Engineered Tumor Model

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Abstract

About 12% of women in the United States will be diagnosed with metastatic breast cancer in their lifetime. Metastasis can occur when a circulating tumor cell (CTC) or a cluster of CTCs breaks off from a primary tumor and attaches elsewhere in the body. One of the main forms of metastatic breast cancer is known as triple negative metastatic breast cancer (mTNBC) and, while treatable, it is not curable. This project aimed to create a 3D tumor microenvironment that can be used to assess the effectiveness of various drug therapies on biopsies from individual patients. To do this, three objectives were identified: create CTC clusters, form a microtissue, and integrate the two within a 3D tumor microenvironment. The hanging drop method was used to create clusters, a tissue ring model was designed to grow self-assembling tissue rings, and 2D and 3D integration models were planned to see the interactions between cancer and breast tissue cells. Experiments were performed to create clusters and showed that the breast tissue cells were able to aggregate into one uniform cluster, while cancer cells aggregated into multiple smaller clusters. A 2D integration model was executed, however the seeded cells did not have enough time to integrate and thus no results were seen. Due to the COVID-19 pandemic, all further testing was unable to be completed. The 3D model was never tested, but the group is hopeful that future work can be done to further examine the possibility of a 3D tumor microenvironment.

Executive Summary

Triple negative breast cancer (TNBC) equates to about 15% of breast cancer cases and 35% of breast cancer deaths. TNBC's name reflects that it is estrogen receptor-negative (ER-), progesterone receptor-negative, and human epidermal growth factor 2 negative (HER2-). The "triple negative" nature of this cancer makes it difficult to treat, resulting in a low life expectancy if diagnosed. There are currently no treatments offered that can specifically and effectively target TNBC. One study in particular found that patients with TNBC were more likely to see distant recurrence compared to other types of breast cancer.

A major problem associated with TNBC is metastasis. Metastasis occurs when cancer cells break off from a primary tumor and travel to other parts of the body, typically through the vasculature. Metastasis allows cancerous cells to attach to other organs such as the liver, kidney, and bones and can grow a secondary tumor in that location. The cancerous cells that spread cancer from the initial site are referred to as circulating tumor cells (CTCs) and, in some cases, form clusters. CTC clusters are hard to isolate and assess, as many isolation methods do not accurately represent the true amount of CTCs present in the body and mechanical methods of isolation break down the clusters in the process. The structure of the clusters makes them difficult to treat because, regardless of whether they are loosely or tightly packed, they do not respond to chemotherapy.

Currently, there are many studies that focus on CTC clusters and their interactions with different tissues found in the body. Many of these studies use murine models to assess the in vivo effects of CTC clusters. Murine models are commonly used in research studies that relate to the human immune system because about 80% of gene expression patterns between mice and humans are the same. In one study, a cancerous biopsy from a human was seeded into a mouse and the metastatic potential of CTCs and CTC clusters was measured. This model was used to determine whether clusters from a localized tumor were more effective than single cells in causing cancers in other parts of the body. These murine models provide insight into how CTCs interact with local tissues, but they do not necessarily provide an accurate model as to how CTCs and human tissue would interface. Further research must be done to observe these interactions in a more representative model.

The main objectives of this project were to assess the integration of cancer cells with normal breast tissue, create realistic CTC clusters, and create a model to support the self-assembly of tissue rings. The team brainstormed different design ideas that would address the objectives of the project. Each idea was compared and ratings were distributed based on how well these ideas met our objectives.

To create realistic CTC clusters, the team decided to use a process called the "hanging drop" method. The team also decided to create a negative mold that could be used to create models that would support the self-assembly of tissue rings. Lastly, a 2D and 3D integration

model of the CTC clusters and breast tissue would be used to assess the interaction between the two cell types.

mTNBC (MB-MDA-231) and breast tissue (CRL-1301) cells were used to create hanging drops. The cells were seeded at multiple densities on the inside lid of a 100mm x 20mm petri dish. The lid was then quickly and carefully flipped and put on top of the petri dish in order for the drops to hang and cells to aggregate at the bottom of the drop. The cells were incubated and checked using a stereo microscope periodically to assess progress in cluster formation.

A final negative model that was used to create molds that could support the self-assembly of tissue rings was 3D printed. A high thermal resin was printed on a stereolithographic apparatus (SLA) 3D printer based on an STL file from our SolidWorks part.

The model's intended use was to be laid into a 24 well petri dish that was filled with agarose, each well containing 2 mL. Once the agarose dried, the model would be removed and a negative print of the model would be imprinted into the agarose. Cells could then be seeded into the agarose mold to form tissue rings.

To assess breast cancer and normal tissue cell 2D integration, a TNBC cell line (MB-MDA-231) and human breast tissue cells were seeded on opposite sides of a 60mm x 15mm petri dish, with a 0.5 cm wide PDMS strip, laying flush against the petri dish, in between them. The cancer cells were previously transfected with green fluorescent protein (GFP) while the breast tissue was stained with Hoechst. Seven petri dishes were made, each with 250K cells seeded on either side of the PDMS strip. At T=0, the PDMS strips were removed from all petri dishes. Images were taken using a Brightfield, DAPI, FITC, and DAPI/FITC filter, on a fluorescent microscope at the designated time points.

Using the tissue ring mold, breast tissue and cancer cells would be seeded together to create rings. Different quantities of each cell type could be seeded to see the effects on the 3D interactions between cancer and tissue cells. Additionally, CTC clusters could be seeded within or on the surface of the tissue rings.

Due to time constraints and the COVID-19 pandemic, only some of the experiments outlined were able to be performed. The team was able to gather results from the creation of clusters and the 2D integration model. The other experiments, creating a tissue ring and observing a 3D interaction, were not completed but had anticipated outcomes.

The clusters made for each cell type formed in about 12 hours but were observed over the course of a week and cell proliferation, confluency of cells, and ability to cluster were all noted. From the images the team gathered, some conclusions were drawn. The breast tissue cell cluster did not grow much in size, but it did form a uniform cluster. The cancer cells grew rapidly, but did not form one consistent cluster. The cancer cluster was broken into multiple small clusters rather than one large one.

After performing the 2D cell integration experiment, we did not get the results we intended. This was because the cells were not able to proliferate quickly enough to fill the 0.5 cm space left by the PDMS. It was noted that the cancer cells proliferate at a much higher pace than

the breast tissue cells, but the team never saw the two interact. To improve this experiment, a smaller PDMS strip must be used so that the cancer and tissue cells can interact.

The negative of the tissue ring mold was created, but never tested. The team assumes that the rings would have been able to self-assemble and that they would be seen by the naked eye.

The 3D tissue ring model with both cancer and tissue cells was never tested. If the team was able to complete this experiment, there are three possible expected outcomes; a homogeneous mixture of cancer and tissue cells, multiple small clusters of cancer cells, or one large cluster of cancer cells. The team additionally hoped to seed individual CTC clusters onto rings containing only breast tissue to observe any interactions between the two. Possible outcomes which we might have seen include tumor growth on the surface of the tissue or cancer cell migration into the tissue.

If a 3D cancer model was successfully created, it could be used to test different drug therapies on tumors. Additionally, this model could be used for personalized medicine, as an individual's tumor and tissue could be cultured on a small scale. Therapies could then be tested on this model to determine which will be most effective in the patient before subjecting the patient to multiple treatments that may or may not work for them.

1.0. Introduction

In the United States, breast cancer is the second leading cause of death in women [1]. More specifically, triple negative breast cancer (TNBC) equates to about 15% of breast cancer cases but 35% of breast cancer deaths. TNBC is estrogen receptor-negative (ER-), progesterone receptor-negative (PR-), and human epidermal growth factor 2 negative (HER2-). Because of this "triple negative" characteristic, TNBC is much more difficult to treat. In fact, no treatments currently exist which can specifically and effectively target TNBC [2]. One study found that TNBC is common in women under the age of 45, and patients with TNBC were more likely to see distant recurrence compared to other types of breast cancer. TNBC cases are more likely to result in death compared to other types of breast cancer (approximately 42% vs. 28%) [3].

A major problem associated with TNBC is metastasis. Metastasis occurs when cancer cells break off from a primary tumor and travel to other parts of the body, typically through the vasculature [4]. This allows the cancerous cells to attach to other organs such as the liver, kidney, and bones and grow a secondary tumor in that location [5]. The cells that spread cancer are referred to as circulating tumor cells (CTCs) and can form clusters in some cases. CTC clusters are hard to isolate and assess, as many isolation methods do not accurately represent the true amount of CTCs at hand and some mechanical methods of isolation break down the clusters in the process [6]. The structure of the clusters also makes them difficult to treat. Tightly packed clusters are even more difficult to treat than loosely packed clusters, and neither respond to chemotherapy [7].

Currently, there are many studies that focus on CTC clusters and their effect on the body. A common trend in many of these studies is the use of murine models to study the *in vivo* effects of CTC clusters. Murine models are commonly used in research studies that relate to the human immune system because about 80% of gene expression patterns between mice and humans are the same [8]. In one study, a cancerous biopsy from a human was seeded into a mouse and the metastatic potential of CTCs and CTC clusters was measured [9]. This model was used to determine whether clusters from a localized tumor were more effective than single cells in causing cancers in other parts of the body.

While research using murine models may provide some insight into the effects of CTC clusters, these models do not accurately reflect how human tissue would interface with these clusters. A human cancerous biopsy that is placed inside of a mouse will most likely react differently than if it had been in the human body. A mouse's immune system is far less advanced than a human's, and the antibodies that would normally resist pathogenic microorganisms in a human merely tolerate these foriegn bodies in murine blood [10]. There lies a gap in the research of CTC clusters because the clusters are not being tested in an accurate, *in vitro* human model.

The goal of this project is to create an *in vitro* model of the tumor microenvironment using tumor derived cells in the context of normal human tissue. The hanging drop method was used to create CTC clusters with a metastatic triple breast cancer (mTNBC) cell line. Interactions

between mTNBC cells and human breast tissue cells (fibroblasts) were studied in 2D, with plans in place to additionally study interactions in 3D. The team also designed a model that is a negative version of a ring mold so that it can simply be pressed into liquid agarose to create the desired wells. Both mTNBC cells and human fibroblasts could be seeded into the agarose wells to form tissue rings. In the future, individual CTC clusters could be seeded onto a ring containing only breast tissue. Our designed model fits into a 24-well plate with four rings per well, allowing for a high throughput screening process. This model could be used to grow patient-specific tumors in order to test the effectiveness of various drug therapies before using them on the patient.

2.0. Literature Review

This section covers the applicable physiology, specifically revolving around triple negative metastatic breast cancer (mTNBC), to understand the motive and decisions made throughout this project. Current engineered cancer models used in research to assess cancer treatments will also be discussed.

2.1. Cancer

The original parameters of determining if something could be deemed “cancerous” were dependent upon if cells had demonstrated either invasive or metastatic characteristics. This remained the case even as time and science progressed, and even when methods were being developed to discover tumors before this point. Tumors, even if discovered early on, were not considered “cancerous” until they fulfilled this very dangerous and concerning potential. This was not changed until the development of stages and grades to define cancer in the 1920s and 1930s. Cancer now has an extreme variety of classifications and definitions, depending on size, location, metastatic potential, etc [11].

