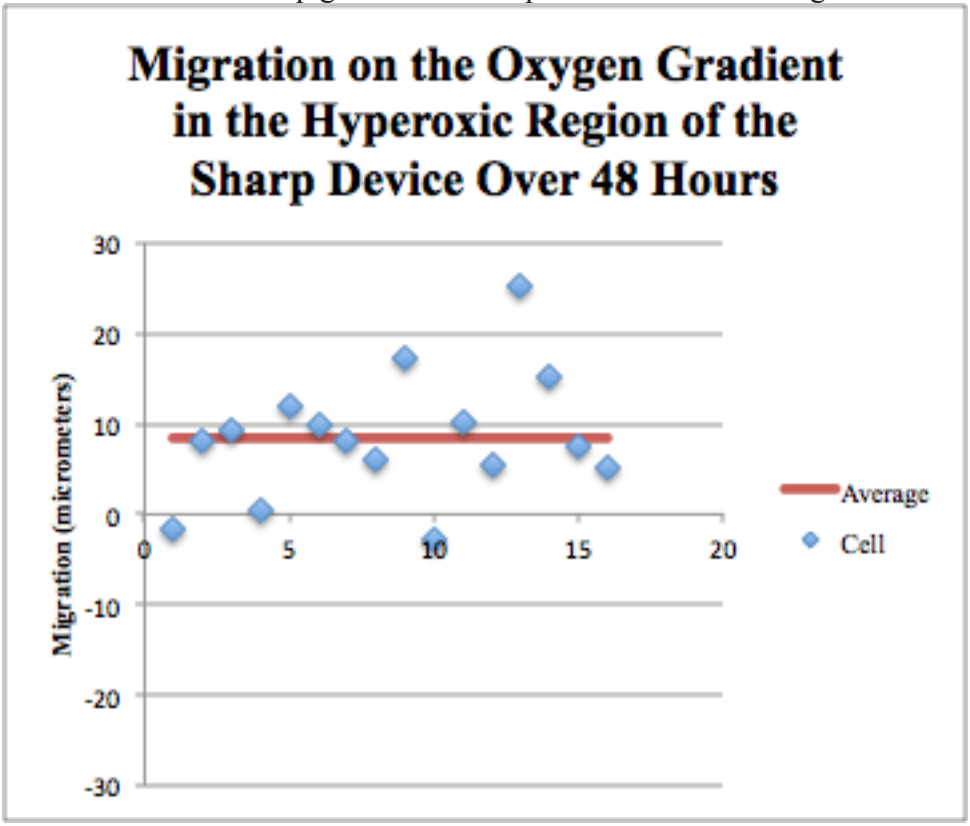


Figure 36: Migration on the Oxygen Gradient in the Hyperoxic Region of the Sharp Device Over 48 Hours. A positive number represents movement into a higher oxygen gradient and a negative number represents movement into a lower oxygen gradient.

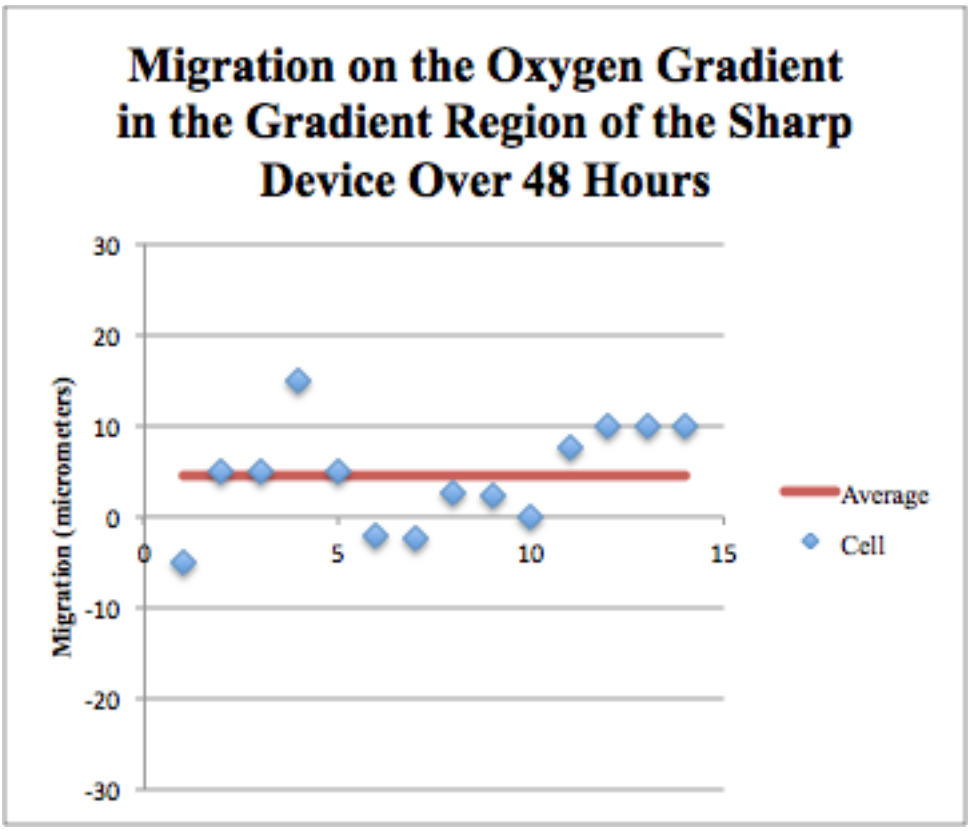


Figure 37: Migration on the Oxygen Gradient in the 100µm Gradient Region of the Sharp Device Over 48 Hours. A positive number represents movement into a higher oxygen gradient and a negative number represents movement into a lower oxygen gradient.

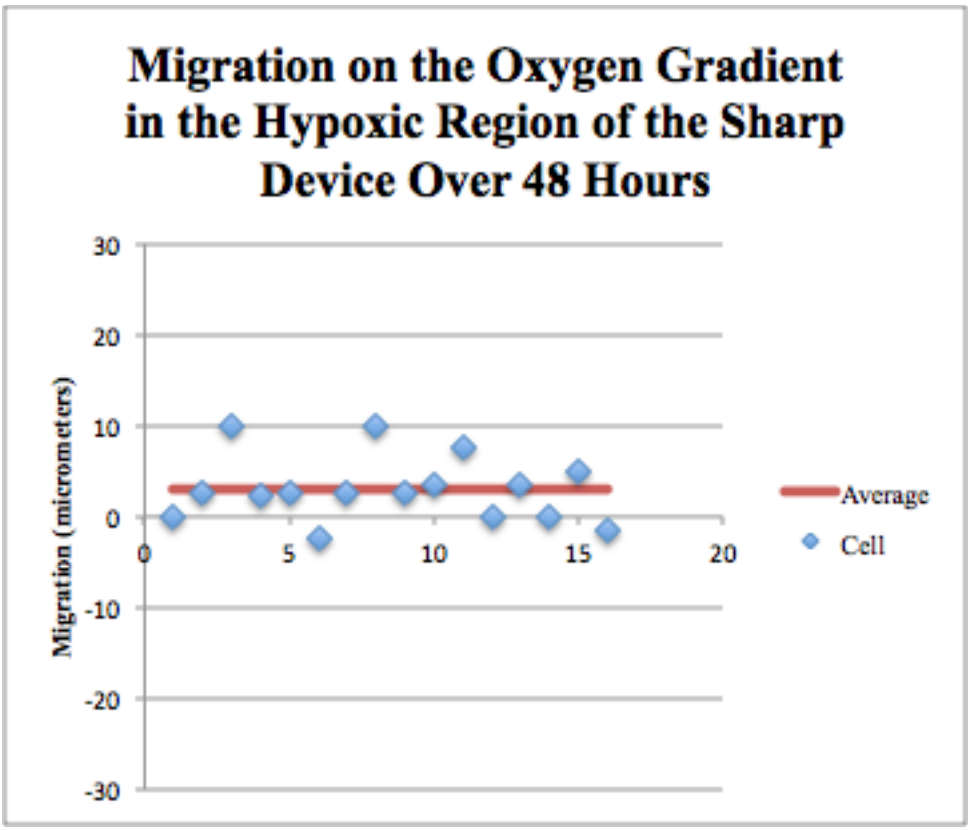


Figure 38: Migration on the Oxygen Gradient in the Hypoxic Region of the Sharp Device Over 48 Hours. A positive number represents movement into a higher oxygen gradient and a negative number represents movement into a lower oxygen gradient.

To make the information of each region clearer than the individual plots in Figures 36-38, the plot in Figure 39 below shows the average migration on the oxygen gradient. The migration in the hyperoxic, gradient, and hypoxic regions can be seen.

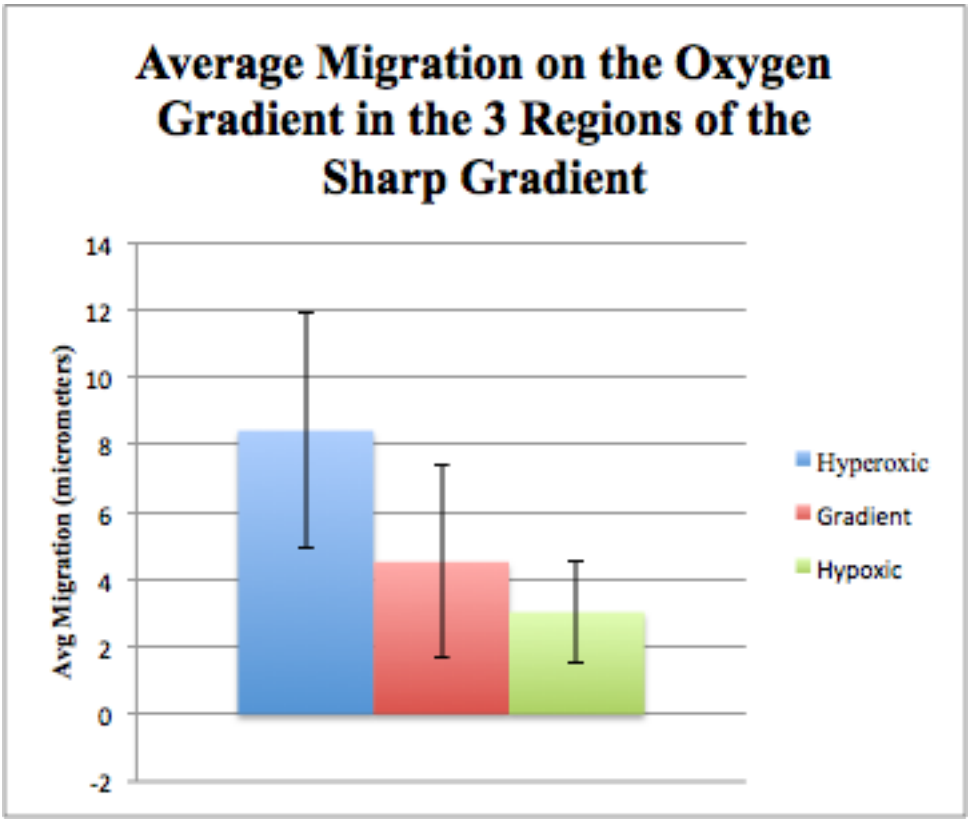


Figure 39: Bar Graph of the Average Migration on the Oxygen Gradient in the three different regions of the Sharp Device Over 48 Hours.

As can be seen in Figure 39, the average migration in all three regions was positive. This means that the cells moved toward more oxygen. The cells within the hyperoxic region moved most; these cells moved to a higher region of oxygen (more hyperoxic). The standard deviation is also plotted within the graph.

5.3.2 Intermediate 700µm Gradient Migration

The data gathered on the migration of the cells in the intermediate environment can be seen below. Again, a positive number means movement to a higher oxygen gradient, and a negative number means movement to a lower oxygen gradient.

Table 10: Intermediate Gradient Migration

Hyperoxic			**Gradient**			**Hypoxic**		
Length 0 hours	Length 48 hours	**Length moved (µm)**	Length 0 hours	Length 48 hours	**Length moved (µm)**	Length 0 hours	Length 48 hours	**Length moved (µm)**
52.78	55.35	**2.57**	77.9	72.93	**4.97**	133.3	138	**4.7**
274	271.5	**-2.5**	83.1	57.8	**25.3**	243.8	246.3	**2.5**
394.6	394.7	**0.1**	85.49	85.45	**0.04**	434.9	439.9	**5**
85.5	93	**7.5**	334.36	339.3	**4.94**	15.1	17.6	**2.5**

63.8	67.86	**4.06**	155.8	145.8	**10**	143.8	145.8	**2**
241.5	248.9	**7.4**	173.4	171	**2.4**	37.7	40.2	**2.5**
115.6	120.6	**5**	88	83	**-5**	62.9	60.3	**-2.6**
135.7	148.3	**12.6**	110.6	105.6	**-5**	334.3	336.8	**2.5**
243.8	238.8	**-5**	148.3	145.7	**-2.6**	346.9	349.4	**2.5**
248.9	246.4	**-2.5**	27.6	50.3	**22.7**	374.6	367.2	**-7.4**
261.6	268.9	**7.3**	55.5	55.8	**0.3**	155.8	163.4	**10.54**
326.8	321.7	**-5.1**	173.4	173.4	**0**	168.5	171	**2.5**
329.3	329.2	**-0.1**	372.1	374.5	**2.4**	513.1	513	**-0.1**
515.3	521.8	**6.5**	384.5	377.1	**-7.4**	384.9	392.3	**7.4**
550.5	543	**-7.5**	90.5	90.5	**0**	377.3	379.5	**2.2**
52.78	55.35	**2.57**	77.9	72.93	**4.97**	133.3	138	**4.7**
Average		**2.022**			**3.537**			**2.449**

For this device, the most migration was observed within the 700μm gradient region. Cells moved an average of 3.537 +/- 9.41μm. They moved higher into the hyperoxic region toward higher oxygen levels, but some moved downwards. A couple cells moved especially far (approximately 20+μm). The cells within the hyperoxic moved an average of 2.022 +/- 5.81μm. A few cells moved down toward the more hypoxic region. The cells in the hypoxic region moved an average of 2.50 +/- 4.06μm. None of the cells in any of the environments moved too much on average. The most migration was seen in the gradient. The data in the table was created into plots for the three different environments of the intermediate gradient. These plots can be seen in Figures 40-42 below.

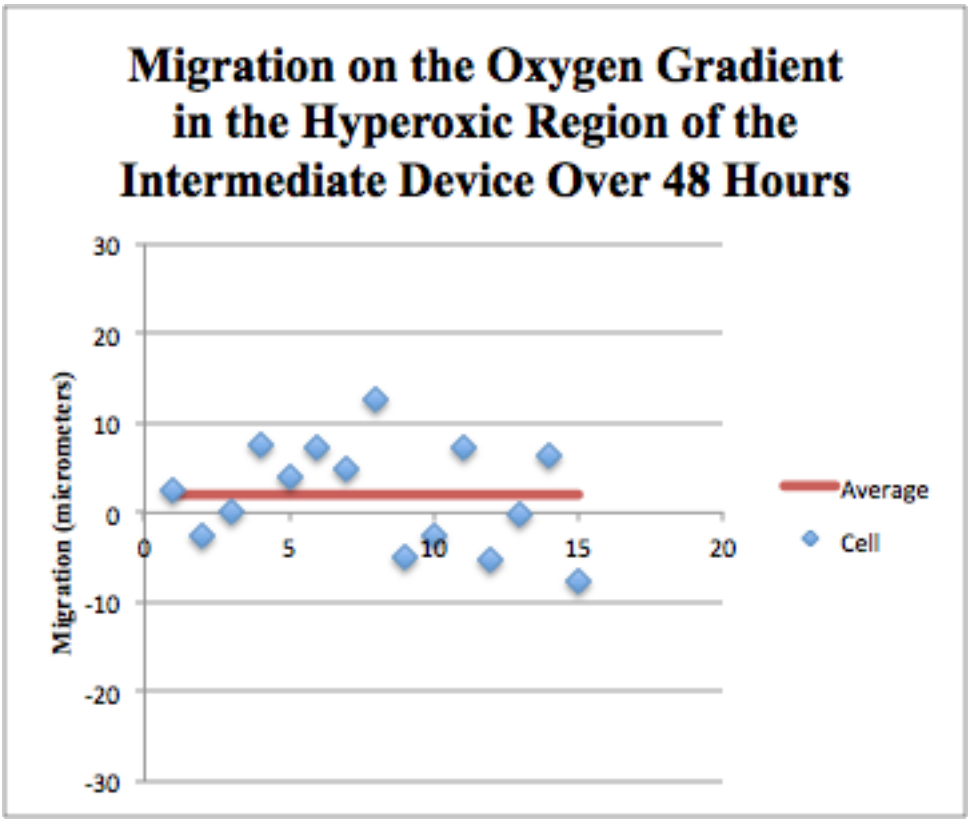


Figure 40: Migration on the Oxygen Gradient in the Hyperoxic Region of the Intermediate Device Over 48 Hours. A positive number represents movement into a higher oxygen gradient and a negative number represents movement into a lower oxygen gradient.