2.1.1. Cancer Metastasis

Although cancer can spread locally to tissue near the site of origin, it can also spread to distant tissue, organs, or lymph nodes of the body. This process, and the means by which the cancer cells travel, is called metastasis. Metastatic cases are often characterized as Stage IV, and are identified as the same type of cancer as in the original location. For example, prostate cancer cells found in the liver would not be called “liver cancer” cells, but rather “metastatic prostate cancer” cells and would be treated as prostate cancer [12].

2.1.2. Occurrence of Metastasis

The process of cell migration and metastasis is long and complicated. It begins with cells growing and strengthening within their own active site, and eventually they invade nearby tissue. From there, the cancer cells grow towards lymph nodes and/or blood vessels and eventually infiltrate the walls of these systems. The cancerous cells then move through the lymphatic system or blood vessels until they encounter something that stops them or allows them to then infiltrate the surrounding tissue wall again. They then enter a new site and, if they have survived this far, can populate and strengthen to create a new tumor and active cancer site in the new location [12]. The cells which leave their original tumor site and move throughout the body, potentially causing metastasis, are referred to as circulating tumor cells (CTCs), discussed later in this chapter.

It can take years, however, for the metastatic cell to grow and become active in this new site. Although it is extremely difficult for cells to survive through this entire process, it is possible with certain body conditions. Common cancers where metastasis is seen, as well as the common end sites of metastatic cancer cells for each, are outlined in Table 2.1. Metastasis is most commonly seen in epithelial cancers [12].

Table 2.1. Common Cancers Associated with Metastasis and Metastatic End Sites, Adapted from the National Cancer Institute

Cancer Type	Main Sites of Metastasis
Bladder	Bone, Liver, Lung
Breast	Bone, Brain, Liver, Lung
Colon	Liver, Lung, Peritoneum
Kidney	Adrenal Gland, Bone, Brain, Liver, Lung
Lung	Adrenal Gland, Bone, Brain, Liver, Other Lung
Melanoma	Bone, Brain, Liver, Lung, Skin, Muscle
Ovary	Liver, Lung, Peritoneum
Pancreas	Liver, Lung, Peritoneum
Prostate	Adrenal Gland, Bone, Liver, Lung
Rectal	Liver, Lung, Peritoneum
Stomach	Liver, Lung, Peritoneum
Thyroid	Bone, Liver, Lung
Uterus	Bone, Liver, Lung, Peritoneum, Vagina

2.1.3. Detection of Metastasis

Metastatic cancer symptoms are specific to the cancer at the active site. For example, patients whose metastatic cells have spread to the bone may experience bone fracture. If the cancer has spread to the brain, headaches and dizziness may occur. The patient might experience shortness of breath if the metastatic cells are located in the lungs [12]. Since symptoms do not disclose any insight into the possibility of a tumor that originated somewhere else in the body,

and do not necessarily imply a tumor in the first place, it is especially difficult to identify metastasis. This is especially concerning when the tumor has developed to, or developed past, a dangerous level.

There are no regular screenings to detect metastatic cells or tumors, since they are not found in their expected location. For example, there is no way that a mammogram will detect breast cancer cells in the liver. However, if they are large enough, metastatic tumors will appear on ultrasounds, PET scans, etc. From there, a laboratory examination after a biopsy or surgery will detect them as being metastatic because cells found in a new location will still display the phenotype and characterization of the cancer cells from the original site [13].

New technology is being developed to detect metastatic cancer cells more quickly and effectively during this examination process. More specifically, a morphology based machine learning technique was investigated to predict metastasis in rectal cancer tumor images. Tumor depth, lymphatic invasion, venous invasion, poorly differentiated clusters, and tumor budding were evaluated. The study showed promising results, as seen in Figure 2.1, where the machine learning process produced a higher area under the curve, regarding a true-positive fraction to false-positive fraction ratio, in comparison with human-based processes. The machine learning technology is being studied and improved to decrease both false-positive and false-negative rates seen in the study, but shows potential to detect and predict metastasis more quickly than the human-based methods currently used [14].

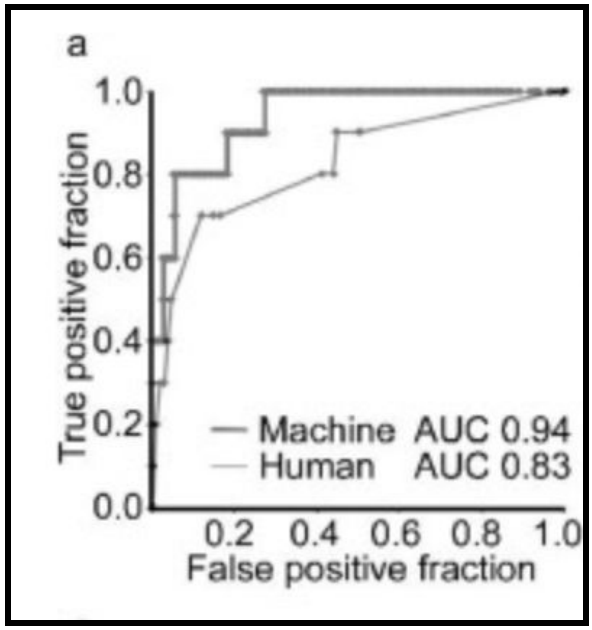

Figure 2.1. Results of a Japanese Machine Learning Study to Predict Metastasis in Rectal Cancer Shows Promising Results [14].

2.1.4. Treatment for Metastasis

Unfortunately, cancer cells become extremely difficult to treat once they become metastatic. Current available treatment aims to slow or stop metastatic tumor growth, rather than prevent metastasis in the first place. Treatment is not necessarily effective in ridding the cancer, but can play a role in prolonging patient life and lessening symptoms for patient comfort and well-being [12]. There lies a gap in treating and killing cancer cells before they become metastatic, or before they fully complete the migration process and the creation of a new tumor.

2.2. Breast Cancer

Breast cancer is the second most common cause of death from cancer in women. Metastasis is the cause of many deaths associated with breast cancer. While metastatic breast cancer is treatable, it is not curable. This makes the five-year survival rate of triple negative metastatic breast cancer, to be explained in Chapter 2.2.1, only 11% [15]. Research must be done to better diagnose and treat this invasive cancer, and ultimately increase the survival rate of these patients.

2.2.1. Classification

A main method for breast cancer classification relies on the receptors that cancer cells do or do not possess. Estrogen receptor positive (ER+) breast cancer is characterized by cells which have estrogen receptors. If the cells do not have estrogen receptors, the cancer is deemed estrogen receptor negative (ER-). Along similar lines, progesterone receptor positive (PR+) breast cancer lines possess progesterone receptors, meanwhile progesterone receptor negative (PR-) breast cancer lines do not. Human epidermal growth factor type 2 positive (HER2+) breast cancer cells possess more than the normal amount of human epidermal growth factor type 2 receptor, whereas human epidermal growth factor type 2 negative (HER2-) breast cancer cells contain a normal amount. HER2+ breast cancer is more likely to spread and divide as compared to HER2- breast cancer [13].

Triple negative breast cancer (TNBC) equates to about 15% of breast cancer cases but 35% of breast cancer deaths. TNBC is ER-, PR-, and HER2-. Due to this “triple negative” characteristic, TNBC is much more difficult to treat compared to other cancers. In fact, no treatments currently exist which can specifically and effectively target TNBC [2]. One study in particular found that TNBC is more common in women under the age of 45 and patients with TNBC were more likely to see metastatic conditions compared to other types of breast cancer. TNBC cases are more likely to result in death compared to other types of breast cancer (approximately 42% vs. 28%) [3].

TNBC's receptor traits, along with the size of the tumor, grade of cell differentiation, distance which the cancer has spread, etc., determine the stage of cancer. The stage of cancer ranges from II to IV and determines the seriousness of the case, next steps for treatment, and ultimately the survival rate of the patient. The receptors (ER, HER2, PR) that cancer cells possess or do not possess specifically play a major role in determining which treatment or treatments to investigate and utilize [13].

2.2.2. Treatment

There currently exists no treatment for metastatic triple negative breast cancer (mTNBC) [1]. This creates obvious concern for those affected by mTNBC. Treatment is being explored and researched, however there exists a high failure rate. This could be due to the lack of available experimentation models which can provide an accurate mTNBC atmosphere.

2.3. Circulating Tumor Cells

Each cancerous tumor consists of millions of cells that harbor genetic mutations. These cells can grow, divide, and invade the local tissue in which they are embedded. Cancerous cells are bound by intercellular junctions that keep the tumor stable and able to proliferate into local tissue. However, some cells can be swept away by the bloodstream or lymphatic system. These cells are known as Circulating Tumor Cells (CTCs). CTCs can dislodge from a tumor in either single cells or a cluster. They can leave their origin tumor and migrate throughout the body, lodging themselves onto new tissues and creating a new tumor.

In one teaspoon of blood, cancer patients usually present about 5 to 50 CTCs. This number is miniscule in comparison to the amount of blood cells in the same sample. Even a small concentration of CTCs is enough to spread cancer throughout the body. A study showed that there is a correlation between the number of CTCs found in a patient's blood and the progression of their disease [16]. Researchers found that breast cancer patients with fewer CTCs in their blood lived longer than those with a higher number of CTCs, proving that CTCs can accelerate the rate at which cancer affects a person.

Isolating CTCs from a patient's blood has proved difficult. Only in the past decade or so have researchers been able to separate CTCs from surrounding blood cells. One of the first established methods of isolating CTCs was based on these cells' physical properties. A blood sample was centrifuged to separate the components (red blood cells, buffy coat, and plasma) into different layers, as seen in Figure 2.2. The density of CTCs caused them to fall into the buffy coat layer, where white blood cells also resided. The CTCs were then filtered out by size, as CTCs are much larger than white blood cells.

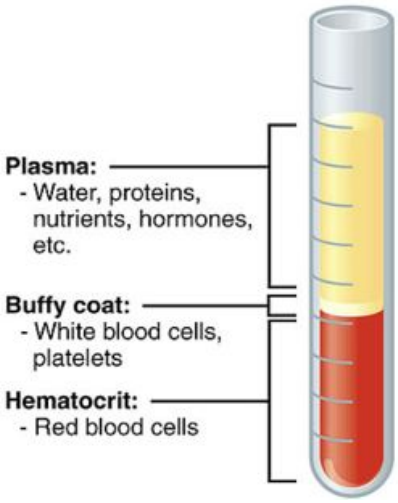

Figure 2.2. Centrifuged Blood Sample Layers [17]

Methods for isolating CTCs have advanced over the years. Currently, CTC isolation techniques are more dependent on targeting epithelial cell-adhesion molecules (EpCAMs), which are proteins that protrude from the outer surface of CTCs and not normal blood cells. Antibodies can bind to these EpCAMs, so CTCs are locked in place. One example of this method being used is in a microfluidic silicon chip that has been designed with thousands of EpCAM antibody-coated microposts [18]. As blood flows through the chip, CTCs bind onto the microposts, allowing for easy capture.