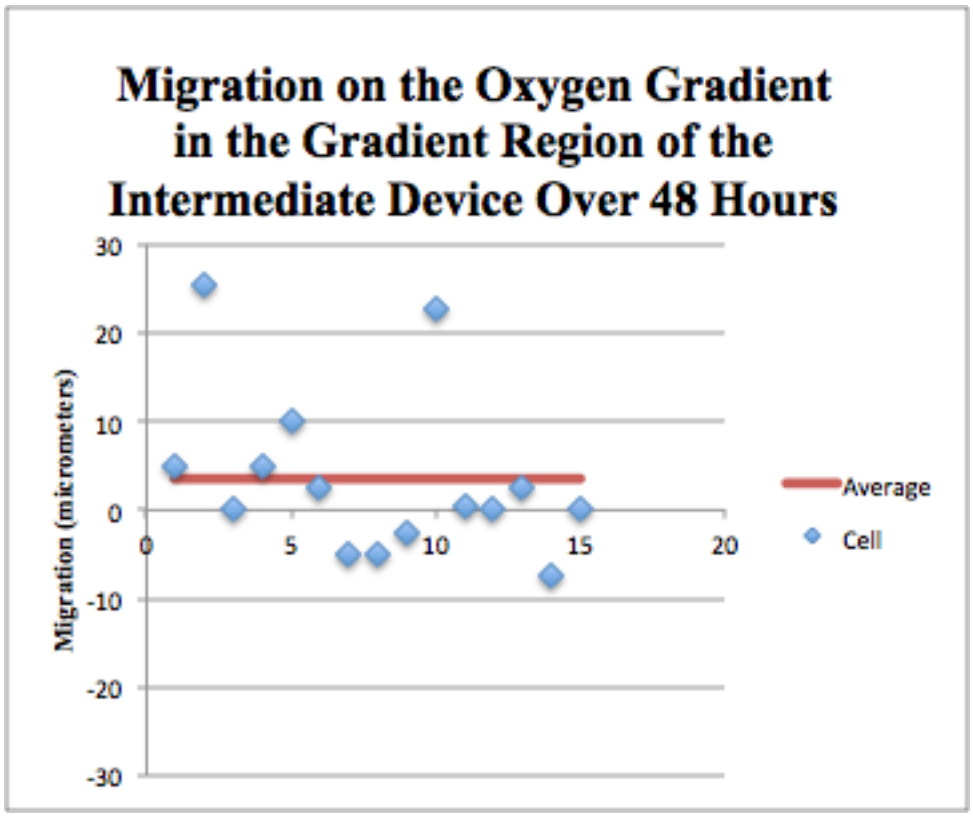


Figure 41: Migration on the Oxygen Gradient in the Gradient Region of the Intermediate Device Over 48 Hours. A positive number represents movement into a higher oxygen gradient and a negative number represents movement into a lower oxygen gradient.

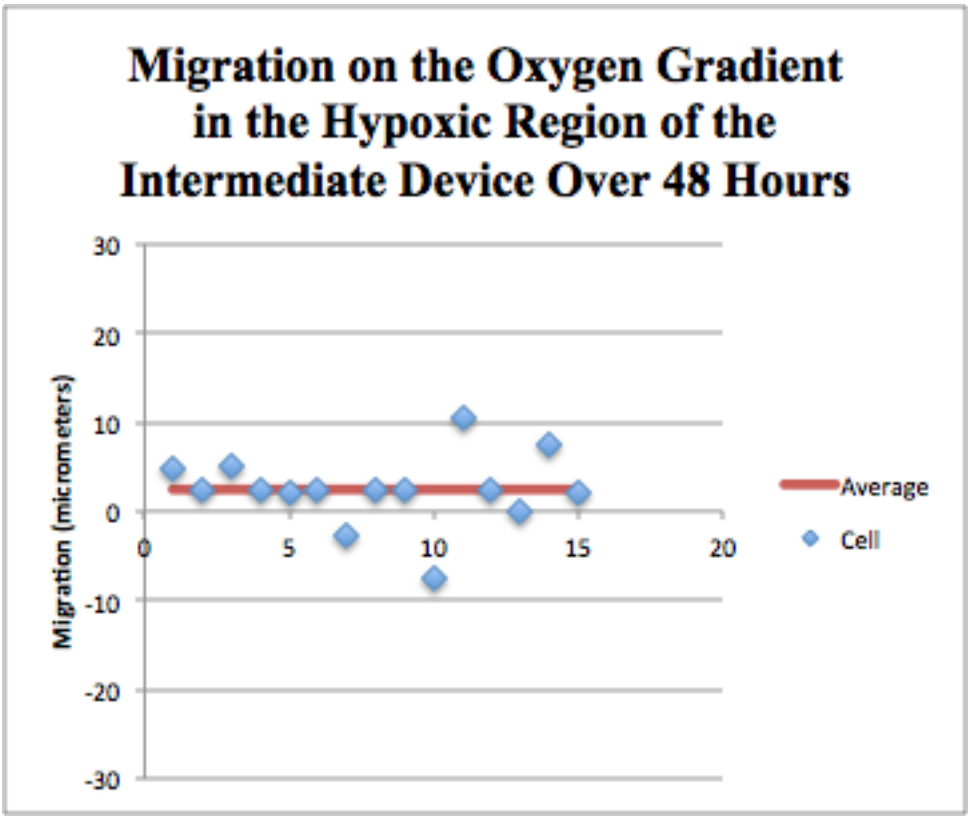


Figure 42: Migration on the Oxygen Gradient in the Hypoxic Region of the Intermediate Device Over 48 Hours. A positive number represents movement into a higher oxygen gradient and a negative number represents movement into a lower oxygen gradient.

Again, to make the information of each region clearer than the individual plots in Figures 40-42, the plot in Figure 43 below shows the average migration on the oxygen gradient. The migration in the hyperoxic, gradient, and hypoxic regions can be seen along with the standard deviation error bar.

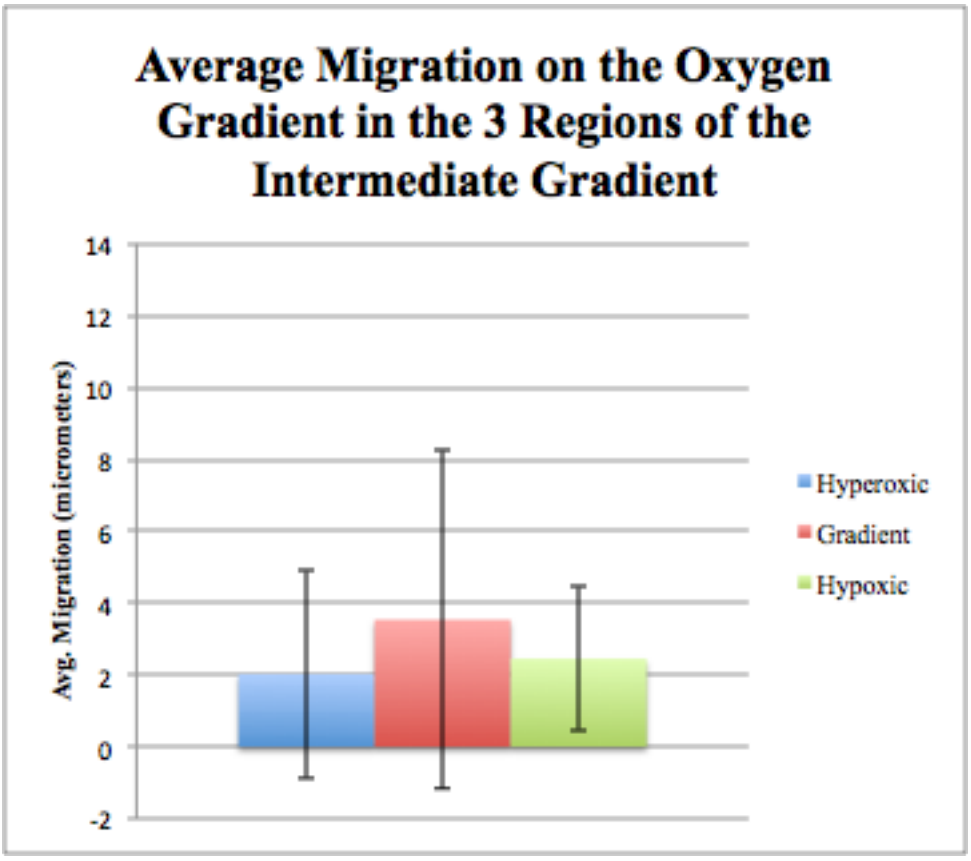


Figure 43: Bar Graph of the Average Migration on the Oxygen Gradient in the three different regions of the Intermediate Device Over 48 Hours.

As can be seen in Figure 43 above, the average migration in all three regions was positive. This means that the cells on average moved toward more oxygen. The cells within the gradient region moved most; these cells moved to a higher region of oxygen. The standard deviation is also plotted within the graph. The standard deviation is particularly high because as can be seen in Table 10, the cells did not move that much on average. Additionally, some cells moved up and some moved down. The movement was much more randomized than was seen in the 100μm gradient. On average, the cells tended to move upwards two to three micrometers.

5.3.3 Shallow 5mm Gradient Migration

Unfortunately, there is no table or plots of migration within the shallow gradient since it could not be observed within the field of view of the microscope. Upon inspection of the cells in the hyperoxic and hypoxic gradient there was little to no movement. Within the gradient it was unclear if there was migration, proliferation, death, or all three.

5.3.4 Overlaid Images of Cells Within Three Different Gradients

Overlaid images of 0 hours and 48 hours can be seen for each of the three gradients. These images were aligned using FIJI.

Figure 44 shows the overlay done for the sharp 100 μm gradient. The yellow cells are from 0 hours and the blue ones are from 48 hours. The large amount of proliferation is visible. The image is not completely aligned; the rotation is slightly off. If it could have been rotated, the migration would have been more obvious.

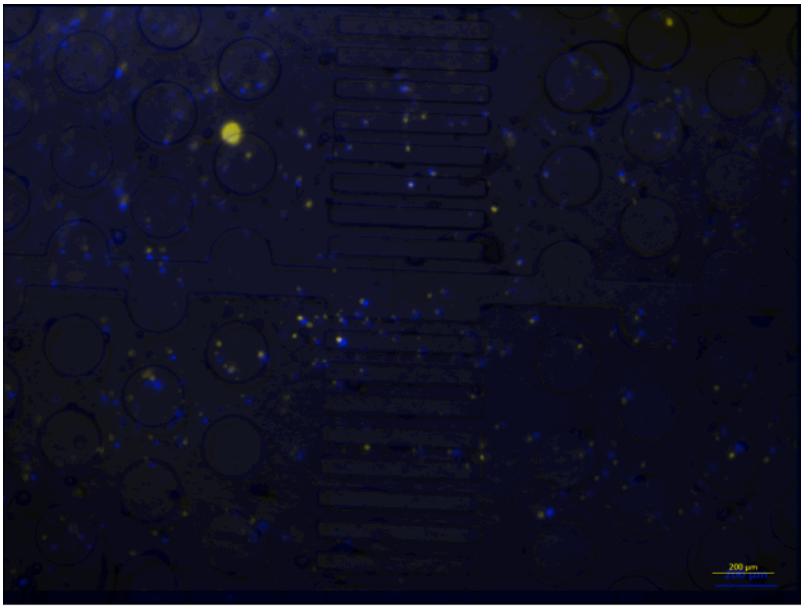


Figure 44: Sharp 100 μm gradient Overlay of 0 and 48 Hours

Figure 45 shows the overlay done for the intermediate 700 μm gradient. The red cells are from 0 hours and the blue ones are from 48 hours. The large amount of proliferation is visible within this device as well. The blue cells tend to move upwards toward the hyperoxic region. (Will fix this one too).

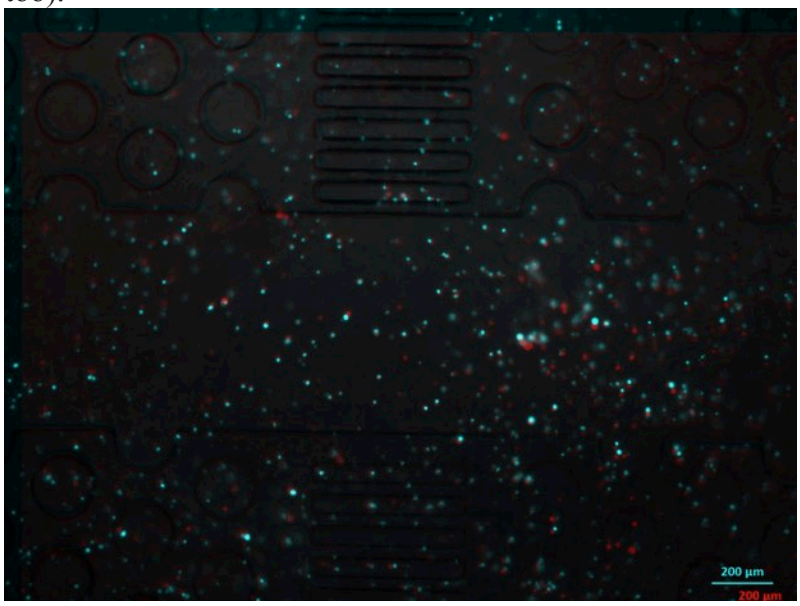


Figure 45: Intermediate 700 μm gradient Overlay of 0 and 48 Hours

Figure 46 shows the overlay done for the shallow 5mm gradient. This is located at the bottom gradient (nitrogen is in the chamber directly below).The red cells are from 0 hours and the green ones are from 48 hours. There are some red cells which shows no movement, but there are also a lot of red and green cells. It is unclear if these cell migrated, because only part of the gradient is being observed and few cells seem to correlate. The ones that do appear as if they moved up. This would go along with the rest of the conclusions- that the cells will move to a higher level of oxygen.

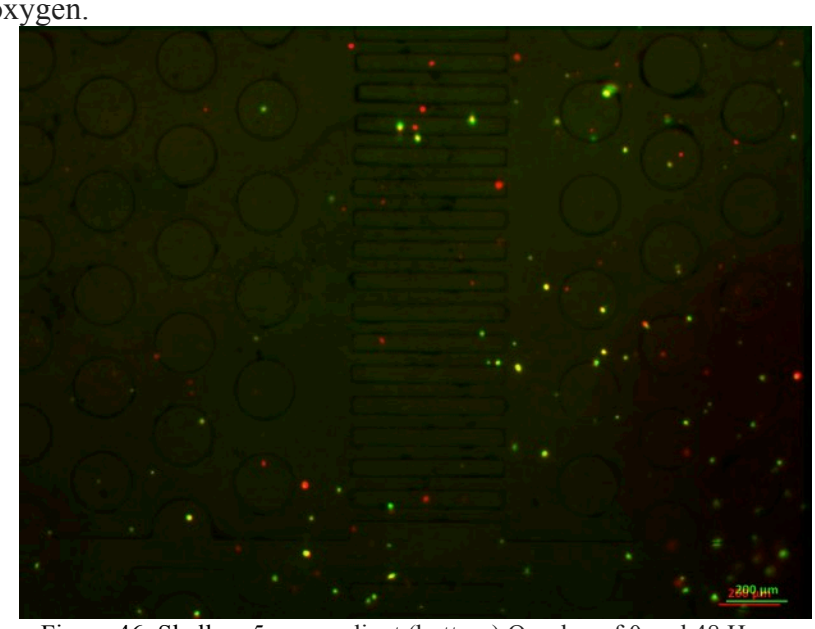


Figure 46: Shallow 5mm gradient (bottom) Overlay of 0 and 48 Hours

The migration and proliferation at the inlets and outlets was also observed. It was found that imaging at these locations had little to no effect on the migration or proliferation; it generally stayed consistent to what was found for each gradient.

6. Final Design & Validation

The final design aspects including its fabrication, components and validation are included in this chapter.

6.1 Fabrication of Devices

A silicon master was created by the microfabrication lab at WPI using the design created on DraftSight. The master was made by photolithography. It can be seen in the Figure 47 below. To create the devices, 100g of silicone base and 10g of curing agent were measured out and then mixed properly in a large weigh boat. The solution was degassed in a vacuum. After fully degassing the PDMS, it was poured onto the silicone master that was contained in a large 140mm X 20mm petri dish. The PDMS was carefully poured in a back and forth motion to minimize the number of bubbles within the master. Then a plastic pipette was used to remove any dust and bubbles that would reside within the device. The dish was then placed in the incubator at 60°C overnight. The silicone master was removed from the oven the following day. To remove the devices from the cured master, a scalpel was used. When cutting the devices out, a circular shape was made and cut in such a way as to ensure that the master was not cut out with the devices. After the devices were removed, they were individually cut out. The next time devices were made, they were made in the same way, except only 50g of silicone base and 5g of curing agent was mixed properly and poured onto the master once degassed.

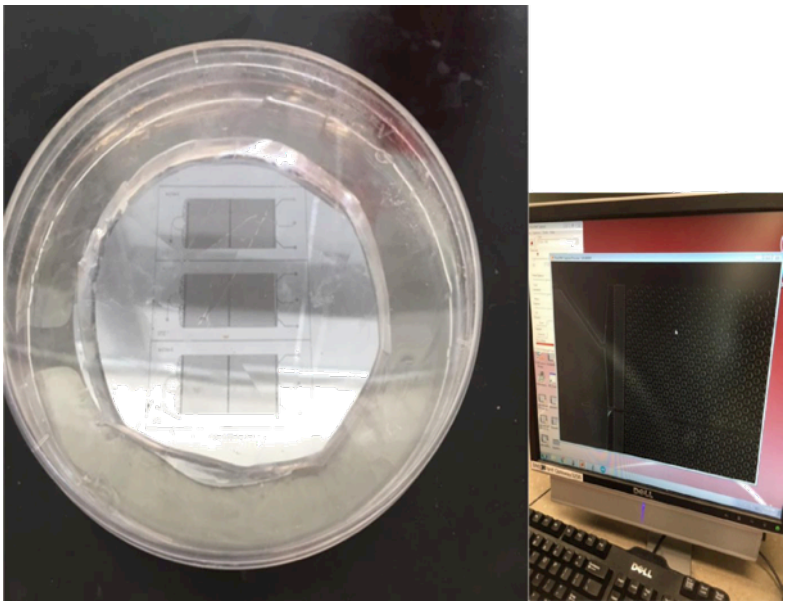


Figure 47: Silicone Master and part of the DraftSight model

Before plasma bonding, the microfluidic device and membrane were cleaned thoroughly with ethanol. Tape was used on the device, slide, and membrane to remove any dust. The first components that were plasma bonded were the devices to the slides. The slide was facing up and the system was facing down. The slide was flipped onto the bottom of the device and was plasma bonded. Next, the device was placed with the system facing up and the membrane next to it on

the tray slide. A drop of ethanol was placed between the membrane and the tray slide, to ensure easy removal from the tray slide.

6.1.1 Fabrication of a 50 μm membrane using PDMS

A membrane will be made out of PDMS which will allow the oxygen of the system to permeate the hydrogel and cells.

In order to make the membrane, the team started by placing a small weigh boat on the balance and measured 5g of silicone base. Then 0.5g of the silicone curing agent was measured. By using a Popsicle stick, the base and curing agent was mixed thoroughly for at least two minutes. Bubbles formed at this point. Once mixed thoroughly, the weight boat was placed inside a vacuum chamber and turned on. Bubbles were observed rising to the surface of the mixture. The spigot was used to release the vacuum slowly to pop any bubbles (some bubbles were blown lightly to pop). This process took about fifteen to twenty-five minutes. A strip of 0.25mm scotch tape was added to the length side of the fluorinated slide, size 75mm x 50mm. The fluorinated slides were placed on aluminum foil, and the fluorinated side was made sure to be facing up. Once there were no bubbles in the mixture the PDMS mixture was then poured onto the slide, slowly in a side-to-side motion to avoid forming bubbles. Once the entire slide was covered in PDMS, the other fluorinated slide was placed on top, which covered the PDMS mixture between both slides. It was important to make sure that the fluorinated slide was facing down. The slides were pressed down on until the PDMS mixture spread evenly between the slides. Used binder clips to keep a tight seal between the slides. Next, the slides were cleaned on the sides of the slides from the extra PDMS mixture and the slides were then placed onto a clean aluminum foil sheet. The aluminum foil was wrapped around the slides and placed in the oven at 60°C which they were left overnight.

Removing 50μm PDMS membrane from the fluorinated slides

The PDMS mixture within the fluorinated slides was taken out of the oven after being left overnight. The aluminum foil was opened up and a straight edge razor blade was used to cut in the corner and gently lift the slide up to avoid damaging the membrane. This was done to each corner to easily remove the top slide without damaging the membrane. Once the top slide had been removed, the PDMS membrane on the bottom slide was cut along the tape edge using the straight edge razor blade; this helped remove the PDMS membrane from the slide. Once the membrane was removed from the slide, it was placed in a safe location to avoid damaging it. The membrane was then measured and kept within a taped petri dish.

6.1.2 Fabrication of Wells

The wells were fabricated similarly to the membranes. A large weight boat was used to mix 70g of silicone base with 7g of silicone curing agent. These were stirred together with a popsicle stick and degassed in a vacuum chamber. After there were no more bubbles, the weigh boat was placed into the oven at 60°C overnight. The next day rectangular squares roughly the same size as the device were cut out. From there, the correct measurements to expose the system were drawn onto the rectangle and cut out using a straight edge razor. The height of the well was then measured.

6.2 Device Sterility

The device was sterilized by rinsing it with ethanol. Dust was removed by wiping it with a kim-wipe and then blowing air onto it. The device, which is plasma bonded to the slide and membrane, a well, and a cover slide of glass was placed in ultraviolet light for approximately two hours before seeding cells and hydrogel.

6.3 Cells Seeded

The number of cells to seed is 200,000 cells. If a plate has been culturing for 5 days, the approximate volume would be 75uL after passaging. This number of cells was selected because it allows room for proliferation and migration to be observed under the microscope. Below is an image that compares 200,000 cells to 2 million.

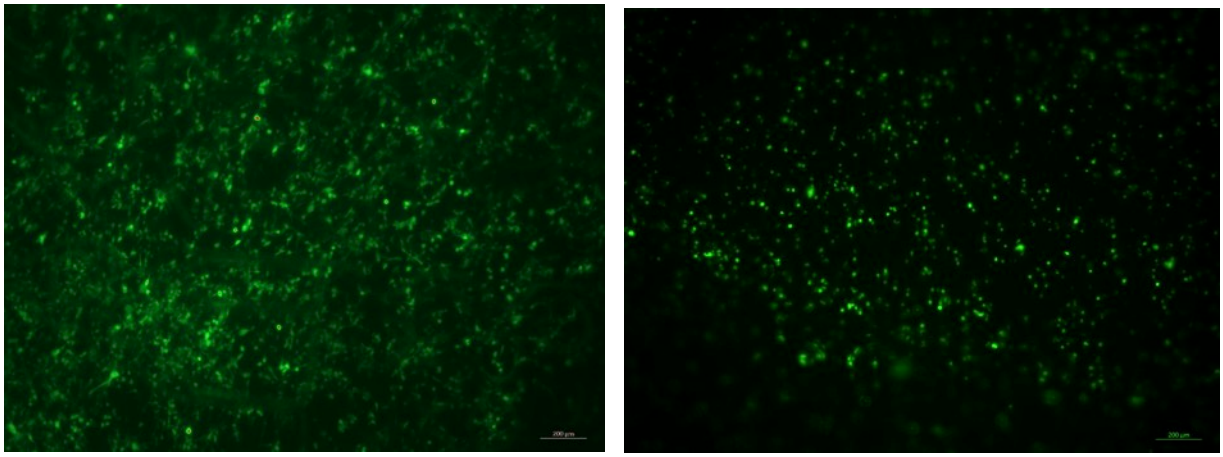


Figure 48: 2 Million Cells Seeded in PureCol EZ (left) versus 200,000 Cells Seeded in PureCol EZ

6.4 Gas Gradient Validation

During the gas test an OceanOptics Ruthenium- coated slide was used to observe the change in oxygen levels. As shown on the image (Figure 24) the brighter area is the decrease in oxygen levels caused by the carbon dioxide being blown onto the slide.

To observe an oxygen gradient in the device, an image was taken before the gases were turned on and another image was taken when the gases were flowing after the gradient was stabilized. The top chamber inlet was compressed air and the middle chamber was nitrogen/carbon dioxide to observe a gas gradient through the device.

6.5 Cell Viability in Chosen Hydrogel

PureCol EZ Bovine Collagen Solution was chosen as the hydrogel to use because preliminary experiments concluded that MDA-MB-231 can proliferate and migrate in the hydrogel. Cells were observed under the blue light of the fluorescent microscope. The GFP of the genome fluoresces under this light. Cells remained viable as the GFP was present and observable (Figure 12).

6.6 Device, Cells, & Hydrogel

200,000 MDA-MB-231 cells were seeded in 1mL of PureCol Ez hydrogel. After the hydrogel solidified, 1mL of media was placed inside the well. The device with all components was placed in the incubator. This included the device, well, hydrogel, cells, media, and glass cover slide. Compressed air was flown through the top chamber and nitrogen gas was flown through the middle chamber or bottom chamber depending on the gradient being produced. Three different gradient experiments were conducted. The experiment ran for 48 hours and media was replenished if necessary. The cells were imaged at 0 hours, 24 hours and 48 hours. Using FIJI, the images were compared to observed migration and proliferation.

6.7 Imaging

The device and cells were imaged using the ZEN program with the Zeiss Axiovert 40 CFL Fluorescent microscope. The FIJI image analysis program was used to analyze the proliferation and migration images, which were saved as ome.tiff.

Proliferation images were analyzed for each experiment at 0, 24, and 48 hours. It was further broken down by the region (hyperoxic, gradient, hypoxic). The fluorescent image was added to FIJI and the background was subtracted through the threshold adjustment. Watershed was added to break up the clumped cells. The particles were then analyzed.

Migration images were analyzed by combining the image of the reference point and the focused fluorescent cells. Initial images from 0 hours were compared to the 48 hour images. Fifteen cells within each region (hyperoxic, gradient, hypoxic) were measured; this measurement went from the middle of the cell to a reference point for both timepoints. The direction of movement was noted and the length difference was calculated. To overlay these images the “align RGB stack” plug in was installed. This allowed the images to be aligned.

6.8 Economics

The team received the MDA-MB-231/GFP/Blasticidin cells from Professor Ambady as a gift. The team was gifted a new master mold with the alternative designs from the microfabrication lab and BME595T course at Worcester Polytechnic Institute. The team purchased a compressed air tank, a nitrogen gas tank and carbon dioxide gas tank at a total cost of $111.00. The team started with a budget of $500 and after purchases ($311) had a final budget of $189 remaining.

The microfluidic device would help in reducing the cost of each experiment. The cost to fabricate one device with its components is $22. The capability to reuse each device helps in reducing the cost of buying multiple devices for experiments. One silicone master can be reused to make multiple devices. The suggested amount of cells, hydrogel and media for each experiment is lower compared to other methods of 3D culturing at a total of $40. Providing a low scaled 3D environment with similar oxygen levels as the body can aid in cell biology and drug treatments. This capability will affect the market by introducing more drug treatments if the device is successful in the research.

6.9 Environmental Impact

The microfluidic device can be reused after it is proper cleaned. This helps decrease the amount of culturing plates thrown away after one use. The device is capable of culturing cells for 48 hours. The cells, hydrogel and media will be disposed of in the biohazard waste at the end of each experiment.

6.10 Societal Influence

The microfluidic device could be helpful in a research environment that requires oxygen gradients that closely mimic *in vivo* environment to further advance cell biology and drug discovery. With further experiments, the microfluidic device can be an important tool for this field of research. As of right now, there are improvements and validations necessary to provide the best outcome. If this device were to be useful in furthering cancer cell biology, alternative treatments for cancer could be discovered. This would greatly affect the population and increase life expectancy. People, their families, insurance companies, doctors, cancer research facilities, etc. will all be impacted by finding a cure for cancer.

6.11 Political Ramifications

The microfluidic device was designed to be used in a research environment that requires an oxygen gradient that closely mimic *in vivo* for cell biology and drug discovery. With this in mind, it would not have an impact to the global economy since the market for this type of research is small. If a new drug discovery is successful in using the device, it will provide a new addition to the market in that treatment field. The political ramifications are likely to be minimal.

6.12 Ethical Concerns

The microfluidic device was designed to closely mimic *in vivo* environment and will help reduce the usage of mice or other animals in testing. The device will provide an alternative method to observe cell biology and drug discovery as long as the cells can be cultured and produced in the design. Ethically speaking, several people may find cures for cancer to be unnatural. If cancer cell biology is furthered and a cure is discovered, there will be less deaths due to cancer. This would cause a population rise. Cancer may be seen to many as a means of controlling the population.

6.13 Health & Safety Issues

The design is made of PDMS which if handled properly will not be toxic to any of the people involved in the fabrication of each device. Cells, hydrogel, and media will have to be handled properly to prevent any contamination in each experiment. The microfluidic device also needs to be properly cleaned and sterilized before beginning each experiment to prevent any contamination from occurring.

6.14 Manufacturability

The only materials needed to manufacture the devices are a silicone master in a 120mm petri dish, a 184 Sylgard silicone kit, a vacuum chamber, and a oven. These can all be bought at once and can be reused except for the 184 Sylgard silicone kit, which would need to be bought as it is used.

6.15 Sustainability

The microfluidic device is made from a silicone master that can be reused to produce many devices. After each experiment, the device can be cleaned properly to be reused by following the cleaning and sterilization protocol. As long as the protocol is followed carefully, the devices should last for 20 to 25 experiments before seeing some deterioration on the membrane. This will benefit the personnel involved by allowing multiple experiments before needing to fabricate more devices from the silicone master.