2.4. Engineered Tumor Models

Engineered tumor models are often used to create a physiological representation of a disease within the body. In biomedical research, tissue engineered tumor models are used to mimic the native tumor microenvironment. Creating tumor models allows doctors and researchers to gain more information on a disease state.

2.4.1. *In Vivo* Models

Animal models have been widely used in translational research for cancer therapeutics. Mice are commonly used because "(a) they are small in size; (b) they are inexpensive to maintain; (c) they reproduce rapidly and have large litters; and (d) they can be genetically manipulated," which makes them advantageous over other mammalian models [19]. There are three main types of rodent cancer models, with one being xenograft cancer models. In this model, human or animal cancer cells are transplanted into an immunogenic mouse under the skin or at the organ of tumor origin. Xenograft models are primarily used for researching drug therapies and imaging techniques. This type of model is relatively inexpensive compared to other types; however, xenograft models do not accurately represent the normal conditions of cancer

within the human body. This is because the immune system of the rodent is compromised and natural reaction to tumors is not present in this model [20].

Other rodent cancer models include chemically or genetically induced cancer models. The chemically induced cancer model is developed by exposing rodents to carcinogens to ultimately cause tumor formation. This method is often time consuming due to the lack of tumors that usually form. In genetically engineered rodent models, a sequence of DNA is injected into the nuclei of fertilized zygotes to integrate this sequence into the genome of the mouse. This sequence will either cause a cell to convert into a tumor cell or will prevent the expression of tumor suppressor genes. Although this method has shown great promise with translational cancer research, the major disadvantage is there is no way to express the genes of interest 100% of the time. For an *in vivo* model to show promise, the animal must not only have similar physiology to humans, but also must be able to accurately represent human body reactions against cancer cells.

2.4.2. *In Vitro* Models

As opposed to *in vivo* models, *in vitro* models utilize cells and tissues outside of the body to mimic normal physiological conditions for tumor microenvironments. In cancer research, these models help scientists study aspects of cancer such as tumor growth, migration, drug delivery, etc. *In vitro* models allow for the control of certain variables such as cell sources, extracellular matrix (ECM), and biochemical cues. *In vitro* models are broken down into different categories in cancer research, as seen in Table 2.2 [21].

Table 2.2. Types of in vitro tumor models separated into four different categories, as well as descriptions of the processes related to cancer growth and progression, adapted from [21].

Transwell-based Models	
Model	**Phenomena**
Migration	Migration, intravasation, extravasation, drug screening
Invasion	Invasion, intravasation, extravasation, matrix remodeling, drug screening
Transendothelial Migration	Intravasation, drug screening
Spheroid-Based Models	
Model	**Phenomena**
Spheroids in media	Growth/proliferation, drug screening

Spheroids in gels	Growth/proliferation, invasion, matrix remodeling, angiogenesis, drug screening
Coculture	Invasion, angiogenesis, drug screening, immune interactions
Tumor-microvessel Models	
Model	**Phenomena**
Predefined ECM scaffold	Invasion, intravasation, extravasation, angiogenesis, dormancy, drug delivery
Microvessel self-assembly	Invasion, intravasation, extravasation, angiogenesis, dormancy, drug delivery

2.4.3. Transwell-Based Models

Transwell-based assays are commonly used in cancer research to model cellular migration and invasion [21]. Migration is defined as any cell movement within the body from one location, such as long ECM fibers or a basal membrane, to another [22]. Invasion is described as the penetration of tissue barriers. An example of this could be cancer cells passing through basement membranes and infiltrating underlying interstitial tissues. While migration occurs on a 2D surface, invasion occurs in a 3D environment [23].

There are three variations of common transwell-based assays: migration assays, invasion assays, and transendothelial migration assays. Migration assays involve the seeding of cancer cells onto the surface of a porous membrane. The cells would then migrate vertically down the membrane towards a compartment with medium, which contains a chemical attractant or a higher serum concentration. Invasion assays follow the same principle as migration assays, but an additional layer of ECM is placed over the porous membrane to block non-invasive cells from migrating through the membrane. The invasive cells, or cancer cells, will migrate through the ECM and can be tracked using fluorometric detection [23]. Transendothelial migration assays use a monolayer of endothelium on top of a porous scaffold. This method utilizes junctions between the endothelial cells and the ECM they produce. This kind of model shows more accurate interactions between cancer cells and endothelium [21]. Figure 2.3 shows the general set up for these three types of transwell-based models.

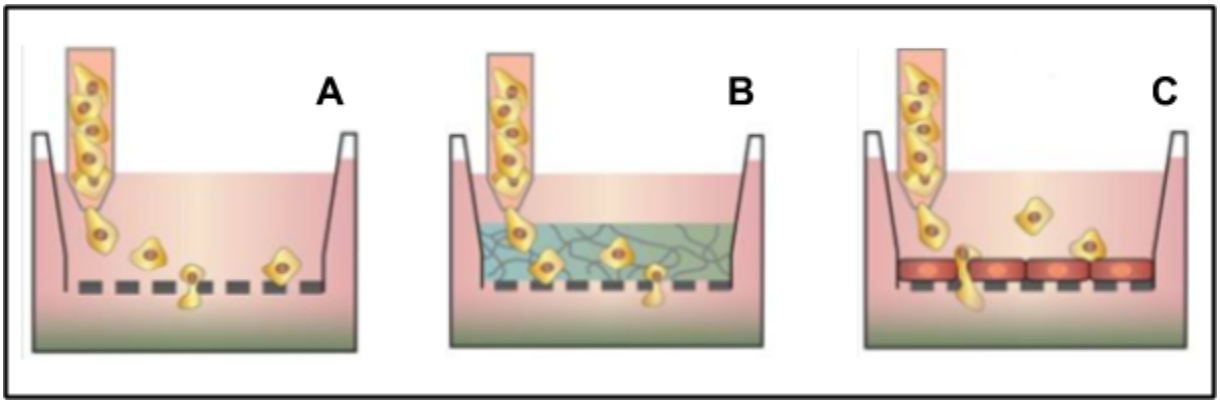

Figure 2.3. Three types of trans-based models. A) Migration Assay B) Invasion Assays C) Transendothelial Assays. Adapted from [21].

2.4.4. Spheroid-Based Models

Spheroids are a collection of cells that are studied in suspension and 3D culture [21], [24]. Spheroids are preferred over 2D culture models because they show a more accurate representation of interactions between different cell types as well as between cells and ECM [24]. In cancer research, spheroids are created using a multicellular suspension, which is composed of the cancer cell line endothelium, fibroblasts, and immune cells. Spheroid-based models are utilized in drug screening, where the effect on the diameter of spheroids is observed with the use of various compounds [25].

There are multiple methods that are used to form cancer spheroids for research. One of the earliest and most common methods used is the hanging drop method, which is shown in Figure 2.4. The hanging drop method involves pipetting a suspension of cancer cells within medium on the underside of a culture plate and inverting it to allow the cells to aggregate to the bottom of the drop [24]. This method allows for the control of the spheroid size and can allow for co-culture with different cell types with relative ease. A disadvantage of the hanging drop method is that there can be a low output of clusters due to difficulties with media changes [21]. Another method for forming cancer spheroids uses microfluidic devices, which are able to continuously create cell spheroids. Using microfluidic devices, however, requires proper equipment to collect and analyze the spheroids [24].

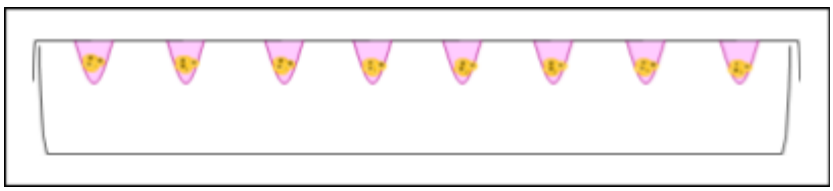

Figure 2.4. Depiction of the hanging drop method, which allows for the creation of multicellular tumor spheroids.

2.4.5. Tumor-Microvessel Models

Tumor-microvessel models are different from most other tumor models because they introduce vasculature to mimic the environment of tumor cells within blood vessels or lymph vessels [21], [26]. A schematic showing the model's set up can be seen in Figure 2.5. This model involves the use of a scaffold device with predefined vasculature that is placed above a glass slide used for imaging. The scaffold is primarily made up of polydimethylsiloxane (PDMS), with diameters ranging between 75 - 250 μm. Media is flowed through the scaffold and fills the vasculature, which is then seeded with epithelium to align the vessel walls. To accurately model the tumor microenvironment, tumors are grown around the surrounding ECM, allowing tumor cell invasion and intravasation using either single tumor cells or tumor cell clusters [26]. Tumor-microvessel models allow for the study of tumor environments with complex vasculature, but there still needs to be more work done to understand the interactions between the vessels and tumor cells that contribute to tumor angiogenesis and proliferation [21].

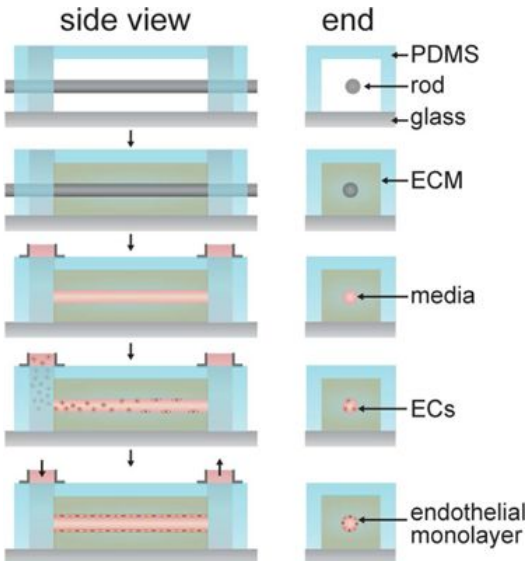

Figure 2.5. Schematic illustration of template synthesis of a microvessel. A single cylindrical channel is formed by casting a solution form of ECM around a template rod. The rod is removed, and the channel is perfused with media and endothelial cells to form a confluent vessel endothelium [26].

The models discussed in Chapter 2.4 allow researchers to represent how tumors behave within the body. The models also allow for testing different aspects of tumor progression. Even though each model can predict certain behaviors of the tumor cells, each model also has drawbacks, such as failing to represent every aspect of tumor growth and metastasis. These drawbacks produce a need for a more physiologically accurate model that can be easily manufactured and maintained to be used in research and future drug therapies and giving a high throughput of results.

2.5. Role of CTCs in Cancer Treatment

CTCs contain genomic information that can give doctors data on the presence and status of a tumor. Isolating and characterizing these CTCs can play a major role in treating the tumor and can also lead to advances in patient-specific treatment. Specifically, the isolation of CTCs can aid in prognosis and predicting survival in metastatic breast cancer patients.

2.5.1. CTCs Predicting Metastasis

Because of a CTC's ability to break off of preexisting tumors and circulate throughout the body through vasculature, a blood sample can be taken from a cancer patient to determine if CTCs are present. Finding CTCs in a blood sample can indicate the presence of a tumor elsewhere in the body [27]. Taking a blood biopsy is significantly less invasive than taking a tumor sample, and can provide just as much information on the cancer through the genotype of the CTCs.

Typically, cancer and metastasis are measured using radiology. Like any technology, radiology has its limitations, including user error and issues with reproducibility [28]. Variability in radiological evaluations due to different interpretations is up to 15%, while variability using CTCs is about 1%. Evaluating CTCs is also beneficial as it can be performed much earlier than radiology. A blood sample can be collected and examined at 3-4 weeks while radiology on the tumor cannot be performed until 8-12 weeks [29]. Data collected from CTCs is a better predictor of the disease state and survival of the patient than currently used radiology techniques.

2.5.2. CTCs in Breast Cancer Diagnosis and Treatment

For patients with breast cancer, survival rates decrease once the cancer becomes metastatic [27]. Prior to the development of a metastatic tumor, CTCs can be evaluated to determine a prognosis. Higher quantities of CTCs lead to a lower chance of survival, as each CTC has the capability of forming a micrometastasis on another organ. The number of CTCs can be quantified before and after chemotherapy treatment. In many cases, the number is reduced after chemotherapy; however, CTCs tend to grow resistant to chemotherapy, thus requiring higher dosages with each round of treatment. In cases where the number of CTCs is not reduced after chemotherapy, the patient's cancer is more likely to become metastatic. In this case, other treatment methods must be tried. The number of CTCs following the first round of treatment is the most important indicator of cancer progression and metastasis [29].

2.5.3. Personalized Medicine

Personalized medicine is when medical treatments are tailored to the patient's needs [30]. Due to advances in genomic technology, doctors now have the ability to gain information on a

patient's disease susceptibility based on their genetics. With these advances, doctors can also gauge how a patient will respond to treatment. A major benefit of personalized medicine is that treatment can be proactive instead of reactive.

The characteristics of each tumor are different, which means that treatments may be effective for some but not others. Characterizing CTCs allows doctors to gain insight on the composition of the specific tumor where the CTCs originated. This allows for the creation of a treatment plan that has been tailored to a specific patient's tumor and is more likely to be effective [27]. Personalized medicine also allows doctors to rule out treatments that are not working for a specific patient.

3.0 Project Strategy

This section summarizes the team's process of establishing a client statement and project objectives. Also, this section acknowledges any constraints and requirements that must be considered during tumor model experimentation. Lastly, a rough schedule of the team's project work is outlined and displayed as a Gantt chart.

3.1. Initial Client Statement

About 12% of women in the United States will be diagnosed with invasive breast cancer in their lifetime. Metastatic breast cancer, seen when breast cancer spreads to other areas of the body, is treatable but not curable. The five-year survival rate of triple negative metastatic breast cancer is only 11%. Methods to create a more accurate prognosis are needed to allow doctors and researchers to develop better treatments against metastatic breast cancer. There is a need for a model that will allow for doctors to more accurately and efficiently test cancer drugs and therapies to determine how they will affect breast cancer in a specific patient. Research must be done to better diagnose and treat metastatic breast cancer and ultimately increase the survival rate of these patients.

3.2. Objectives & Constraints

Our main objective was to test parameters that play a role in the creation of a 3D cancerous tissue model which could futuristically be used to assess mTNBC treatments. Firstly, we wanted to test and assess the integration of breast cancer cells with normal breast tissue cells. Before continuing on with the composition of a 3D tissue model, we firstly needed to compare the rates of growth between cancerous and normal breast tissue cells. In this, we seeked to assess cell behavior and function of these cell types once combined.

Secondly, we wanted to induce the formation of clusters for single cell types, as well as clusters containing both cell types. We wanted to see how well cells cluster depending on the cell type or combination of cell types, also depending on the number of cells seeded into the cluster. This objective would allow us to create the visible clusters which would be found in a final 3D tissue model, and would give us insight into the cluster's composition and formation.

Finally, we needed to determine the culture methods and tissue ring model parameters that best support 3D tissue formation. We aimed to adjust a current ring mold fixture in order to make the molding process more efficient and to ensure a structure that encouraged tissue-cancer cluster formation. We needed to establish which phase in the cell cycle cells should be seeded so that they can successfully form a ring.

Since our ring mold was an updated version of a currently used ring mold, we

additionally needed to make sure that our improved mold would allow for high ring reproducibility. This would ultimately maximize throughput and the amount of drug screening which can be completed with the model. The tissue ring model should therefore fit into a welled tissue culture plate, with multiple rings to be created in each well. The mold would also need to properly comply with well plate dimensions and withstand autoclave conditions to ensure sterilization. The mold needed to be a proper size, big enough so that models are structurally sound and do not rupture from the force of their own compaction, but small enough so cells throughout all layers of the ring receive enough nutrients. The well plate would be filled with agarose as a biomaterial in order to avoid cellular adherence to the mold.

The completion of these objectives was difficult, however, due to a number of constraints. Our team was working under a limited amount of time, with only 28 weeks to design, plan, and complete our project ideas. Along with this, each team member had an additional job, courses, extracurriculars, and other external commitments to maintain. We were also working in a limited laboratory space, with limited supplies and resources. We were allocated a budget to help ease this problem, however this was also limited.

3.3. Design Requirements

In the completion of our project, we needed to take the standards, guidelines, and expectations of the International Organization for Standardization and the Food and Drug Administration into consideration.

3.3.1. International Organization for Standardization (ISO)

164 members representing 164 countries form The International Organization for Standardization (ISO), an organization that provides internationally enforced industry standards. A variety of fields, including technology, food safety, and healthcare, are monitored by ISO standards and regulations [31]. Although not all ISO specifications applied to our project work, there are some that were relevant to our project's goal and future work. Standards to consider for our project include, but are not limited to, ISO 13845, ISO 20916, and ISO 11737-2:2009.

ISO 13845, specific to the medical device industry, outlines "the quality management requirements for manufacturers of medical devices." The standard does not set fixed specifications, but rather encourages quality assurance, risk management, and manufacturing controls to be set in place to ensure effectiveness, safety, and customer satisfaction [32]. Although it does not apply to what was accomplished in the scope of our project, ISO standard 13845 would need to be considered and followed in the design, manufacturing, and regulation of our 3D printed ring mold if this design was used moving forward. The tumor model which we aim to work towards also generally constitutes as a medical device, according to ISO [32].

ISO 20916 is a standard which is specifically applicable to our model; It outlines regulations for studies which utilize tissue or other specimens from human subjects. The standard

is not meant to assess a clinical process' success in its results, but rather provides instructions for proper setup of the clinical process and guidelines to be followed throughout. More specifically, the standard aims to protect patient rights, safety, dignity, etc. ISO 20916 also aims to ensure that samples are properly pulled from a patient and properly tested to acquire "reliable and robust results," since samples are limited and valuable [33]. Generally speaking, ISO 11737-2:2009 regards the creation, validation, and maintenance of all medical device sterility processes and will be relevant in the sterility of our ring mold and cell culture performance [34].

3.3.2. Food and Drug Administration (FDA)

The Food and Drug Administration (FDA) is a United States federal organization that ensures the safety and quality of drug products, food products, medical equipment, etc. Our proposed model fits under the FDA's medical device classification of an "... implant, in vitro reagent, or other similar or related article... intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals..." [35]. In order for our ring mold fixture and overall cancer model to reach the market and be used in diagnostics and treatment, it must first undergo a validation process overseen by the FDA and ultimately acquire FDA approval.

3.3.3. Good Cell Culture Practice (GCCP)

Throughout the duration of our testing, Good Cell Culture Practice (GCCP) was used. GCCP is a term used in human and animal cell and tissue culture to indicate that proper techniques were used and standard principles were followed. These "good" practices revolve around the characterisation and maintenance of essential characteristics, quality assurance, recording and reporting, safety, education & training, and ethics [36]. More specific practices performed in our experimentation include, but are not limited to, the keeping and updating of a lab notebook, proper and safe cell culture techniques, sterile and proper equipment use, etc.

3.4. Revised Client Statement

The most common treatment currently used for patients with triple negative metastatic breast cancer (mTNBC) is chemotherapy, providing little to no success. The average length of survival after a diagnosis for those who receive treatment is only 13.3 months. Researchers require a more accurate and efficient method for testing mTNBC treatment which can be more effective for patients. A patient-specific model combining healthy tissue with cancerous tissue is needed to increase the survival rate of patients.

3.5. Project Approach

Considering the objectives which we set and the limited time to achieve them, our group created a Gantt chart and a detailed work breakdown structure in order to ensure that the team stayed on track. The Gantt chart created can be seen below in Figure 3.1. Our team additionally held regularly scheduled (weekly) meetings with our project advisor, as well as with each other, to ensure team management and communication. We met with and communicated via email with supplemental advisors when their principal knowledge in a subject was needed. All laboratory practices were performed in Goddard Hall 006 of Worcester Polytechnic Institute. Our team kept an up-to-date laboratory notebook with our activity and progress.

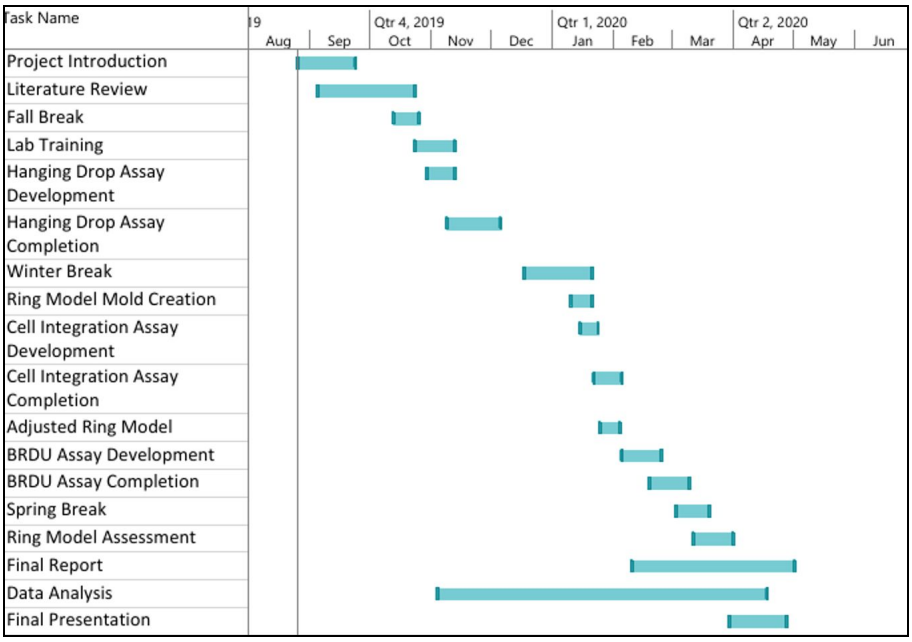

Figure 3.1. Project team's Gantt Chart.

4.0. Design Process

This section will cover the team's design process and the development of our experimentation to improve the treatment for mTNBC. We established what was important and needed in our final design, and outlined experiments and methods to fit these needs. We established our final design and the deliverables for our project.