6.16 Final Design Discussion

In the final design, proliferation and migration were observed within the different oxygen gradients. While three gradients were tested, the best design was the 100 micrometer sharp gradient. The most proliferation occurred within the hyperoxic region of this gradient and migration in the hyperoxic region was furthest, but there was obvious migration within the gradient and hypoxic regions as well. Although the cells could migrate on the Z-plane of the hydrogel making it difficult to focus on the same cells, the proliferation percentage and migration average in each region of the device were calculated. To avoid this complication, it is a possibility to stain the cells that were first seeded. This will allow for easier tracking when focusing under the microscope and observe if the cells are in the same plane. Once the initial cells are seeded, using a different stain on the hydrogel would help track of any proliferation. New proliferated cells should express the new stain it was exposed to and observed under the microscope. These are a few improvements to better validate proliferation and migration was observed in the oxygen gradient.

7. Discussion

This chapter discusses the results of the gas tests and cell response in the three different gradients.

7.1 Device

The objectives of Chapter 3 were accomplished throughout the year. The team tested three different oxygen gradients: a shallow, an intermediate, and a sharp gradient. A new microfluidic device was designed and created to produce the 700 micrometer gradient. It was believed that this gradient would produce the best results; however, the sharp gradient was deemed the best as the most migration and proliferation occurred. The hydrogel used was able to simulate a 3D environment effectively.

Different gases were able to flow through the microfluidic device. The nitrogen gas produced a hypoxic environment and the compressed air produced a hyperoxic environment. Between these two chambers, a gradient was produced. The oxygen gradients could be observed and quantified based on the amount of fluorescence that was observed under the microscope. It would have been nice to have more accurate quantifications, but the fluorescent slide worked well. The other devices to measure oxygen were too expensive and out of budget.

The microfluidic devices allowed simultaneous observation of the cancer cells in the hypoxic, gradient, and hyperoxic environments. This allowed the team to directly test the viability, proliferation, and migration within one device.

The fabrication of one device cost about $22, which means it was cost efficient. This includes the PDMS device, PDMS well, PDMS membrane, hole puncher, and glass slide. Once fabricated all of these components are reusable. This does not include the cost of the master. To run an experiment would cost approximately $47. This includes the device components, the cells, PureCol EZ hydrogel, Complete Media, and the pipette, and gas components such as the tubing, needles, pins, and luer locks. These prices do not take the gas tank costs or the costs to run the incubator into account. Additionally, a fluorescent slide and microscope are needed to analyze the cells, but again, these are not included. For more information on the prices of the experiments see Appendix D.

It was predicted that the cancer cells would thrive in a high oxygen environment and would proliferate the best under these conditions. From the experiments it was determined that the MDA-MB-231 breast cancer cells grow best in hyperoxic (high oxygen) environments and will migrate to this higher level of oxygen when in a sharp enough gradient.

7.2 Gas Test on the Fluorescent Slide & Within the Device

The gas test on the fluorescent slide produced results as expected. The carbon dioxide flow can be clearly seen and the shape of the fluorescence was also expected.

While it took many hours and iterations to capture relevant, useful images of the gas flow within the device, the images in Figure 26 perfectly represent the gradient produced for the 100 and 700 micrometer gradients. The thickness of the membrane affected how long it took for the gradient to be established. As membrane thickness increased, time to establish the gradient increased. The device was pressed directly onto the glass fluorescent slide, which could be a reason why the diffusion time was longer. Instead of diffusing through both the membrane and

through the thin chamber gaps, the slide made it more difficult to diffuse through the membrane, since it could only reach the slide. It would have been helpful to create a gas test simulation of the gradient through the hydrogel. This could have been tested with water, however, it was too difficult to make a really thin well that effectively held the water within the device. This could have been done if the microscope did not observe the device from the bottom-up; using an inverted microscope would have been much easier for this task. It would have been helpful to confirm that the 5mm gradient was produced.

The precise oxygen sensors were outside of the team's budget. These would have been useful to further confirm that the gradients seen in the COMSOL models and gas tests within the device were being produced. This would have quantified the oxygen gradients. Overall, the oxygen gradients were successfully produced in the device.

7.3 Proliferation, Migration, and Viability

After testing across a shallow 5mm gradient and a sharp 100μm gradient, COMSOL modeling and research led the team to create an intermediate 700μm gradient. It was predicted that the cells would have a more gradual gradient to respond to than in the 100μm, but sharp enough for the cells to acknowledge a sense of the gradient as opposed to in the 5mm gradient. Within the trials run, it appeared that the sharp gradient produced the most migration and proliferation of the three gradients. The cells within the hyperoxic region of the sharp gradient migrated approximately 8.4μm toward an even higher oxygen level on average. Some cells were able to migrate close to 25μm. It is possible that the cells were able to easily signal the sharp change in oxygen concentration. The intermediate gradient had migration, but it was not as much as was seen in the sharp gradient. This could be because the gradient was too wide for the cells to really get a sense of the change in oxygen concentration. If that was the case for the intermediate, then it further concludes why we did not observe clear migration in the shallow gradient. The cells within the intermediate gradient tended to have more randomized movement (as the standard deviation bars are quite large). From the results it appears that the cells moved to a higher level of oxygen on average. Some cells within the gradient moved to a more normoxic region of the device. This could have been because these oxygen levels closely resemble what is seen in the body, or the cells may not have been able to effectively sense and respond to the gradient. This would cause more randomized, natural movement.

Research shows that cells will migrate around four micrometers in twelve hours in a 2D gradient (Chang et al, 2014). Cells moved linearly through the gradient, but it was also observed that they moved up and down within the hydrogel. This could not be measured. Also, some cells showed side-to-side movement within the gradient. These values were not calculated since there was no movement to a different gradient. Since the cells were observed under the 5X magnification, this small amount of movement is difficult to clearly see. When calculating the migration lengths using FIJI, the scales were set to the same and extreme caution was taken in making sure the line went from the middle of the cell to the reference point of choice. With this being said, there still could have been some human error.

The most proliferation was also seen within the hyperoxic region of the sharp device (experiment #1) with an increase of approximately 167%. The population more than doubled. Extreme care was taken to make sure that the images taken at each time point closely resembled the image taken at the previous timepoint. Close care was taken in ensuring that the images were straight and even (same amount of hyperoxic chamber visible as hypoxic chamber). In

experiment #1's case (or in any of the cases), it is possible that the high amount of proliferation was correlated to a slight shift in the image. If more of the hyperoxic region was imaged, that means that less of the hypoxic region was imaged, and would thus look like an increase for the hyperoxic region and a decrease for the hypoxic region. This is why alignment was so important. The gradient region would be unaffected by this though, because it was always in the middle of the image. There also could be some human error involved in calculating the proliferation on FIJI. For each image the threshold had to be adjusted. These values were kept relatively the same, but each image was different because the brightness of the cells/image varied. Great care was taken in making sure that all cells were properly visualized for the cell count; this meant that not all thresholds were the same. It is odd that the cells in the hyperoxic region of the shallow gradient did not proliferate. This could be due to image alignment, that less cells were in the region, or that cells did not migrate there, and therefore did not increase the cell number within the region.

Migration measurements were not affected by misalignments of images, but the overlay images of 0 and 48 hours were. When using FIJI, the images could be aligned side to side and up and down, but they couldn't easily be rotated. If the image was rotated a few degrees, the migration in these overlays would be more visible.

Only a few experiments were run for each gradient because of time constraints. Some experiments failed. These included: a leaking well and a hydrogel that peeled away from the bottom of the device because it was too thin. More experiments would be useful to further confirm the data and conclusions. Additionally, moving the device outside of the incubator for an hour out of the forty eight probably was not enough to affect the growth and migration of the cells, but it may have.

Through this project, breast cancer cell biology was furthered. Even though migration was slight, it can be observed that cells will migrate to a higher oxygen level of up to 21% if it is available. Additionally, cells will proliferate more in these conditions. It is likely that cells in a lower oxygen environment (hypoxic) have less "fuel." At lower oxygen concentrations than normal, cell function is affected. Cancer cells can grow in hypoxic conditions, but that does not mean that it is a preferred condition. The fact that there was slight proliferation in the hypoxic regions proves that cancer cells can grow in low oxygen.

8. Conclusions & Recommendations

This chapter summarizes the findings and the conclusions of the project, and the recommendations for further improvement.

8.1 Conclusions

Oxygen gradients were produced and created in the designed device. These gradients were apparent in the sharp and intermediate gradients. There was a visible difference in color between the channel with nitrogen gas (hypoxic) and the channel with compressed air (hyperoxic). Within the middle of these chambers was a gradient that produced a color change comparable to what was modeled in COMSOL. Additionally, the region of the sharp gradient had a much quicker shift in color than the intermediate gradient; this makes sense. The oxygen gradient in the 5mm gradient could not be accurately imaged using the microscope, since the 5mm gradient was outside the 2mm range of view that could be see under the 5X magnification, however, it was assumed that this gradient was produced.

The breast cancer cells were properly cultured over a duration of five months; no contamination was ever observed.

The migration and proliferation in the device was less than expected, but it could still be observed. The most migration overall was seen within the sharp 100μm gradient. Overall each cell moved approximately 5μm to a higher oxygen gradient. The hyperoxic region had the most migration. On average cells moved about 8.5μm to an even higher oxygen level; some cells moved as much as 20μm within this region. The gradient saw more migration to a higher level of oxygen than the hypoxic region; the cells within the gradient moved an average of 4.5μm, and the cells within the hypoxic region moved an average of 3μm. It is likely that the cells within a higher region of oxygen had more fuel to allow them to migrate and proliferate. There was migration within the intermediate 700μm device, but it was less than what was seen in the sharp. Cells within all regions of the device migrated an average of about 2.75μm to a higher level of oxygen. The shallow gradient had movement, but it was unclear if it was from proliferation or migration, since the whole 5mm gradient could not be observed at once under the microscope. There was also movement within the hyperoxic and hypoxic regions of the device, but again it was unclear if the cells had moved out of the region, proliferated, or even died.

As far as proliferation goes, it was concluded that the most proliferation occurred within the hyperoxic regions on average. These oxygen levels were higher than what is normally seen in the body. It makes sense that cancer cells would find this higher oxygen environment attractive since they use a lot of oxygen. Additionally, the oxygen levels within the incubator are approximately 17% oxygen (in a 5% CO2 incubator) (BioSpherix, n.d.). This means that the cells are used to proliferating in this higher level of oxygen. There was little to no proliferation seen in the shallow gradient; the cell numbers remained relatively the same. There was much more proliferation seen in the intermediate and sharp gradient devices. Within the hyperoxic regions, the cell count increased by approximately 60-70%. In the gradient region of these devices, there was a cell count increase of approximately 20%. The hypoxic regions had a cell count increase of only 5%; this can conclude that there was little to no proliferation in this region.

Overall, the 100μm device had the most proliferation and migration observed. Lastly, all devices are reusable and can be used to culture other cell lines. Different gases, or even small volumes of liquid can potentially be flown through the device.

8.2 Recommendations

In order to build upon the achievements of the device and the results that were found during this project, there are a few recommendations for future work. Using an expensive oxygen sensor would allow for the oxygen levels to be quantified more precisely. These were outside of the budget, but would further confirm that the oxygen gradients were produced especially if they could be done within the hydrogel.

It would be useful to better control the atmospheric oxygen interference. The incubator has an oxygen concentration of about 17%, so it would be useful to use a hypoxic incubator that contains much less oxygen. Utilizing an incubator that has only 5% oxygen would be a drastic difference. Additionally, the device was covered by a glass slide on both ends, however, the sides were remained untouched. Since it is possible that oxygen could have diffused through the PDMS (even though it was thick), the device can be entirely encased in glass for the future. Using media that is oxygen free would also decrease interference.

The cells were observed under the 5X magnification lens. This was because the device and all the added components made the device quite thick. A thinner device could be made in the future; this could allow the cells to be focused on under a higher magnification such as 10X. Additionally, cells could be more uniformly distributed throughout the gel.

The migration and proliferation were difficult to accurately observe since cells under a higher magnification couldn't be observed, and it was difficult to properly align the reference point at each time point. A way to live track the cells in the incubator for the future would prove beneficial, since the device would remain stationary.

Testing cell proliferation and migration in each gradient device more times and for longer than 48 hours could enable more growth and movement to be seen. More data would allow for more statistically significant conclusions.

Future groups can use the devices produced to observe different cell lines and can flow different gases or fluids through, to observe those effects on cell proliferation, migration, and viability.

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Appendix A- Cell Culturing

Passaging Protocol

1. Observe cells under microscope to determine confluency (75-80%)
2. Aspirate media in plate
3. Add 5mL of DPBS(-) with serological pipette and then aspirate DPBS (-) after rinsing the cells
4. Add 3mL of 0.25% Trypsin-EDTA
5. Place on the heating plate at 37°C for 10 minutes (observe under microscope to see cell detachment)
6. Add 2mL of complete media to neutralize Trypsin
7. Suspend the 5mL in a 15mL conical tube
8. Remove 1mL for cell counting
9. Centrifuge the 15mL conical tube at 200g for five minutes
10. After centrifuge aspirate without disturbing the cells
11. Add 1mL of cells onto a sterile culture dish and add 10mL of fresh media
12. Observe cells under microscope and place in incubator to culture

Passaging Table of Cultured Plates

The team started out culturing one plate. For B-term break the cells were frozen and four plates were replated.

Passage Tables:

1 Plate

Date	Passage Number	Cells/mL
November 3, 2016	0	500,000
November 7, 2016	1	2,250,000
November 11, 2016	2	2,750,000
November 16, 2016	3	2,412,500
November 21, 2016	4	2,807,000
November 27, 2016	5	2,682,500
November 30, 2016	6	1,732,500
December 3, 2016	7	1,465,000
December 6, 2016	8	1,250,000
December 10, 2016	9	1,277,500
December 12, 2016 (Freeze Cells)	10	500,000

4 Plates

Date	Passage Number	1-1 (Cells/mL)	1-2 (Cells/mL)	2-1 (Cells/mL)	2-2 (Cells/mL)
January 17, 2017	11	680,000	780,000	1,020,000	780,000
January 21, 2017	12	1,397,500	1,412,500	935,000	1,216,000
January 25,2017	13	1,545,000	1,362,500		1,945,000
January 28, 2017	13			2,722,500	
January 29, 2017	14	1,670,000	2,642,500		2,110,000
January 31, 2017	14			1,720,000	

February 1, 2017	15	1,832,500	2,075,000	2,000,000	1,965,000
February 4, 2017	16	1,110,000	1,690,000	2,310,000	1,940,000
February 8, 2017	17	2,162,500	2,322,500,	2,570,000	2,495,000
February 11, 2017	18			1,517,500	
February 12, 2017	18	2,190,000	2,380,000		2,117,500
February 19, 2017	19			1,905,000	897,500
February 22, 2017	19	2,470,500	2,990,000		
February 25, 2017	20			3,050,000	2,950,000
February 27,2017	20	1,797,500			
February 28, 2017	20		2,482,000		
March 1, 2017	21	2,030,000	1,575,000	2,260,000	2,577,500
March 12, 2017	22	2,006,000	2,312,500	2,000,000	2,452,000
March 17, 2017	23	2,495,000	2,757,000		
March 20, 2017	23			2,065,000	2,177,500
March 22, 2017	24	1,942,000	2,600,000		
March 25, 2017	24			2,025,000	2,332,500
March 27, 2017	25	2,565,000	2,560,000		
March 30, 2017	25			2,440,000	2,052,500
April 1, 2017	26	3,110,000	3,132,500		
April 3, 2017	26			1,542,500	1,992,500
April 5, 2017	27	2,255,000	2,102,500		
April 8, 2017	27			1,140,000	1,457,500
April 11, 2017	28	2,680,000	2,447,500		

Media Protocol for 50mL of 10% FBS Media

1. Add 44mL of DMEM in a sterile 50mL conical tube
2. Add 0.5mL of Glutamine
3. Add 0.5mL of PennStrep
4. Add 5mL of Fetal Bovine Serum
5. Mix by gently inverting the 50mL conical tube multiple times
6. Store in refrigerator when not in use