4.1. Needs Analysis

A major issue with developing a treatment for mTNBC is that there is no experimental model to thoroughly study the disease. Through research of previous disease models, we were

able to identify gaps in these models and create a model to potentially fill those gaps. The animal models currently used to study breast cancer have not been able to accurately mimic how human patients would react to current therapeutics. There are also *in vitro* models that are used to study breast cancer and mechanisms that can cause the disease. These models, however, do not include all the complex components of a tumor in its microenvironment. This would include aspects such as immune cells, ECM proteins, and other cell types that are a part of the tumor's microenvironment within the human body.

Our group believed that in order to more effectively treat mTNBC, it would be best to create a model that could mimic the patient's response to potential therapeutics. This would give reliable data and the model could also be used for clinical trials in the future. The requirements for this model were evaluated using the Pugh Method, which is a widely used ranking system used in evaluating designs [37]. A matrix weighing the needs for our design can be seen in Table 4.1.

Table 4.1. Design requirements and assigned weights for creation of a tumor model.

Requirement	Weight
Contains normal tissue and cancerous cells that were retrieved from the patient	5
High throughput for drug screening results	5
Easily manufactured	4
Includes complex components, such as immune cells, necessary for tumor microenvironment	3
Represents three dimensional in vivo interactions between cancer and tissue	4

Each of these requirements were weighed on a scale of 1 to 5 to determine their importance in the design, with 1 being not important and 5 being very important. Containing the patient's cancer cells was ranked as very important because it was believed that this requirement would allow for more accurate drug screening, and using the patient's cells could allow for a more personalized approach. Having a high throughput would allow for more results from drug screening. To create a patient-specific disease model, it is believed that the model should be able to be manufactured with great ease. The goal of representing three dimensional interactions between cancer cells and normal tissue was discussed in our initial evaluation, but was ultimately given a score of 4 due to it adding more complexity to the disease model for the scope of our project. However, adding other components from the tumor microenvironment into our model would allow for the model to mimic the environment seen *in vivo* as much as possible.

4.2. Design Concepts

This section will discuss our team's reasoning and process in developing our project design. Previous work and different design stages were reviewed to help influence the teams choices. This section will also give an overview of different ideas and preliminary work that went into our final design.

4.2.1 Preliminary Data

Previous work has shown the feasibility of using cell self-assembly to create tissue models. Professor Marsha Rolle at Worcester Polytechnic Institute has studied the use of human vascular smooth muscle cells to create a tissue engineered blood vessel. This work showed the effectiveness of creating rings using cells which produce their own extracellular matrix The principal investigator of this research hopes that it could eventually be used for vascular disease modeling and drug screening [38]. This study shows that there is potential to create a three-dimensional tissue model utilizing cell self-assembly.

Another study performed in Professor Raymond Page's Laboratory at Worcester Polytechnic Institute conducted research using skeletal muscle tissue rings to develop to create muscular tissue and an *in vitro* model for different muscular disorders [39]. Initial work showed that the tissue rings would rupture after a short period of time in culture. To combat this issue, cells were seeded into ring-shaped wells made of a 2% agarose hydrogel and remained in culture using media supplemented with adult horse serum. This media caused the cells to exit the cell cycle and stop cell proliferation. This culture method was used to drive the cells into forming their own extracellular matrix and reduce proliferation.

The wells that were used as the tissue mold contained a post in the middle of the well to help the cells assemble into the rings. Also, the posts were designed at an angle. The study tested angles of 85 and 87.5 degrees to assess the effects of the ring formation, and showed that the 87.5 degree posts yielded the best results for ring formation and their duration in culture [39].

The model developed in Professor Page's laboratory was able to show signs of the correct phenotype for skeletal muscle *in vivo* without the use of a scaffold material. This study lays the foundation for future work revolving around the creation of a diseased tissue model to allow for drug screening and other applications for personalized medicine, which is something that our team's research is working towards. This dissertation's work shows the feasibility for our designs, and provides insight that could help us to create a self-assembled tissue model for use in breast cancer research and treatment.

4.2.2 Conceptual Designs

To develop a treatment for mTNBC, there were different avenues that our group discussed. The team discussed using a diagnostic tool to be able to help patients, but it was believed that using a disease model will help to discover and assess therapeutics for years to come. The *in vitro* models were believed to be better suited than other options to model breast cancer because they would have the potential to be used on a more personalized level and can be easily manufactured. Figure 4.1 maps out our considered concepts to help with treatment of mTNBC.

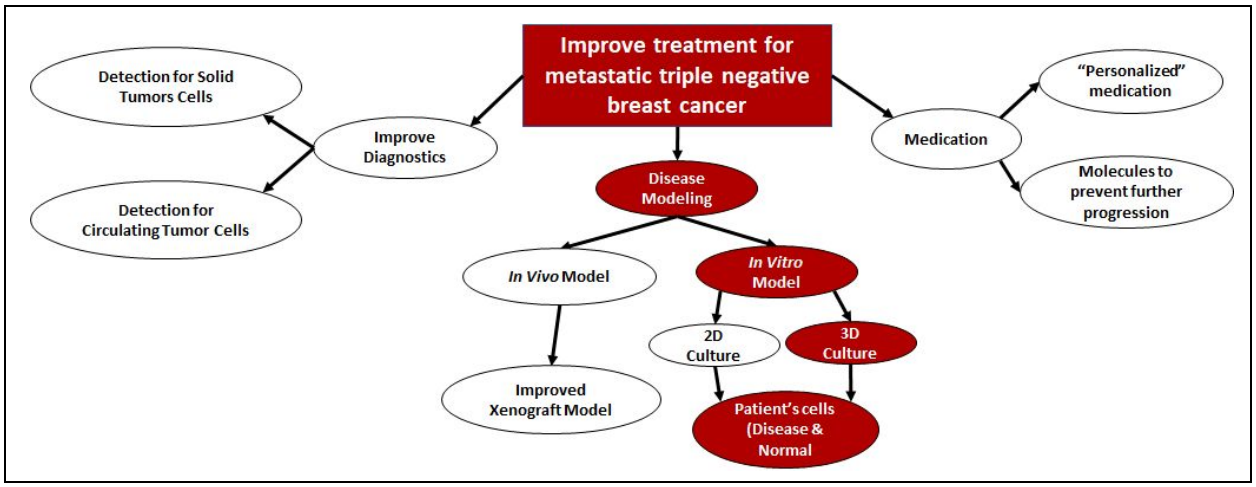

Figure 4.1. Conceptual map to look into potential tools to be used for treatment of mTNBC.

4.3. Alternative Designs

At the beginning of the design process, a few methods were considered for creating clusters and tissue as well as studying interactions between the two. It was important to evaluate options in order to choose the methods that best fit the goal of this project.

4.3.1. Cell Clusters

A common method to create cell clusters is through cell aggregation. One specific technique uses spheroid formation medium and embeds cells in a methyl cellulose matrix [40]. Cells are cultured in a 96-well plate designed to be cell repellent to prevent cells from attempting to adhere to the bottom of the plate. Within the first 24-48 hours, the cells should form clusters that can be removed from the wells. Similarly, the clusters can be formed by embedding cells in a collagen matrix. This method provides a very tunable approach to create the desired 3D cell clusters. While working with the methyl cellulose and collagen, it is very important that the mixtures are homogeneous and no outside particles are in the matrix solution. Figure 4.2 depicts this process.

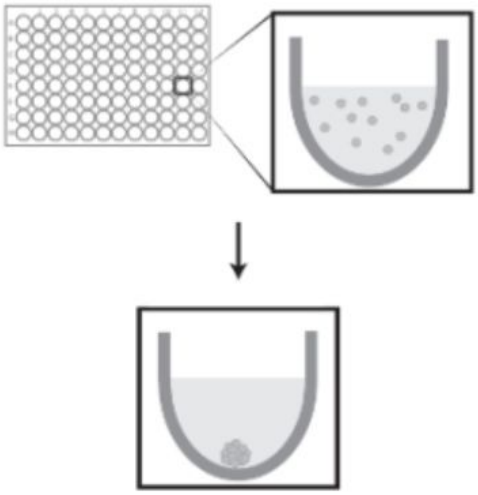

Figure 4.2. Schematic of cellular aggregation within a methyl cellulose matrix [40].

Cell clusters can also be formed through a more natural process using cell-cell interactions. This method has been used for creating clusters containing at least two different cell types [41]. Initially after seeding, the multiple cell types in this method naturally begin to aggregate. When the cells come in contact with each other, they begin to express more cadherin, which is a cell adhesion molecule that functions to bind cells to one another. The cadherin molecule is expressed on a cell's surface, and the cadherin interactions between cells cause the cells to tightly aggregate. This method, however, relies heavily on the cells following a natural process, one which is out of researchers' control. A schematic depicting this process is shown in Figure 4.3.

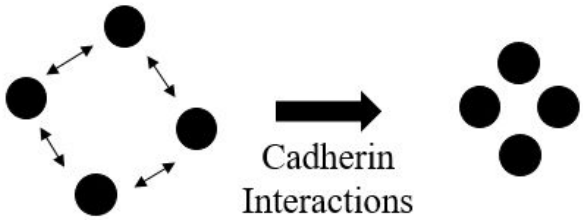

Figure 4.3. Schematic depicting cadherin interactions between cells leading to aggregation [41].

4.3.2. Tissue

Scaffolds made from various natural and synthetic materials play a large role in tissue formation and regeneration [42]. Using a scaffold allows the researcher to fine tune material properties to influence cell growth and tissue formation. A scaffold is typically porous to allow for cell migration in and throughout the scaffold as shown in Figure 4.4. Cells are seeded onto the scaffold where they adhere and begin to secrete their ECM. Ideally, the scaffold will degrade in time and a 3D mass of cells and matrix that roughly resembles a tissue will remain. An issue with this approach is that nutrients may not diffuse to the middle of the scaffold and the cells in

this region will die. Additionally, the scaffold material must maintain its structure long enough for the cells to develop their matrix, but must degrade to allow for a cell-only tissue. Using a scaffold also requires additional materials and more time for creating the scaffold. Ideally, tissue formation moves away from using scaffolds.

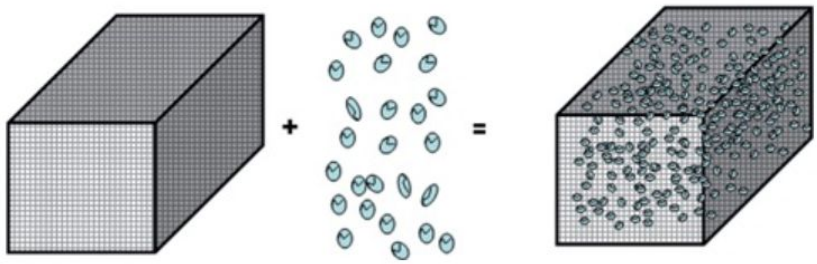

Figure 4.4. Schematic of cells combined with a porous scaffold [43].

Cellular self-assembly methods can also be used to create tissues. For example, one study utilized a well containing a 2 mm agarose post in the center [44]. A high concentration of cells (500,000 cells/mL) was seeded into the well and, over the course of four days, a ring formed around the post as shown in Figure 4.5

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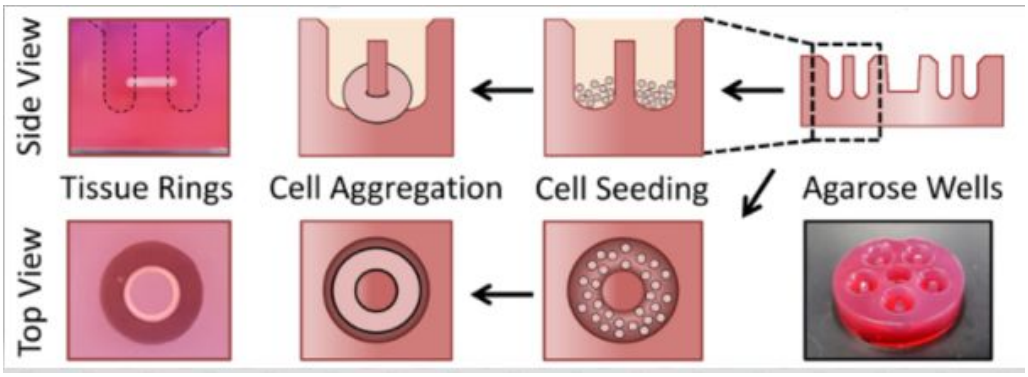

Figure 4.5. Tissue ring seeding process and cellular self-assembly [44].

These rings can then be removed from the wells for analysis. Cells used for this method are often smooth muscle cells, as they exhibit contraction and will move to contract around the post. This method does not require a scaffold and the use of agarose is beneficial as cells will not stick to it. Using such a high cell seeding density, however, tends to be costly as well as leads to necrotic tissue. The cells within the ring are tightly packed and those touching the post in the center receive little to no nutrients, resulting in cell death [39]. The post is at an 85 degree angle, making it difficult for the formed ring to migrate up the post during contraction. This causes the ring to settle at the bottom of the well, which makes the ring difficult to remove from the agarose. In this case the removal from agarose results in the breaking of rings until the researcher is familiar with the procedure.

4.3.3 Integration

It is important that the tissue cells and cancer cells interact with each other as they would inside the body. One way to monitor cell type interactions is on a 2D surface such as a cell culture dish. In this method each cell type is plated on either side of a dish and are then allowed to grow into each other. Cell morphology changes as well as cell growth can be monitored. Unfortunately, this method lacks the 3D complexity that is representative of *in vivo* conditions. A 3D model can be used to add in the 3D complexity component that allows for a more accurate cancer model. Both models are shown in Figure 4.6.

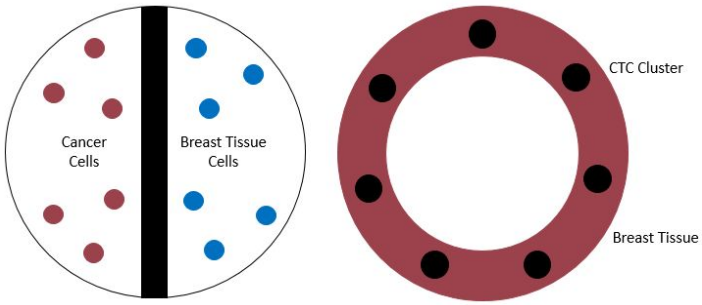

Figure 4.6. Schematic showing a possible method for 2D (left) and 3D (right) integration.

4.4. Final Design Selection

After considering various alternative designs, the team decided on how we would meet the previously stated objectives. To create realistic CTC clusters, the team decided to use the hanging drop method. The team also decided to use a ring model to create tissue. And finally, a 2D and 3D integration model of the breast CTC clusters and normal breast tissue were chosen to assess the interaction between the two cell types.

The hanging drop method was chosen over other alternative designs because it forces the cells to aggregate while still producing a realistic cluster model. The group wanted to be able to easily replicate the clusters and this method provided a consistent way to create the clusters. This method was also beneficial because it was easy to produce and allowed for multiple clusters to be made at once. A decision matrix comparing how alternative design ideas met the identified design requirements can be seen in Table 4.2.

Table 4.2. Decision Matrix for Cell Cluster.

Characteristics	Cell-Cell Interactions	Hanging Drop	Embedded Cells
Uniform Cluster	3	3	4
Self Aggregate	4	4	3
Replicable	2	5	3

Ease to Make	4	5	2
TOTAL	**13**	**17**	**12**

Key	1: Bad	2: Poor	3: Average	4: Good	5: Great

Tissue rings were chosen as the method to produce tissue samples that mimic normal tissue found in the body. The ability for cells to self-assemble in the tissue rings was very beneficial to the team, as the cells would only need to be seeded to make the rings. This method was also replicable and easy to do, making it a worthwhile approach for the team. A decision matrix comparing all design ideas for making tissue can be seen in Table 4.3.

Table 4.3. Decision Matrix for Tissue.

Characteristics	Scaffold	Rings
Uniform Tissue	3	3
Self Assemble	4	4
Replicable	3	5
Ease to Make	4	5
TOTAL	**14**	**17**

Key	1: Bad	2: Poor	3: Average	4: Good	5: Great

Having an ultimate goal of combining the clusters and the tissue to create a 3D tumor microenvironment for the project, the team decided that testing the interactions of the cancer and tissue cells was needed on a 2D scale first so that proper hypotheses could be made. Once this testing was done, a 3D model containing both the hanging drop cluster and the tissue rings, would then be used to see the integration of cancer cells into normal tissue. Both models are easy to make and show how the tissue and cancer cells interact. While the 3D model is a more accurate representation of a tumor microenvironment, the 2D option is easy to assess and replicable for further testing. The decision matrix used to decide which methods to use for creating a tissue and cancer cell interactive model can be seen in Table 4.4.

Table 4.4: Decision Matrix for Tissue and Cancer Cell Interactions.

Characteristics	2D Model	3D Model
Accurate of Tumor Microenvironment	2	5
Interactions	4	4
Replicable	5	3
Ease to Make	4	4
TOTAL	**15**	**16**

Key	1: Bad	2: Poor	3: Average	4: Good	5: Great

4.5. Final Designs

We chose the hanging drop method, outlined in Appendix A, to create clusters of cells. We wanted to see how these cells self-assembled into clusters and see the interaction of these cells. We planned to test 3T3 (mouse fibroblasts), MB-MDA-231 (TNBC), and CRL-1301 (human fibroblasts) cells to create hanging drops and then observe the drops using a stereomicroscope. We planned to seed cells in droplets on the inside of the lid of a 100mm x 20mm petri dish. We then would flip the lid and put it onto the plate. We would incubate the cells and check their progress periodically to see how the clusters were forming.

To create tissue rings, we first needed to create a negative model of the mold we wanted. The final model for the tissue rings was a 3D printed, high thermal resin model. Figure 4.7 shows the final SolidWorks model created.

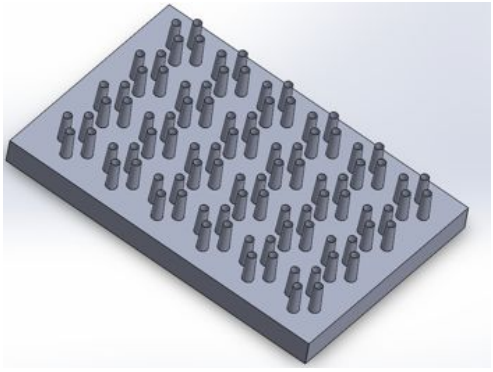

Figure 4.7. Tissue Ring Model.

This model's intended use was to be pressed into a 24 well plate with wells containing agarose. When the agarose solidifies, a negative print of the model will be imprinted into the plate. The model allows for 4 rings to be made per well, completing our objective of being able to easily produce multiple rings at once. The entire protocol for making the tissue rings can be seen in Appendix B.

While wanting to do both a 2D and 3D model of the tissue and cancer cell interactions, we needed to complete the 2D model first. We decided to set up a time-based experiment where we observed the proliferation and integration of cancer cells and breast tissue in a 2D model. We needed to label cells for observation under a microscope, so we decided breast tissue cells would be stained with Hoechst and the cancer cells were previously transfected with GFP. We then wanted to create a 0.5 cm thick PDMS barrier in the middle of a 60mm x 15mm petri dish and seed cancer cells on one side and breast tissue cells on the other. We would do this in 6 plates so that we would have an appropriate amount of time points. The test would begin when we peeled away the PDMS barrier from all plates at T=0. Each plate would be imaged at a subsequent time point using a fluorescent microscope with a Brightfield, DAPI, FITC, or DAPI/FITC filter. A full protocol can be seen in Appendix C. An image of the set up for this method can be seen in Figure 4.8.

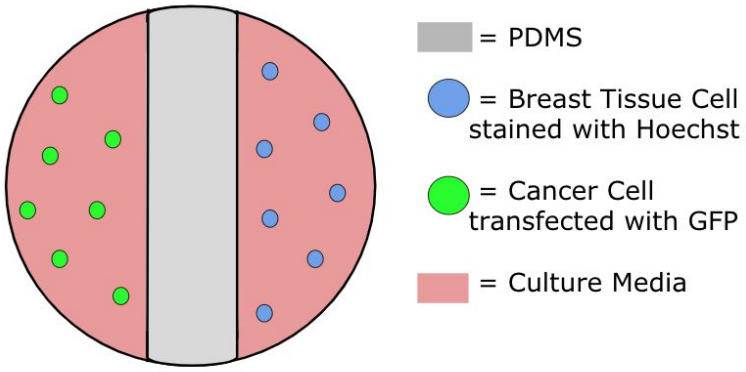

Figure 4.8. 2D Cell Interaction Set Up.

After the 2D model was complete, the 3D model would be made. Using the tissue ring mold, breast tissue and mTNBC cells would be seeded together to create rings as outlined in Appendix B. Different quantities of each cell type could be seeded to see the effects on the interactions between cancer and breast tissue cells. One option would be seeding the cancer cells and the breast tissue cells together. Another option would be to seed a CTC cluster onto a breast tissue ring. And one last option would be to seed a CTC cluster within the tissue ring. These different methods are shown in Figure 4.9.

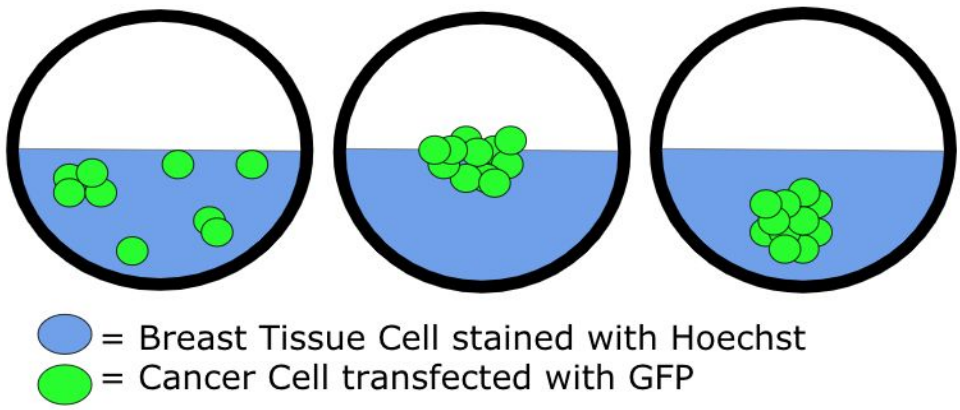

Figure 4.9. Different seeding methods of the 3D Tumor Model.