Appendix B- Cleaning the Microfluidic Device After Use

Cleaning Protocol for Microfluidic Device After Experiment

1. Place device in hood and place the cover slide in a new petri dish
2. Aspirate any remaining media inside the well
3. Remove well from device and place with cover slide
4. Remove the hydrogel from device using a pipette tip (1000µL) into another clean petri dish
5. Place device with well and cover slide
6. Pour 5mL of isopropyl alcohol onto the device. Make sure to release all over the device
7. Pour 2mL of isopropyl alcohol onto the cover slide and well, similar to the device. Let them submerged for five minutes
8. Dry all components with a paper towel and place in a new petri dish
9. Pour 5mL of ethanol on all components in the new petri dish and let them submerged for five minutes
10. Dry all components using a paper towel and place them in a new petri dish for storage
11. If device will be used the same day, place the petri dish with all components under UV Sterilization oven for two hours to sterilize. If it would not be used the same day, sterilize the same way on the day of usage.
12. Aspirate the petri dishes with isopropyl alcohol and ethanol
13. Dispose of all petri dishes in the biohazard waste

Appendix C- COMSOL Simulations

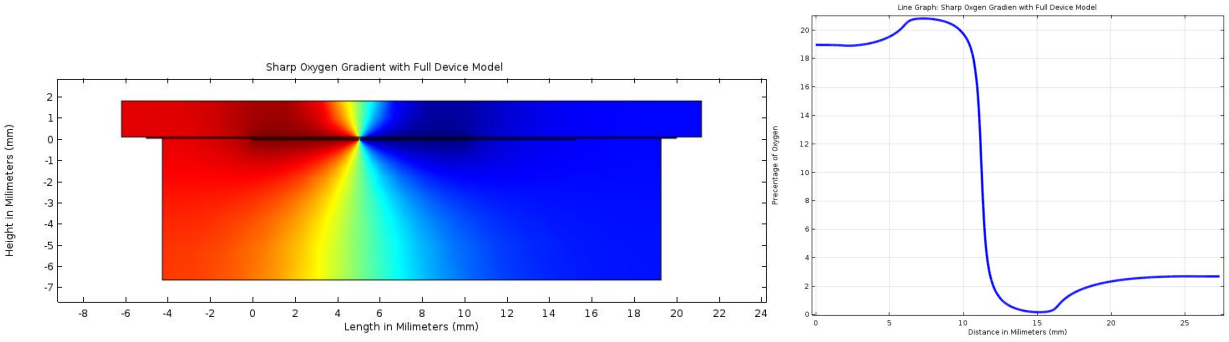
The following are models on COMSOL. Using the hydrogel, membrane and chambers to model the oxygen gradient.

Parameters:

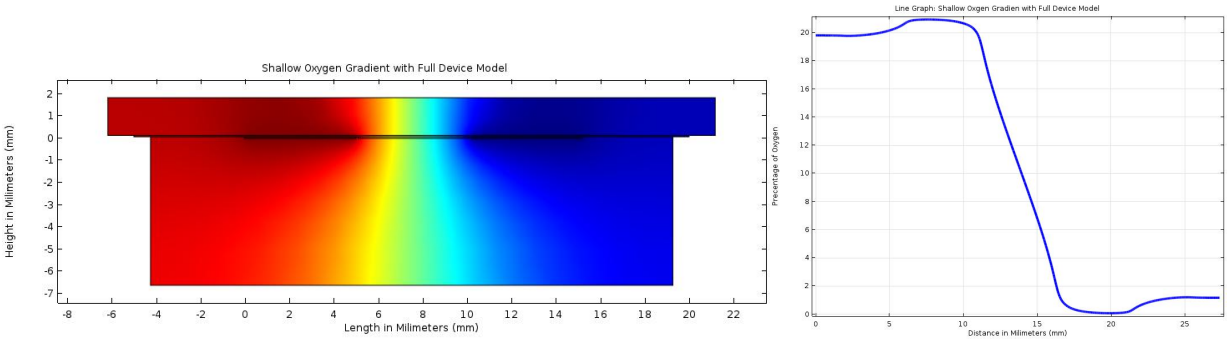
- Hydrogel Dimensions: 27mm X 1.7mm(Thickness)
- Membrane Dimensions: 25mm X 0.0635mm(Thickness)
- No flux boundary all around the system
- Diffusivity of Oxygen: $3.25*10^{-9}[m^2/s]$
- 1.97×10^{-9} $[m^2/s]$ (Ferrell & Himmelblau, 1967)
    - Simulates diffusion of oxygen in the hydrogel
- Three Chambers: 5mm X 0.1mm each
- 100µm gap between chambers
- 21% oxygen in one chamber
  0% oxygen in one chamber
- Line plots were taken at 200µm above the membrane
- The bottom of the device was not used for modeling because it was presumed that it would not affect the oxygen gradient modeled. As can be seen below, analysis of the slope of the gradient was equal when comparing the models of the system with and without the bottom of the device.

Complete Device

Sharp Gradient in Complete Device

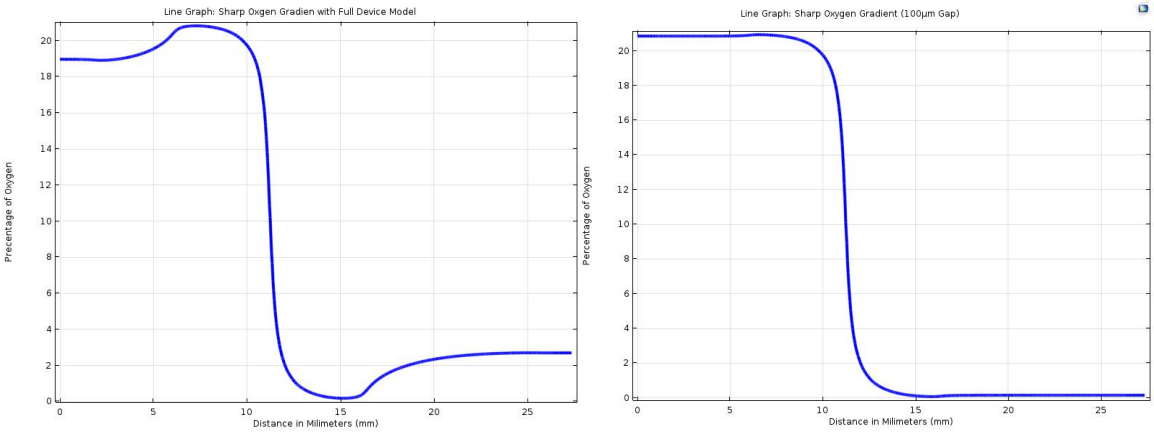


Shallow Gradient in Complete Device

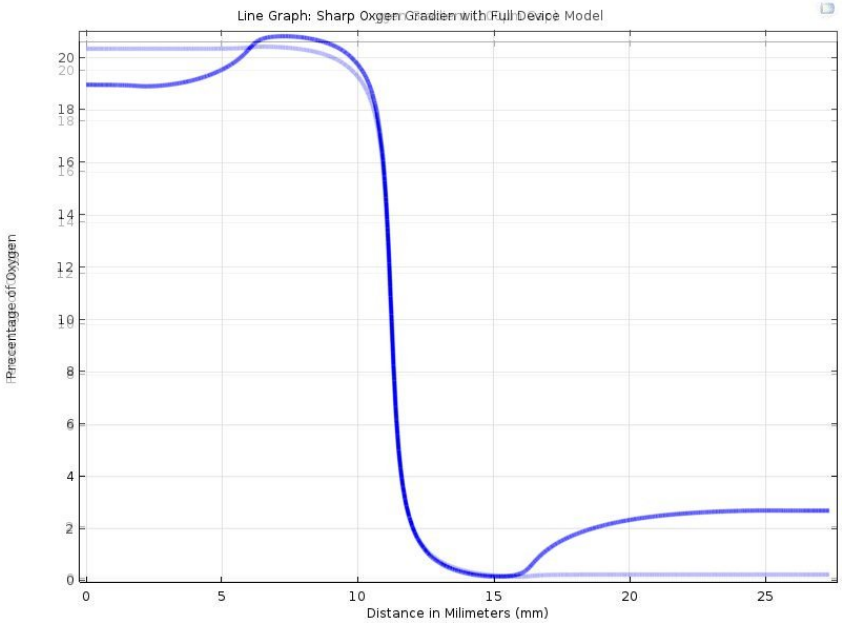


Full Device Compared to Displayed Models

Sharp Gradient of Full Model compared to sharp gradient of displayed model of paper

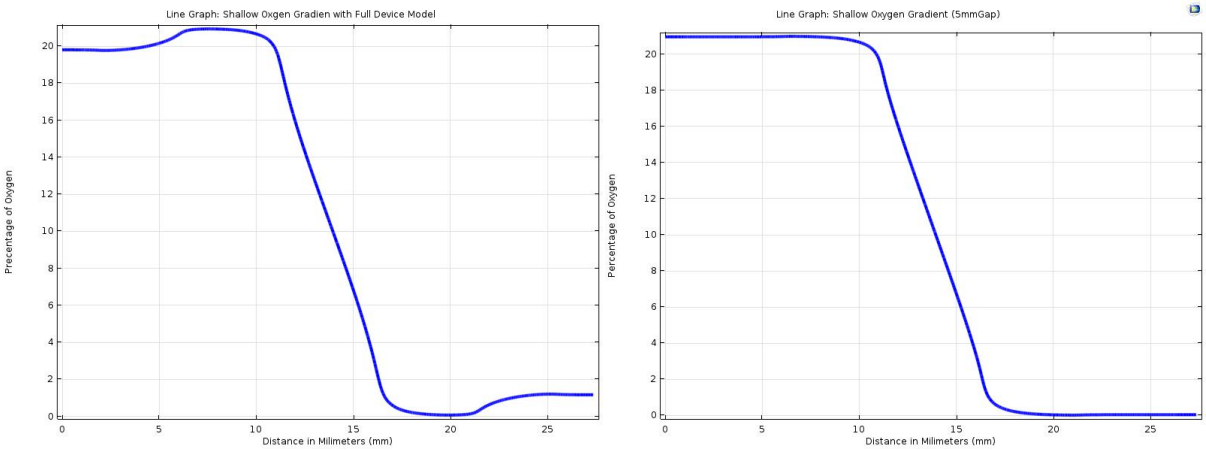


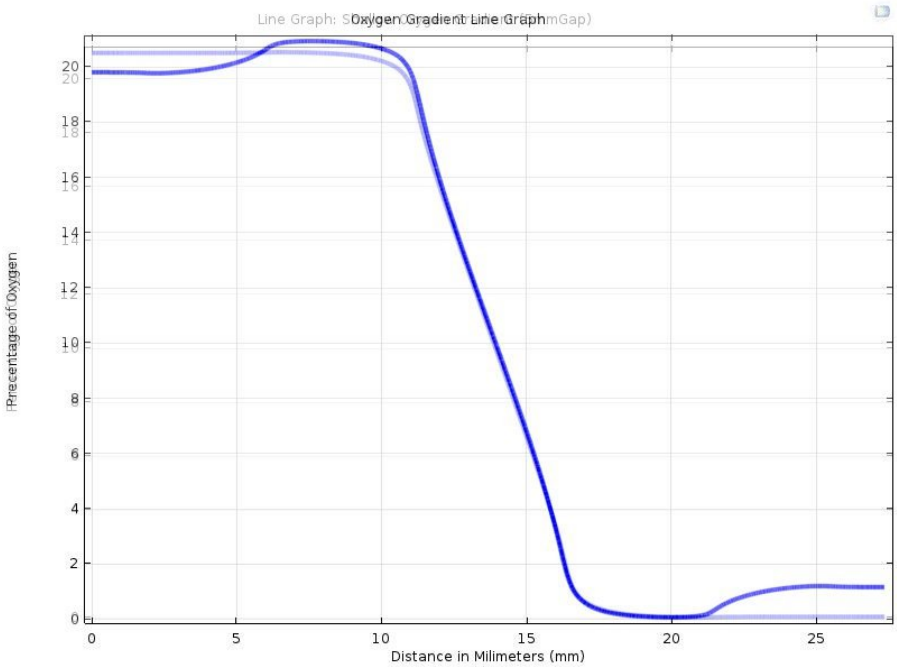
Overlay:



As can be seen, the gradient slopes are equal.

Shallow Gradient of Full Model compared to shallow gradient of displayed model of paper

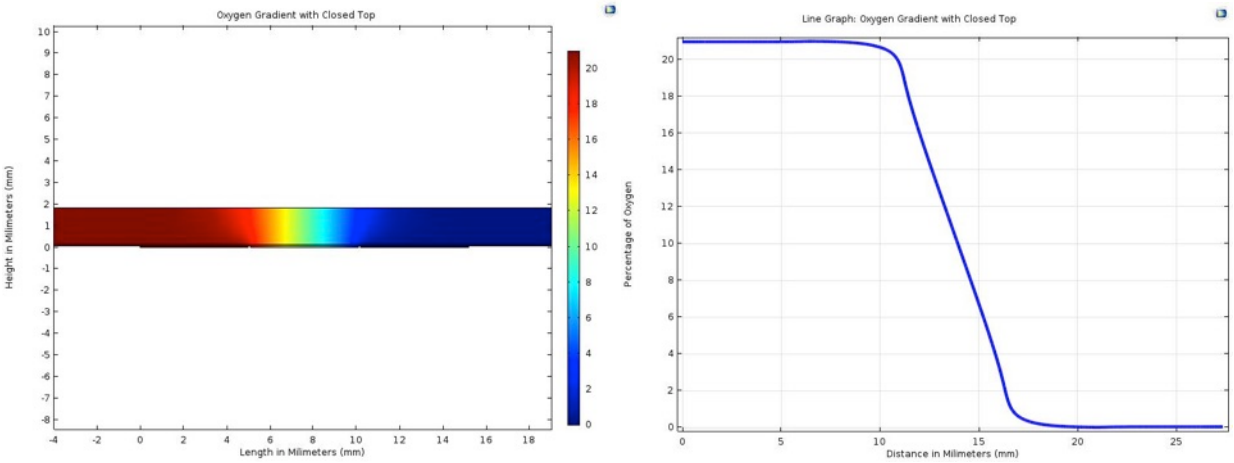




As can be seen, the gradient slopes are equal.

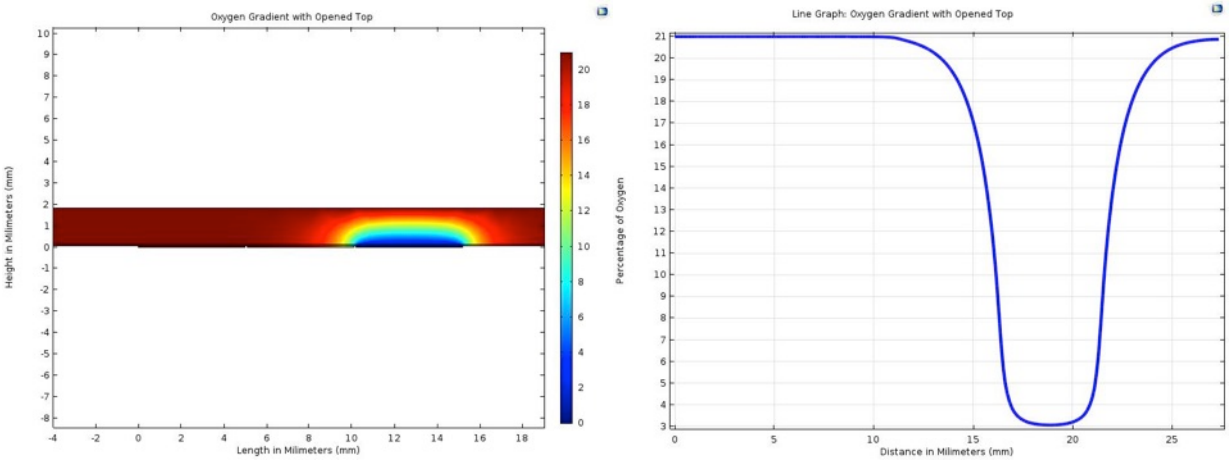
Open Top Vs Closed Top

Closed Top



The max slope of the oxygen gradient is: 3.38 $\%O_2$/mm.

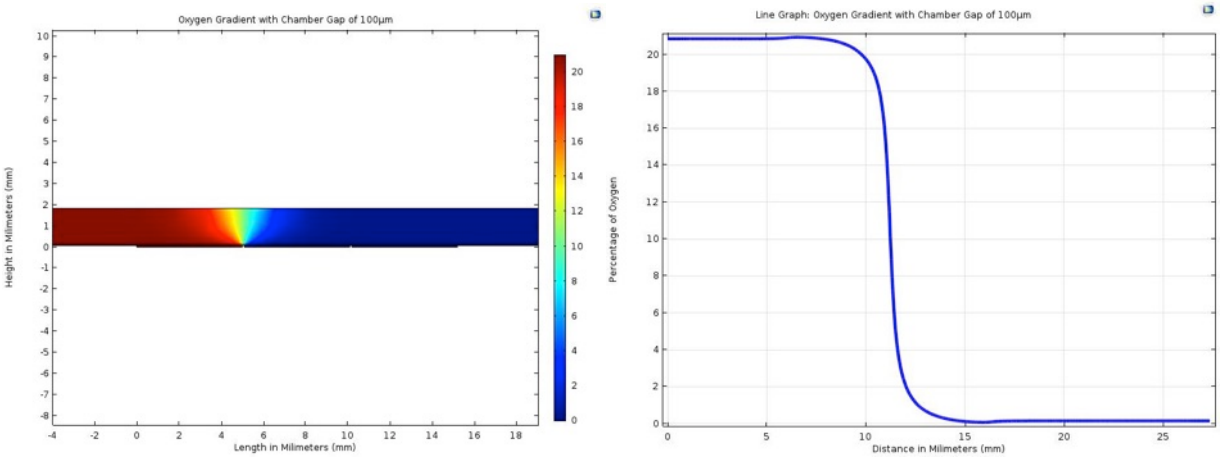
Opened Top



The max slope of the oxygen gradient is: 6.5 $\%O_2$/mm

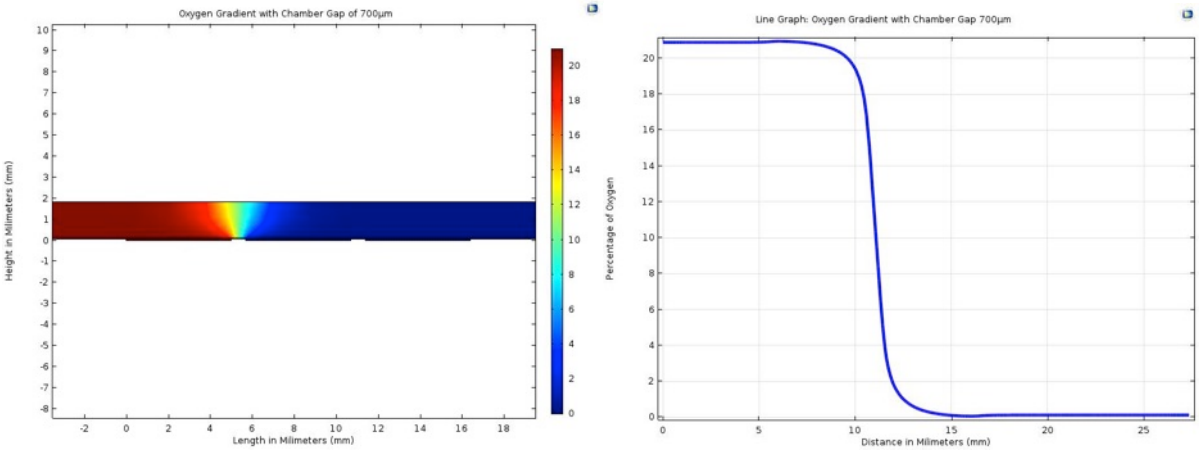
Distance Between Chambers

100µm



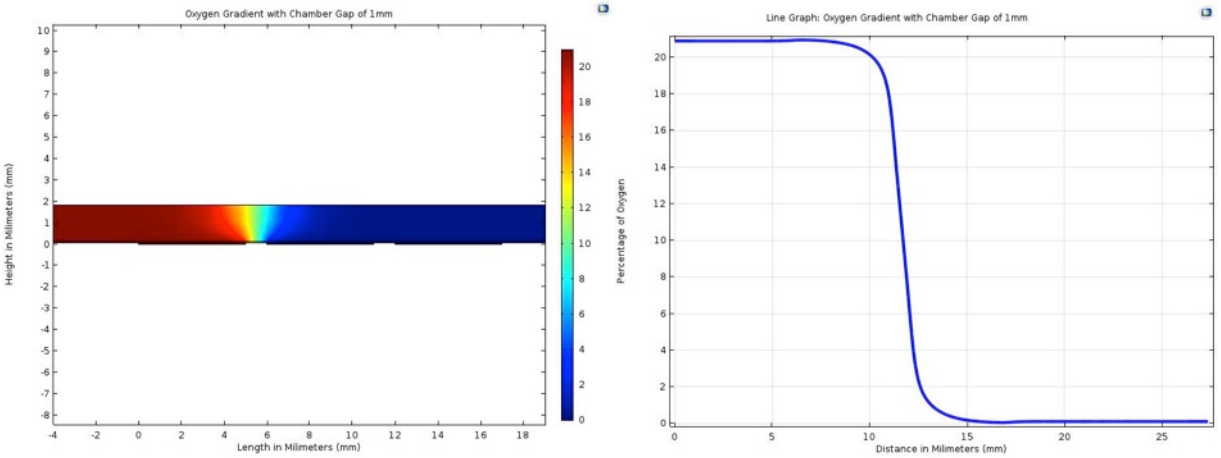
The max slope of the oxygen gradient is: 25 $\%O_2$/mm

700µm



The max slope of the oxygen gradient is: 14 $\%O_2$/mm

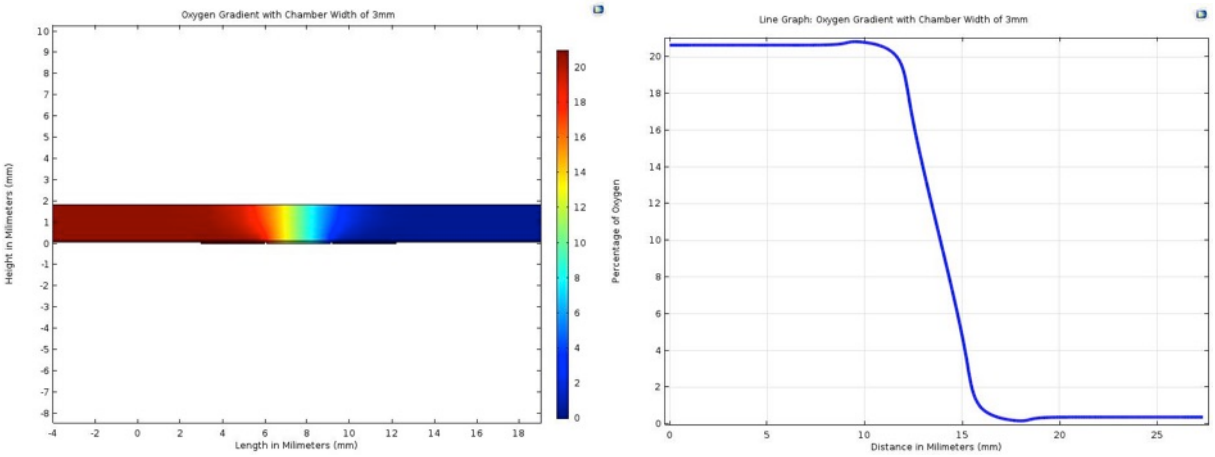
1mm



The average max slope of the oxygen gradient is: 7.20 $\%O_2$/mm

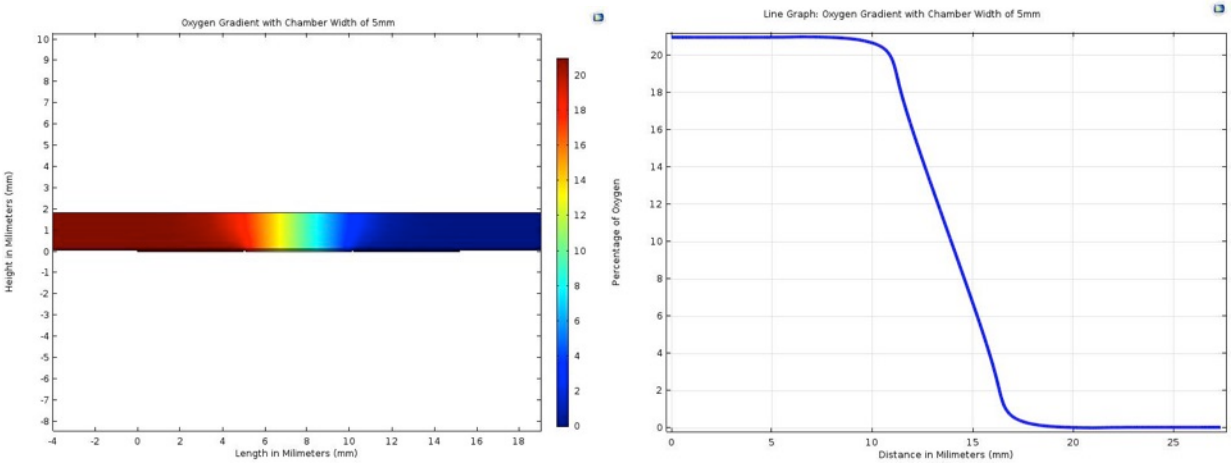
Width of Chambers

3mm



The max slope of the oxygen gradient is: 4.75 $\%O_2$/mm

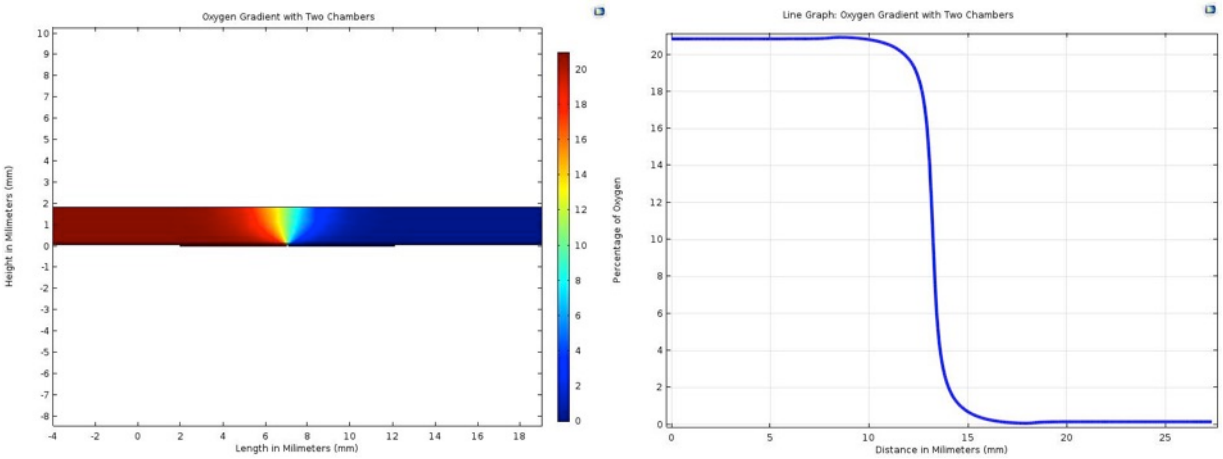
5mm



The max slope of the oxygen gradient is: 3.16 $\%O_2$/mm

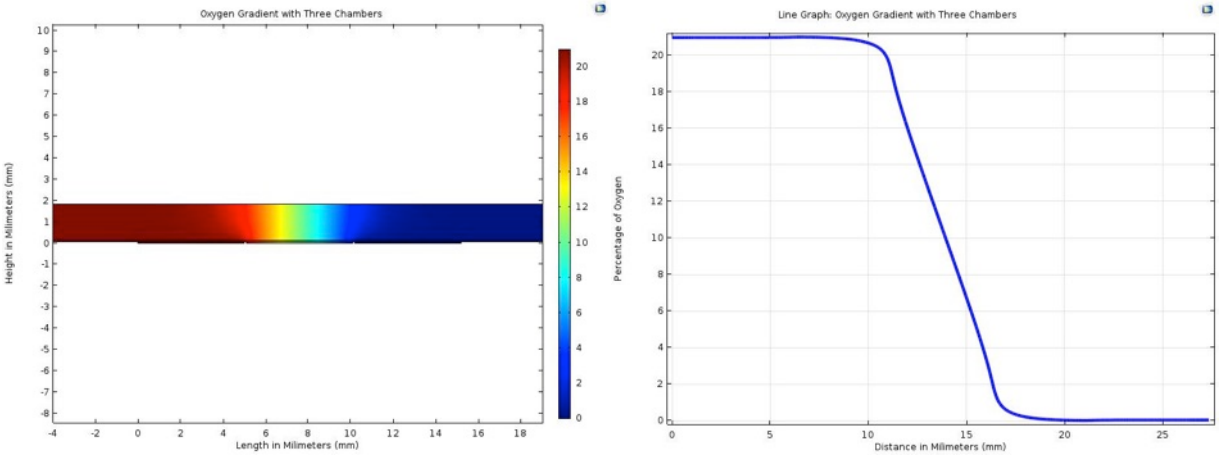
Two Chamber Device Vs Three Chamber Device