5.0. Final Design Verification

This chapter will outline the results from the testing described in the previous section.

5.1 Making Clusters Using the Hanging Drop Method

After choosing the hanging drop method to create the clusters, an initial experiment was conducted to ensure that the method could be successfully performed by all group members and could produce the desired results. The first set of cell clusters was made with mouse fibroblast cells (3T3s), as they are a less costly, versatile, and plentiful cell type to practice with. Figure 5.1 below shows a 3T3 cell cluster containing 1000 cells. In our experimentation, 3T3 cells successfully aggregated into a tight cluster, and the best results were seen with clusters made with 1000 cells/drop.

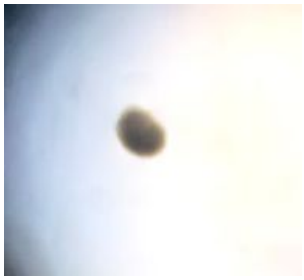

Figure 5.1. 3T3 cell cluster through a stereomicroscope.

Our next experiments tested the ability of the human breast fibroblasts (CRL-1301) and mTNBC cells (MB-MDA-231) to form clusters individually at 1000 cells/drop. Figure 5.2 shows the resulting clusters formed by these two cell types. As expected, the fibroblasts formed a tighter cluster than mTNBC cells.

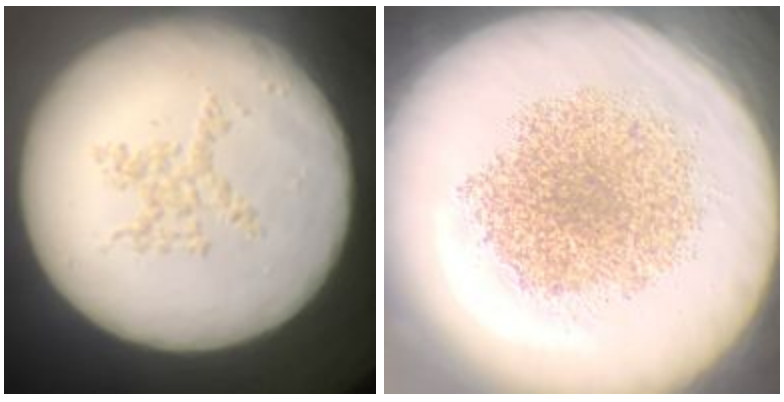

Figure 5.2. Human fibroblast cluster (left) and TNBC cluster (right) through a stereomicroscope.

5.2 2D Cell Integration

For the 2D integration experiment, the interactions between human breast fibroblasts and mTNBC cells were evaluated in a petri dish. Figure 5.3 shows the results of this experiment.

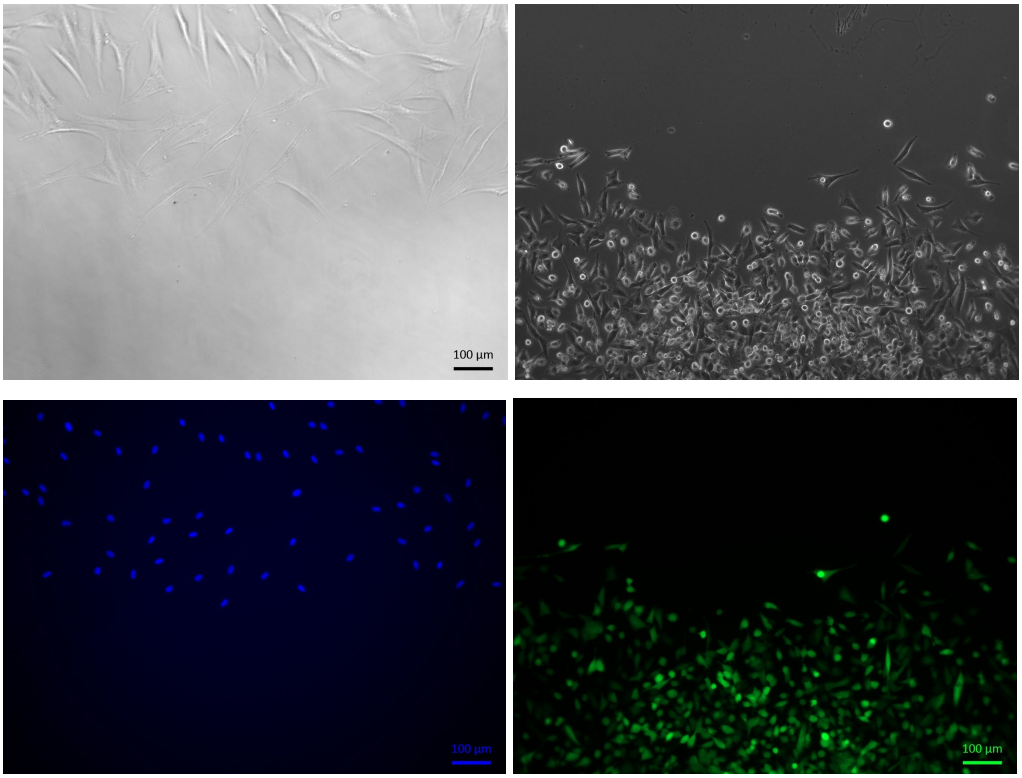

Figure 5.3. Brightfield image of human fibroblasts (top left). Brightfield image of TNBC cells (top right). DAPI image of human fibroblasts (bottom left). FITC image of TNBC cells (bottom right).

Unfortunately, the two cell types never interacted, so no conclusions were able to be drawn from this experiment. The mTNBC cells proliferated faster than the fibroblasts, but that was expected due to the nature of cancer.

5.3 Planned Work

Due to extenuating circumstances, some of the planned experiments were not able to be carried out. Section 5.3 outlines two proposed experiments that the team was unable to execute, but we recommend that they be performed in the future.

5.3.1 BrdU Assay

A BrdU Assay should be performed to determine when the human fibroblasts and mTNBC cells exit the cycle and are no longer proliferating once cultured in reduced serum (in

this case adult horse serum). It is thought that cells that have exited the cell cycle will form more stable rings as the cells will produce more of their own ECM. The experimental design can be found in Appendix D. It is expected that a majority of the cells of each cell type will cease proliferation after four days in the reduced serum [39].

5.3.2 Ring Formation

We ultimately wanted to test the ring model that we designed with the human fibroblasts and mTNBC cells. The proposed experimental design is outlined in Appendix E. It is expected that the ring mold used would effectively create wells, and seeded cells will be able to aggregate around the posts to form rings. It is expected that the rings containing cells cultured with adult horse serum (AHS) would exhibit less necking or thinning on one side [39]. Rings would be made with the cancer cells, tissue cells, and a combination of the two. Ultimately, rings with a combination of the two cell types are desired. If those rings did not form successfully, though, then the inhibiting cell type can be determined. To differentiate between the two cell types within the ring, a cell-specific marker can be stained for using immunohistochemistry after tissue processing. Due to the low cell count within the ring, it is expected that there would be little tissue necrosis at the section of the ring contacting the post. If the rings containing both cancer and tissue cells form successfully, then there are three possible outcomes for the cells' interactions: a homogeneous mixture of cancer and tissue cells, multiple small clusters of cancer cells, or one large cluster of cancer cells.

6.0. Final Design Validation

Our main objectives were to assess the integration of cancer cells with normal breast tissue, create realistic CTC clusters, and create a model to support the self-assembly of tissue rings. While working towards completing our objectives, we kept in mind how aspects of our project met the standards outlined in Chapter 3.3. The standards we considered for our project included, but were not limited to, ISO 13845, ISO 20916, and ISO 11737-2:2009. We were able to meet ISO 11737-2:2009 with our tissue ring model because it was made to be sterilized in an autoclave. We also performed sterile cell culture practices, meeting ISO 11737-2:2009. Other ISO standards that would be taken into consideration if our project was able to be manufactured or performed in clinical trials include ISO 13845 to ensure quality during manufacturing, and ISO 20916 to provide instruction for proper setup of the clinical trial process.

FDA standards were considered and must also be met in the future completion of our project to ensure that it is safe. The FDA has an approval process for all medical devices before they go to market. Since our project would be considered a "medical device," it would need to pass the FDA regulations before being a manufacturable product. GCCP was followed for the duration of our project, but must also be upheld while testing our project to ensure proper and safe cell culture techniques using sterile equipment. This must be done to validate our project and hold it to the standards mentioned above.

6.1. Economics

While the results from the experiments in the scope of our project do not have much of an impact on the economy of everyday living, the completed project as a whole would drastically affect the market. If our project was successful in creating a model that could be used for drug therapy of individual patients, it has the potential to provide a less expensive alternative to current solutions. Kim Farina stated in a 2010 article that, "During 2010, the United States spent an estimated $125 billion on cancer care. Breast cancer spending accounted for 13% ($16.5 billion) of all direct medical spending on cancer in 2010..." [45].

We hope that our product would cut down cancer market spending as a whole and make treatments more affordable for the average patient. This would cause a socioeconomic shift in that patients who normally couldn't afford expensive cancer treatments now have access to a less expensive option. Our project would provide a drastic economic impact on patients who could not previously afford treatment. The goal is that our project would provide an *in vitro* model that can yield more effective cancer treatment on a patient specific basis.

6.2. Environmental Impact

There are environmental risks associated with our ideal project model. The first being that it would be used for personalized medicine, so each model would be considered a single use device. Each well plate containing the models would be specific to one patient and have that patient's cells in it, meaning that it must be disposed of as biohazardous waste after each use. Currently, the most common material for well plates is polystyrene or other non-biodegradable plastics [46]. Our final product idea, although very beneficial in cancer diagnosis and treatment, would waste a variety of plastics due to the use of cell culture single use devices such as pipettes, conical tubes, or petri dishes, which could have a negative effect on the environment if they are not being discarded correctly. There are regulations in place to dispose of these materials properly, though, mitigating these issues. While wasteful, our project does not introduce any new biohazardous threats to the environment.

6.3. Societal Influence

The societal influence that our ideal model would have could be drastic in the cancer community. More middle to lower class families could afford treatment and a cancer diagnosis would become less scary. Currently, the stigma around a cancer diagnosis is that it is a death sentence, especially with diagnoses such as metastatic breast cancer. If future research and experimentation into a 3D cancer model is successful, there could be a shift in how people view their prognosis since treatments would potentially be more successful when patient specific.