Two Chambers



The max slope of the oxygen gradient is: 25.0 $\%O_2$/mm

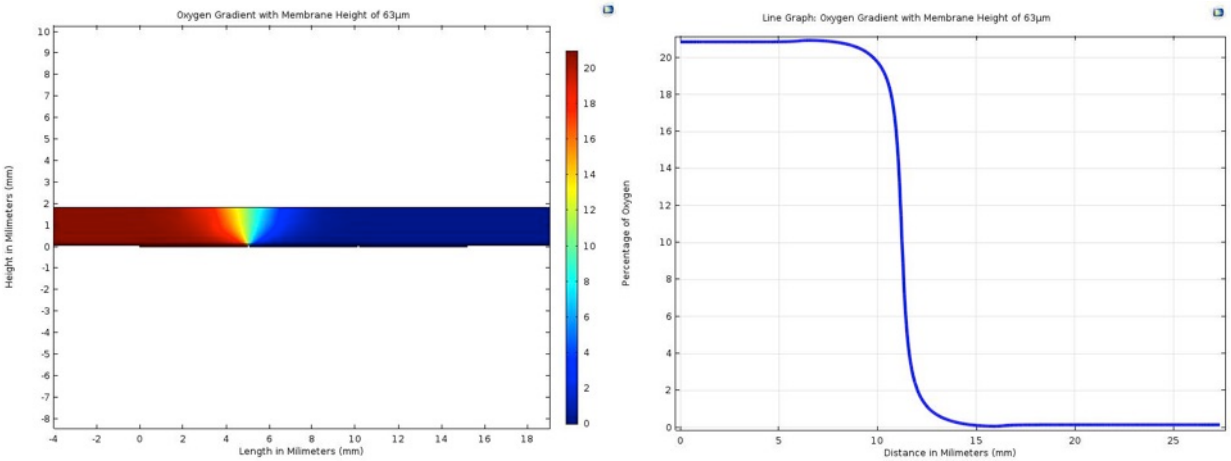
Three Chambers



The max slope of the oxygen gradient is: 3.16 $\%O_2$mm

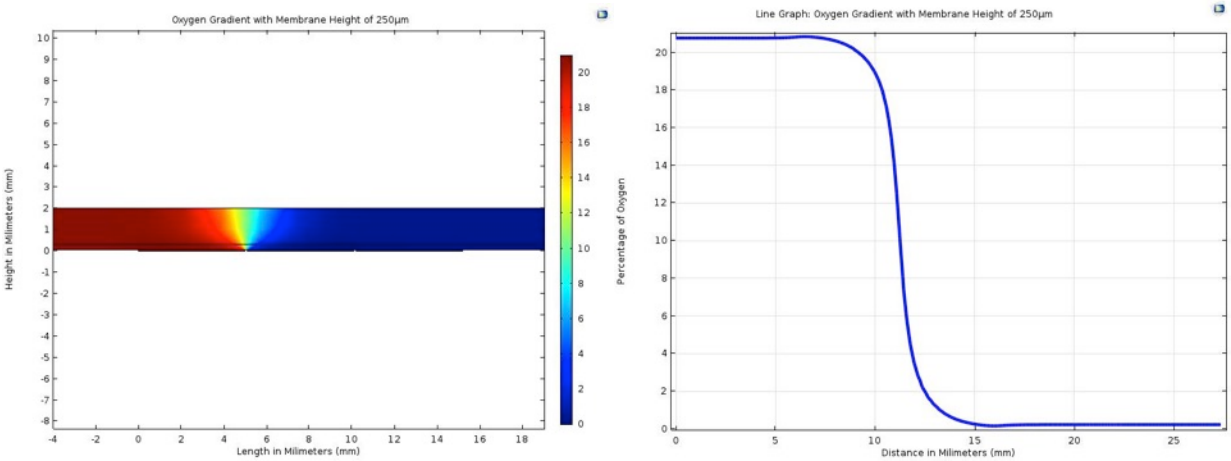
Membrane Height

63µm



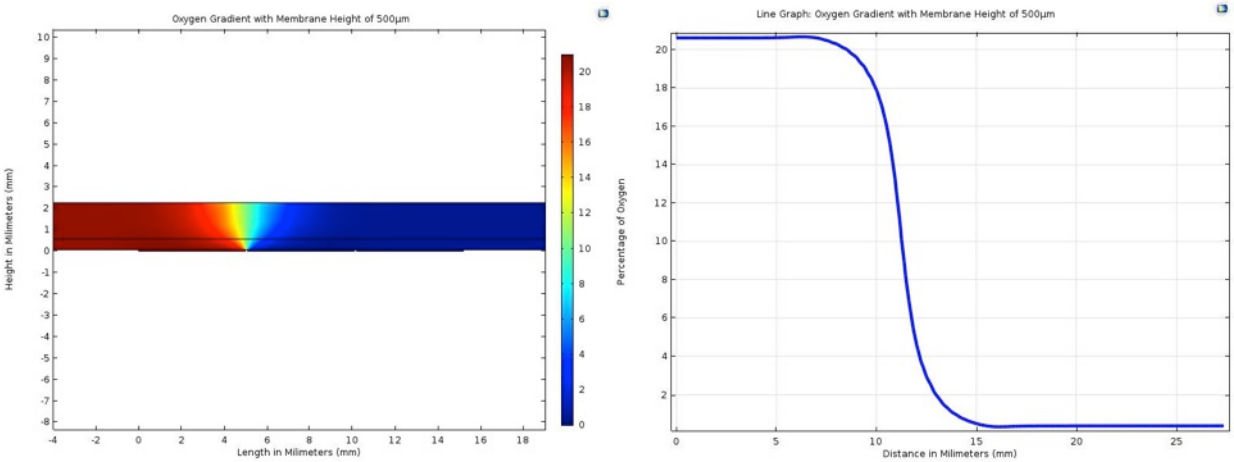
The max slope of the oxygen gradient is: 25.0 $\%O_2$/mm

250µm



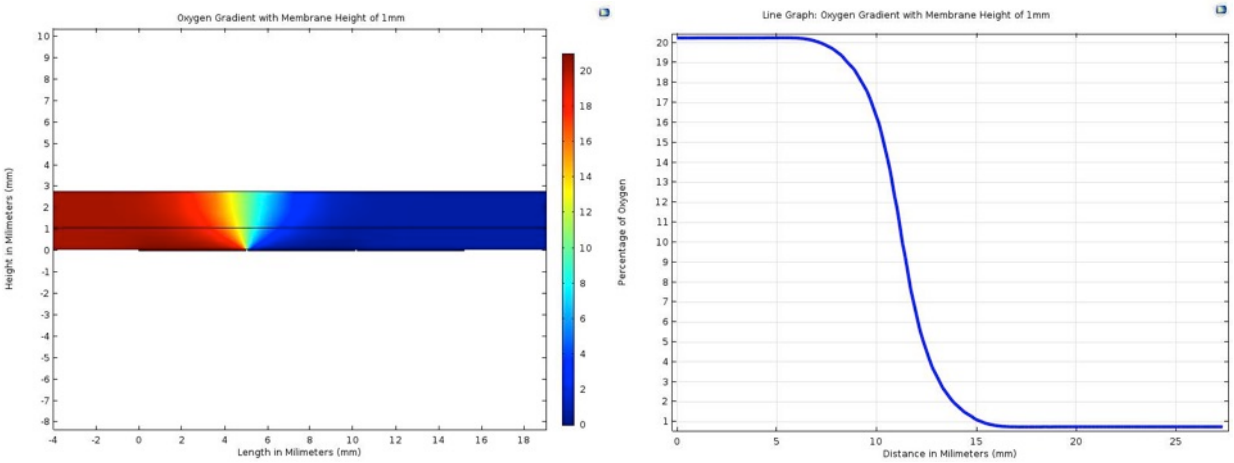
The average max slope of the oxygen gradient is: 7.00 $\%O_2$/mm

500µm



The average max slope of the oxygen gradient is: 4.8 $\%O_2$/mm

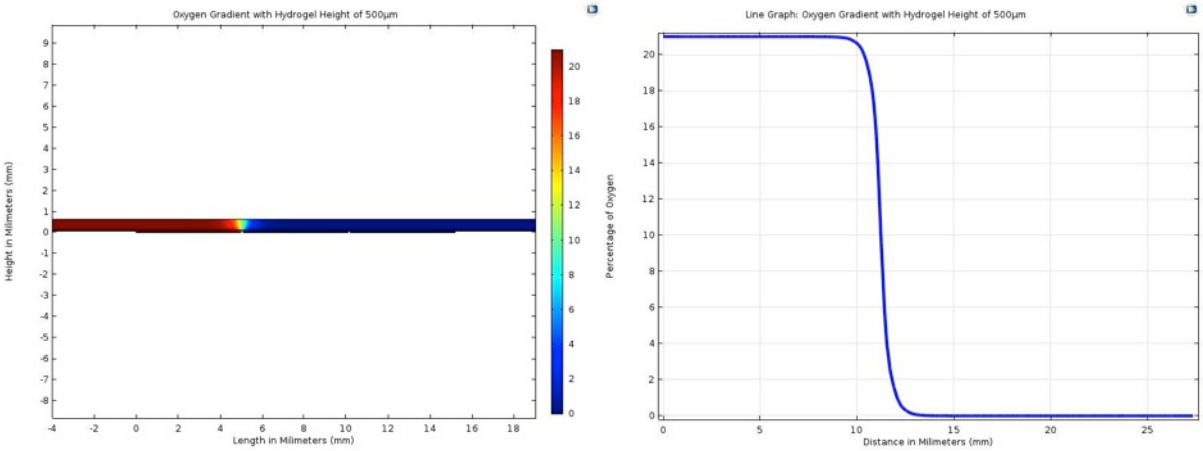
1mm



The average max slope of the oxygen gradient is: 3.33 $\%O_2$/mm

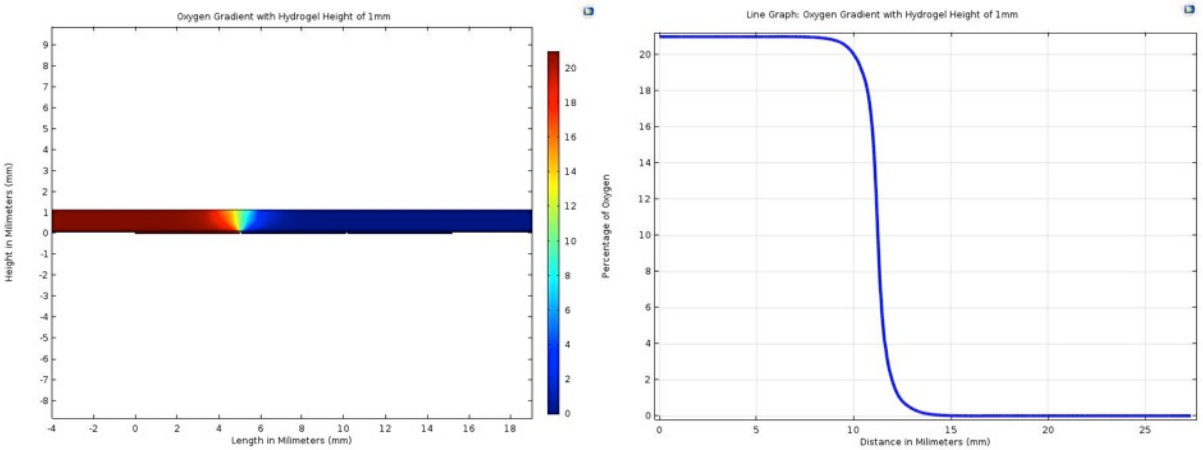
Hydrogel Height (Sharp)

500µm



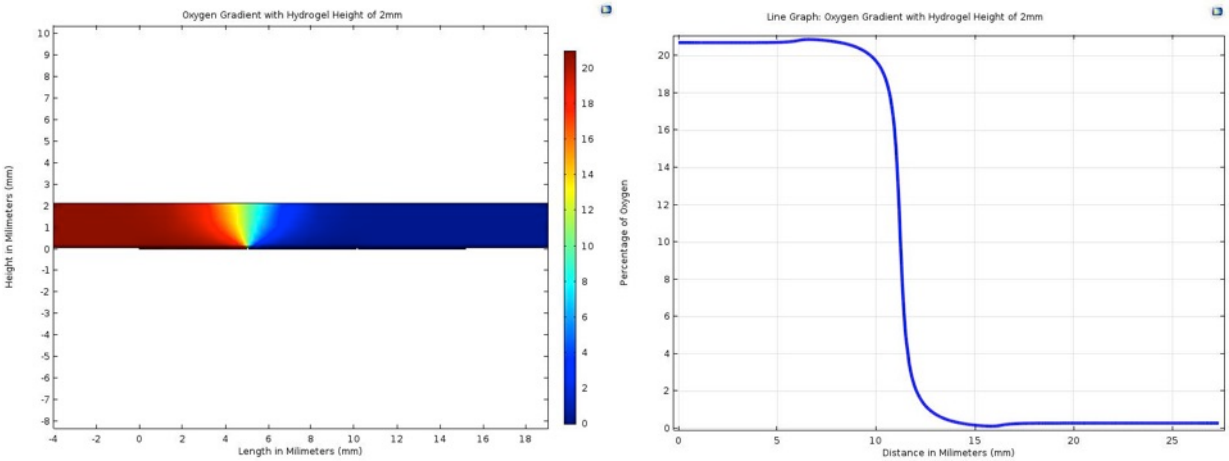
The average max slope of the oxygen gradient is: 9.75 $\%O_2$/mm

1mm



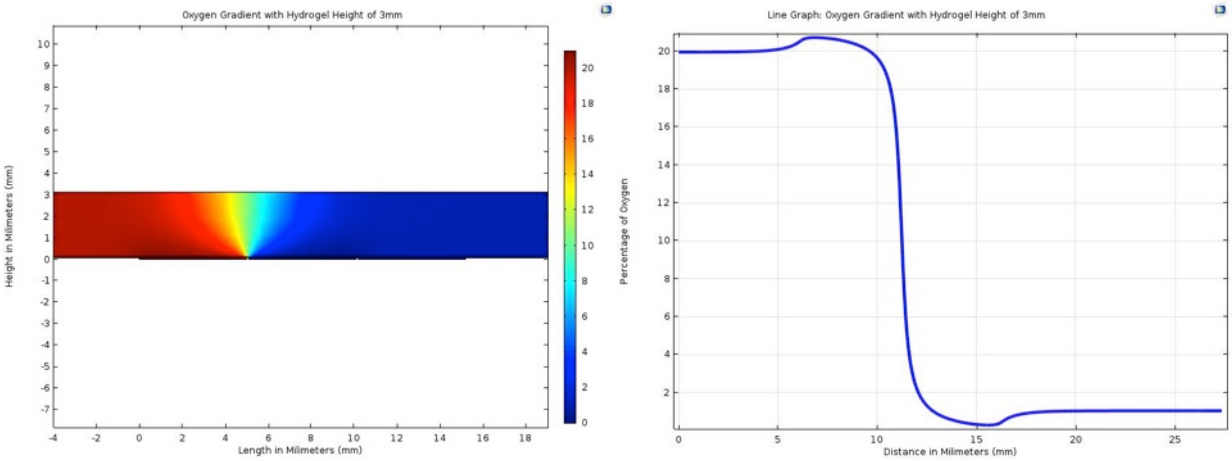
The average max slope of the oxygen gradient is: 9.5 $\%O_2$/mm

2mm



The average max slope of the oxygen gradient is: 9.0 $\%O_2$/mm

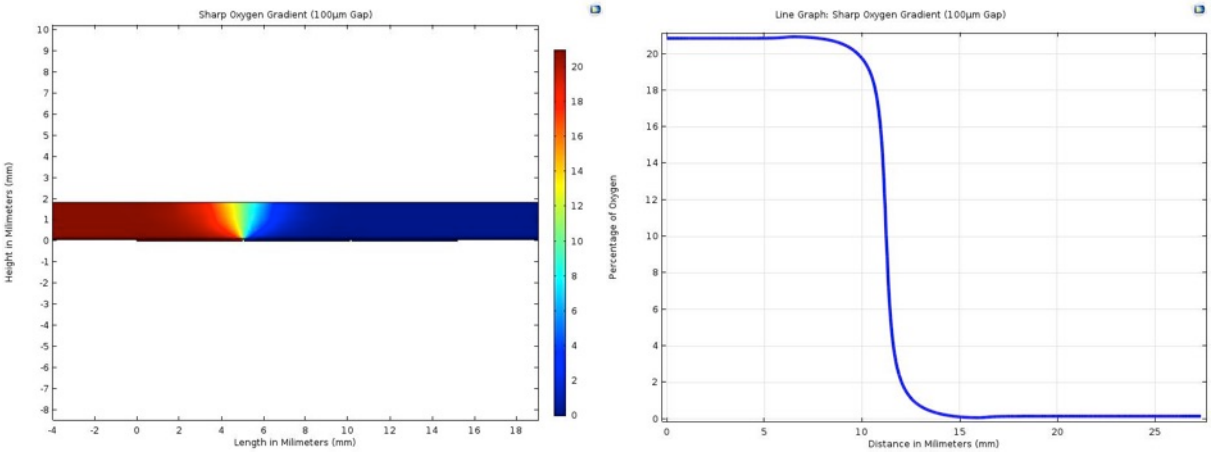
3mm



The average max slope of the oxygen gradient is: 8.75 %O_2/mm

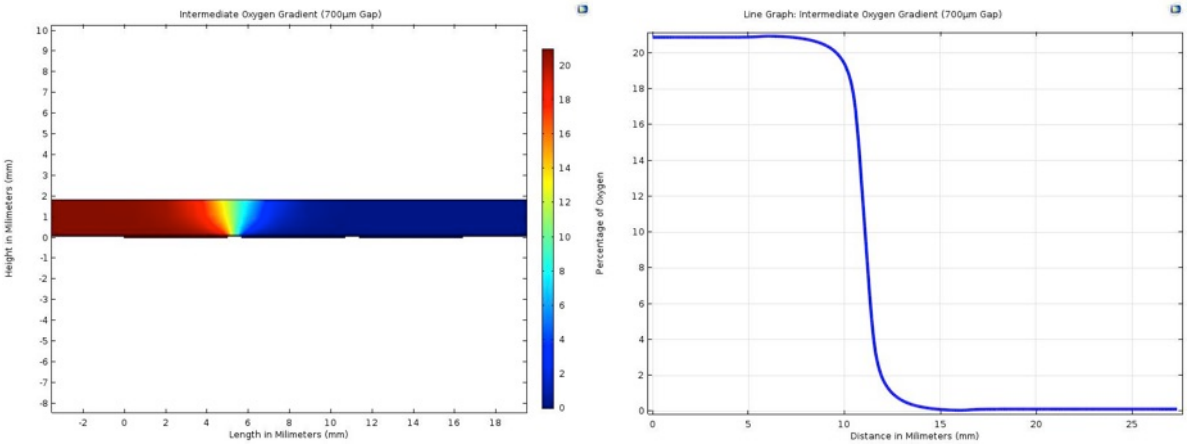
Gradients

Sharp Gradient (100µm)



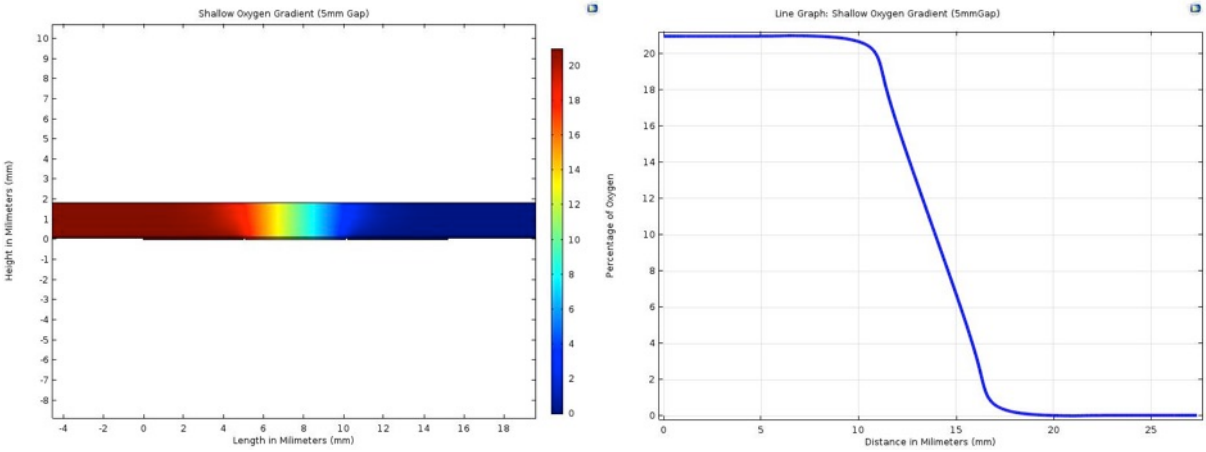
The max slope of the oxygen gradient is: 25 $\%O_2$/mm

Intermediate Gradient (700µm)



The max slope of the oxygen gradient is: 14 $\%O_2$mm

Shallow Gradient (5mm)



The max slope of the oxygen gradient is: 3.1 $\%O_2$/mm

Appendix D- Financials

Complete Item Price Table:

Item	**Price**
MDA-MB-231/GFP/Blasticidin	$395.00 (Gifted)
Cell Culture Dishes	$1.74 each (SL219)
Pasteur Pipets	$0.20 each (SL219)
Serological Pipets (5mL)	$0.21 each (SL219)
Serological Pipets (10mL)	$0.71 each (SL219)
Serological Pipets (25mL)	$0.67 each (SL219)
Compressed Air Tank, Nitrogen Gas Tank and Carbon Dioxide Gas Tank	$111.00 in Total (Purchased)
Dulbecco's Modified Eagle Medium (DMEM) (500mL)	$33.23 (SL219)
L-Glutamine (200mM) (100mL)	$25.21 (SL219)
Trypsin-EDTA (0.25%) (100mL)	$12.52 (SL219)
Penicillin-Streptomycin (100x) (100mL)	$29.10 (SL219)
Fetal Bovine Serum (100mL)	$165.00 (SL219)
Tygon Tubing 1/4in (6.35mm)	$6 each Foot (30.2cm) (Borrowed)
Pin	$0.10 each (Borrowed)
Needle	$0.31 each (Borrowed)
Sylgard 184 Silicone KIT (0.5kg)	$60 each (Borrowed)
PureCol EZ (35mL)	$355.00 (SL219)
Pipette Tip (10µL)	$1.00 each (SL219)
Pipette Tip (200µL)	$1.00 each (SL219)
Pipette Tip (1000µL)	$1.00 each (SL219)
Master Fabrication	$360.00 (Gifted)
Plasma Cleaner	$1,234.00 (SL219)

Oven	$214.00 (SL219)
PDMS Hole Puncher (Biopsy Punch)	$8.00 (Borrowed)
Incubator	$11,150.00 (SL219)
Ultraviolet Sterilization Oven	$75.00 (SL219)
OceanOptics Ruthenium Coated Fluorescent Slide	$275.00 (Borrowed)

Gifted Items Table (no charge):

Item	**Price**
MDA-MB-231/GFP/Blasticidin	$395.00
Master Fabrication	$350.00

The team received the MDA-MB-231/GFP/Blasticidin cells from Professor Ambady as a gift. The team was gifted a new master mold with the alternative designs from the microfabrication lab and BME595T at Worcester Polytechnic Institute.

Borrowed Items Table:

Item	**Price**
Tygon Tubing 1/4in (6.35mm)	$6 each Foot (30.2cm)
Pin	$0.10 each
Needle	$0.31 each
Sylgard 184 Silicone KIT (0.5kg)	$60 each
PDMS Hole Puncher (Biopsy Punch)	$8.00
Ocean Optics FOXY Ruthenium Coated Fluorescent Slide	$275.00

These items were borrowed from advisor Albrecht's lab and from Elyse Favreau. Any leftovers were returned.

Salisbury Lab 219 Table:

The lab cost was $200 in total ($100 per student) and we were able to use the following items.

Item	**Price**
Cell Culture Dishes	$1.74 each
Pasteur Pipets	$0.20 each
Serological Pipets (5mL)	$0.21 each

Serological Pipets (10mL)	$0.71 each
Serological Pipets (25mL)	$0.67 each
Dulbecco's Modified Eagle Medium (DMEM) (500mL)	$33.23
L-Glutamine (200mM) (100mL)	$25.21
Trypsin-EDTA (0.25%) (100mL)	$12.52
Penicillin-Streptomycin (100x) (100mL)	$29.10
Fetal Bovine Serum (100mL)	$165.00
PureCol Ez (35mL)	$355.00
Pipette Tip (10µL)	$1.00 each
Pipette Tip (200µL)	$1.00 each
Pipette Tip (1000µL)	$1.00 each
Plasma Cleaner	$1,234.00
Oven	$214.00
Incubator	$11,150.00
Ultraviolet Sterilization Oven	$75.00

Began with a budget of $500. After purchases totaling $311, a budget of $189 remained.

Estimated Prices
The price to manufacture our device is about: $22.This includes the PDMS device, PDMS well, PDMS membrane and hole puncher.

Experiment price each: $39.71.This includes device fabrication, cells, hydrogel, media, 2 pipette tips, and 1 serological pipette.
Experiment with gas flow: $46.53. This includes device fabrication, cells, hydrogel, media, 2 pipette tips, 1 serological pipette, 2 Tygon tubing, 2 needles, 2 pins and 2 luer locks.

Appendix E- Standard Operating Procedure For Creating and Observing Human Breast Cancer Migration in Microfluidic Device

1.0 Purpose
To describe the procedure to properly set-up an oxygen gradient experiment on the device and ensure safety.

2.0 Scope
This procedure applies to any personnel that wants to do an oxygen gradient experiment on the device.

3.0 Responsibility
All personnel that utilizes the device to observe cell proliferation and migration within an oxygen gradient.

4.0 Definitions

PDMS- Polydimethylsiloxane
UV- Ultraviolet
FBS- Fetal Bovine Serum (a component of complete media)
FIJI- Fiji is just imageJ (image analysis program)

5.0 Materials
Device
PDMS Membrane
(2) Slide
PDMS Well
Petri Dish (60mm)
Petri Dish (120mm)
MDA-MB-231/GFP/Blasticidin (Breast Cancer Cells)
Pipettor
Pipette tip (200µL)
Pipette tip (1mL)
10% FBS Complete Media
1 mL of PureCol EZ
Incubator set to 37 degrees Celsius
Fluorescent microscope
(2) Pins
(2) 1m long Tygon Tubing with a diameter of 6.35mm
(2) 15.24cm long tubing with a diameter of 1mm
(2) Needle
(2) Luer lock
Compressed air tank
Nitrogen gas tank

Ultraviolet sterilization oven
Ethanol
Isopropyl alcohol
Fluoride slide

6.0 Procedure

1. Clean the plasma bonded device with isopropyl alcohol and ethanol.
2. Blow air onto the device to clear the dust.
3. Ensure all dust is removed by placing tape on the device and peeling it off.
4. Plasma bond the bottom of the device to a glass cover slide.
5. Plasma bond a membrane, particularly one that is around 63.5µm in thickness to the top of the device. Make sure this is clean and free of dust by using ethanol and tape. Add a drop of ethanol to the fluorinated slide under the membrane to allow for easy removal.
6. Place the plasma bonded device, well, and glass cover slide inside a 60mm petri dish and sterilize by putting them in a UV sterilization oven for at least 60 minutes. Ensure that the the UV light is hitting the components on all sides.
7. While the device is being sterilized, attach one Tygon Tubing to the compressed air tank regulator. Attach a luer lock that has a needle attached to the other end of the Tygon Tubing. Attached to the needle is a small tubing with a diameter of 1mm. Attached to the 1mm diameter tubing is a pin that will be inserted into the inlet of the device.
8. Attach one Tygon Tubing to the nitrogen gas tank regulator. Attached to the other end of the Tygon Tubing is a luer lock that has a needle attached. Attached to the needle is a small tubing with a diameter of 1mm. Attached to the 1mm diameter tubing is a pin that will be inserted into a different inlet of the device.
9. Turn the gases in each tank on. The pressure reading should be approximately 14kPa.
10. To make sure that the flows are the same, put the pins of each needle in a beaker of water. The flows should look the same.
11. The gases can be turned off for now.
12. Passage the MDA-MB-231/GFP/Blasticidin cells following the passaging protocol in Appendix A and calculate the volume needed for 200,000 cells.
13. After resuspending the cells from passaging, make sure to plate 1,000,000 cells.
14. Remove the plasma bonded device and components from the UV sterilization oven. Make sure the petri dish is closed and bring into the hood.
15. Get PureCol EZ from the refrigerator and make sure to keep it on ice
16. Place the well properly on the device such that the channels are visible, but the inlets and outlet are covered.
17. Add 0.5mL of PureCol EZ to the device.
18. Ensure uniform distribution of the gel throughout the bottom of the well/top of the device by moving it around; the gel will slowly move. Make sure it covers the entire system.
19. Add the calculated amount of µl to the PureCol EZ. Do this slowly and add drops throughout the top of the gel. Tilt the device around to more uniformly distribute the cells.
20. Add the remaining 0.5mL of PureCol EZ inside the well.
21. Allow at least 1 hour for the gel to solidify by placing the device in a petri dish into an incubator at 37 degrees Celsius.

22. After the gel has solidified, take images of the reference points and cells at various locations within the device. The reference point should be taken in brightfield, and the cells should be taken under the blue fluorescence (since they fluoresce green). Do not move the device when shifting to take images of the reference point and cells. They need to remain stationary to properly observe the migration and proliferation. The images taken typically include the top and bottom reference points (middle of the device), and the inlets and outlets. To properly observe migration, make sure that the microscope is focused on part of the device that can see the nitrogen chamber, the compressed air chamber, and the gradient in between. Make sure the reference point images are straight and even.
23. When saving, make sure the images are saved as ome-tiffs and that there are scale bars on the images.
24. Add 1mL of 10% FBS complete media to the well.
25. Place the glass cover slide on top of the well.
26. Put the device into a 120mm petri dish.
27. Put the two tubings from the gas tanks into the back of the incubator. Add a stopper to ensure that the temperature and carbon dioxide from the incubator do not escape.
28. Sterilize the pins that will be inserted into the device with alcohol.
29. Put the device into the incubator and insert the gases into the appropriate inlets
    1. For a sharp gradient put the gases into the adjacent chamber inlets
    2. For a shallow gradient put the gases into opposite chamber inlets
30. Note which inlet contains the nitrogen gas and which contains the compressed air. This will be important for the analysis.
31. To be sure that the gases are flowing through the device a pin connected to a beaker of water can be used. Bubbling will ensure that the gases are flowing.
32. After 24 hours image the same exact locations as the prior day and save. If needed add media to the device.
33. Put the device back into the incubator and attach the tubing into the appropriate inlets.
34. After 48 hours image the same exact locations again.
35. If satisfied with the results, the device can be cleaned following the protocol in Appendix A.
36. The images taken will be compared using the FIJI image analysis program to observe for cell proliferation and cell migration.
    1. Fluorescent images of cells at each time point (0, 24, 48 hours) are used for observing cell proliferation
        1. Load the image into FIJI
        2. Go to image → type → 16-bit
        3. Go to image → adjust → threshold (adjust it so that all the cells are red and the background is black). Click apply.
        4. Go to process → binary → watershed
        5. Count the particles by clicking analyze → analyze particles
    2. To observe migration load in the black and white reference point image and the focused fluorescent image of the cells at that point
        1. Overlay these two images through image → overlay → add image
        2. This will be done at each time point

3. The 0 and 48 hour image are compared using the line and measure tool
    1. Set the scale to 0.53 pixels = 1 micrometer
4. Measure particular cells to the same location on reference point of both images
5. Record the distance and which way the cell migrated

7.0 Attachments

1.) Passage protocol (Appendix A)

1. Observe cells under microscope to determine confluency (75-80%)
2. Aspirate media in plate
3. Add 5mL of DPBS(-) with serological pipette and then aspirate DPBS (-) after rinsing the cells
4. Add 3mL of 0.25% Trypsin-EDTA
5. Place on the heating plate at 37°C for 10 minutes (observe under microscope to see cell detachment)
6. Add 2mL of complete media to neutralize Trypsin
7. Suspend the 5mL in a 15mL conical tube
8. Remove 1mL for cell counting
9. Centrifuge the 15mL conical tube at 200g for five minutes
10. After centrifuge aspirate without disturbing the cells
11. Add 1mL of cells onto a sterile culture dish and add 10mL of fresh media
12. Observe cells under microscope and place in incubator to culture

2.) 10% FBS media production protocol (Appendix A)

<u>Media Protocol for 50mL of 10% FBS Media</u>

1. Add 44mL of DMEM in a sterile 50mL conical tube
2. Add 0.5mL of Glutamine
3. Add 0.5mL of PennStrep
4. Add 5mL of Fetal Bovine Serum
5. Mix by gently inverting the 50mL conical tube multiple times
6. Store in refrigerator when not in use