Another societal influence that our project may have is the understanding that cancer is unique to each patient and that diagnosis and treatment must also be. We currently live in a world where there are limited options when it comes to dealing with mTNBC cancer. Our project provides another approach to treatment for people to consider. A personalized drug therapy test on a patient’s tumor cells allows doctors to explore the best possible treatment. It also allows for more experimental drugs to be tested without the ethical ramifications of doing *in vivo* animal testing.

6.4. Political Ramifications

The global cancer market value is expected to grow from $128.1 billion in 2018 to $182.0 billion by 2023 [47]. Companies that produce cancer drugs and therapies profit immensely off of patient treatment. With this cancer model, treatments will be tried on a much smaller scale before being tried on a patient. If the patient were to receive one successful treatment rather than a few unsuccessful ones, their treatment costs will decrease. While

company profits may decrease slightly, we predict that the surplus of cancer treatment options on the market will prevent companies from experiencing much of an initial change in profits.

Currently, the standard of care is used to treat patients, but in most cases of mTNBC, that care is ineffective. This model will not only be used to determine successful treatments, but also unsuccessful ones. The proposed model may reach a conclusion that a specific drug or therapy does not work and that statement would be met with opposition from the stakeholders who profit from it.

In countries with similar health care infrastructure to the United States, this model could be adopted since necessary resources are available. In third-world countries that are often lacking specialized technology and expendable resources, this model will unfortunately most likely not be an available option for patients.

6.5. Ethical Concerns

While our project may serve as a solution to ethical problems such as drug therapy testing on patients, there are still ethical concerns that surround our project. Personalized medicine as a whole is an up and coming science that is not backed by a lot of clinical data. While seemingly promising, some people are concerned with the outcomes of such technology. One issue at hand is the way this technology may relate to how different social or ethnic groups are perceived. Also ethically speaking, a new cancer model on the market could cause a shift in how resources are created and distributed within the healthcare system. This shift may disadvantage some patients in select social contexts [48]. Another issue that may arise is making sure our project is not overpromised to patients as a cure to cancer. While beneficial to drug screening, our project will never be fool-proof and patients may not see the results they expect. Making sure patients are aware that our project's approach to guided therapy may not work for some patients is very important. Lastly, the experiments outlined in our project would need to be carefully taught to any researcher working with cells. If a person who had not been taught these methods properly were to err when screening different drug therapy options, it could cause the treatment chosen to not benefit the patient.

6.6. Health and Safety

As mTNBC has such a low survival rate due to a lack of effective treatments, our proposed cancer model has the potential to improve and save lives. With this model, doctors would be able to find the best treatment for a specific patient while also eliminating treatments that would not work. Eliminating treatments deemed ineffective for the specific patient will subject the patient to fewer treatments. Side effects of cancer therapies can be harsh, so it is important to not subject the patient to more harm than necessary. Using this model could allow doctors to find the most effective therapy quickly, which would help improve patient quality of

life. One possible health and safety concern is that finding an effective treatment, even with this model, may take some time. Depending on the severity of the cancer, the patient being treated may not have a lot of time for adequate research to be performed, so the standard of care may need to be used.

6.7. Manufacturability

The rings and cancer/tissue samples used within our model are only able to be manufactured in small batches since biopsies and samples must be taken from each individual patient. The process of creating the engineered tumor model itself is relatively simple and could be replicated easily with the correct supplies. Drug therapy testing on the tissue models could be done on a small scale in research labs, but would need to be outsourced to a testing facility if performed with large quantities of models.

The overall manufacturability of the tissue ring mold device is relatively affordable and easy. The 3D printed tissue ring mold costs $0.55 to make. It is made from a high thermal stereolithography (SLA) resin and the 3D printer requires a special tank to hold the resin. If this fixture was brought to market, it would be easy to translate the current printing process into a manufacturing setting. Our fixture could be manufactured using injection molding processes. The cost of the device would decrease if that were the case, and this could be distributed on a mass market scale.

6.8 Sustainability

Our model would serve as a single use device, meaning that it would cause waste, but not waste that isn't already being disposed of in the current market. Every patient being tested would need their own well plate, cell culture supplies, and lab space. Once samples were done being tested, supplies would be discarded into biohazard bins. The only way for this to be a sustainable product would be to have all of the needed supplies readily available and enough biohazard waste space to discard used supplies.

In addition to the plastic wastes discussed in Chapter 6.2, our single use model would create a need for the creation of a job market for people to tend to the cells as they are tested with drug therapies. Additionally, scientists would need to be hired and trained to properly interpret testing results. If there aren't enough workers available to use our model, this product would not be sustainable.

Lastly, our project would need larger lab spaces to manufacture the cultures of our project. Construction of new laboratories may be needed for this type of analysis. These new buildings could be constructed with modern green building standards and state of the art energy efficient power systems to remain sustainable.

7.0. Discussion

While our 2D integration assay may not have given us the results and data pertaining to cancer-tissue integration we had hoped, we were able to see differences in the rate of growth of these two cell types. Breast cancer cells proliferated and spread across the bottom of a petri dish at a much faster rate when compared to the movement of normal breast tissue cells. Ideally, this experiment would be repeated with adjusted parameters, such as a smaller PDMS barrier and an increased original cell density seeded. This new setup would most likely yield results regarding cancerous and normal cell interactions on a 2D level, which would be very useful information in predicting cell behavior within a final 3D model.

In our hanging drop assays, 3T3 fibroblasts created clusters regardless of seeding density (10, 100, or 1000 cells/drop). Clusters created with 10 or 100 cells/drop, however, were smaller in size compared to a 1000 cell/drop cluster before cells proliferated and the cluster was equal in size to a 1000 cell/drop cluster. Additionally, clusters seeded at a smaller density were less visible to the naked eye or under a stereoscope. For these reasons, we concluded that clusters originally seeded with 1000 cells/drop are the most ideal for our model. Since 3T3 fibroblasts created compact, aggregated, and uniform clusters, it was expected and shown that normal breast cancer tissue cells would do the same.

Unfortunately, the breast cancer clusters which we attempted to form by the hanging drop method did not create the uniform clusters seen by 3T3 cells and human breast fibroblasts. The breast cancer cells (seeded at 1000 cells/drop) formed smaller aggregates of clusters rather than one desiried large cluster. We know that cancerous cells are able to form clusters, as seen by circulating tumor cells in the body, so this assay should be revisited and adjusted to promote cancerous cell cluster formation. From there, these clusters could be placed into the final 3D model to represent *in vivo* CTCs. Although our team was unable to assess cluster formation when cancerous and normal breast tissue cells were seeded into the same drop, we anticipate that these two cell types would form one uniform cluster because the fibroblasts would promote cluster formation. Using our results from our 2D integration experiment, however, we also anticipate the cancer cells in this cluster to grow more rapidly and overpower neighboring tissue cells, to an extent. This is not necessarily a negative result, since these conditions are realistic to human cancer site conditions. It was also hypothesized that seeding the same number of cells into a hanging drop, but in a smaller volume of medium, would create a less rounded and more steep drop, producing more tightly packed clusters. Future work aims to test a smaller drop volume, and also aims to seed both cell types in the same cluster in order to see if the fibroblasts encourage cancer cell aggregation.

Our final ring mold model was 3D printed using a high thermal resin, a material which is able to be sterilized in an autoclave. The mold was designed to fit in a 24-well plate with four posts within each plate, creating opportunity for high reproducibility and throughput. These

properties would futuristically allow for the quick and easy creation of many 3D tissue models to test a variety of drugs simultaneously, leading to faster and patient-specific diagnosis and cancer treatment consensuses. Although our mold was designed, it was not able to be tested due to time constraints. Further testing would determine whether mold speculations were acceptable for proper cell self-assembly and ring formation. Luckily, this is something that can be easily adjusted within CAD once ideal sizings were determined.

Although limitations within our experimentation supplemented by time constraints due to the Coronavirus Pandemic prevented us from creating a final 3D tissue model, we believe that our findings brought us closer to doing so. While our objective of assessing cancerous and normal tissue cell interaction was unfortunately not met, most objectives, including the sterilization and high throughput of a tissue ring mold and the formation of clusters, were completed. The experimentation performed in this report created opportunity for the continuation of our project, and furthers the potential of a 3D model for use in cancer drug screening and personalized medicine.

8.0. Conclusions and Recommendations

Based on the results expected from the proposed work, it is anticipated that the discussed model composed of breast tissue cells and mTNBC cells will accurately represent mTNBC. This model could broaden the scope of cancer research by taking a personalized medicine approach. Future work on the model will improve the accuracy of the model itself as well as its ability to predict treatment success. Our ring mold model has the ability to produce four rings per well in a 24-well plate, which allows for a high throughput process that enables the screening of multiple drugs on each patient's own cells. After the future completion of a 3D tumor model, further research should be done to compare the model to the current 2D and 3D cancer disease models to ensure that its effectiveness is comparable or better. A 3D tumor model does not necessarily need to be specific to breast cancer, and could instead incorporate any type of solid tissue cancer cells. Additionally, other components of the tumor microenvironment such as immune cells or signaling molecules could be incorporated to improve *in vivo* representation. We believe that, with further research and experimentation, the creation of an *in vitro* 3D tumor model mimicking the microenvironment seen *in vivo* is possible in the near future, and could drastically improve the treatment and cancer research advancement.

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Appendix A

Hanging Drop Method

Adapted From Foty, 2011 [49] and Professor Page

Purpose:

Create cell aggregations of desired cell type.

Materials:

- DMEM supplemented with 10% FBS
- PBS
- 0.25% EDTA Trypsin
- Cells cultured to ~85% confluency
- Petri Dishes (100mm x 20mm)

Procedure:

1. Follow the subculturing protocol outlined in Appendix F.
2. Following the cell count and centrifugation, resuspend the cells to 50,000 cells/mL.
    a. This will give you the desired 1000 cells in 20µL.
3. In a 100mm x 20mm petri dish add 7mL of PBS.
    a. This is to control humidity during culture.
4. On the inside of the lid, pipette 20µL of the cell suspension in rows, careful to maintain a drop shape.
5. Once the drops have been made, flip the lid in a swift, fluid motion onto the petri dish.
6. Observe the cells in the following days using a stereo microscope to confirm cell aggregation.

Appendix B

Tissue Ring Formation

Adapted From: Professor Marsha Rolle [44], Professor Raymond Page, and Jason Forte [39]

Purpose:
Create a tissue ring out of the cell type of choice.

Materials:

- Agarose Powder
- DMEM without supplements
- 24-well plate
- Pipette aid
- 5mL sterile pipettes
- Thermal resin ring mold
- Cells of choice at ~85% confluency
- Autoclave
- 50mL glass bottle

Procedure:

1. In a 50mL bottle add 50mL of DMEM without supplements. Add 1g of agarose powder and mix to create a 2% agarose solution.
2. Place the thermal resin ring mold in an autoclave pouch and autoclave on the correct cycle.
3. Autoclave the 2% agarose solution on the correct cycle and transport to the sterile cell culture hood.
4. Open the 24-well plate inside the hood and, using the pipette aid, pipette 2mL of agarose into each well. Move quickly so the agarose doesn't set.
5. Remove the ring mold from the pouch without touching the side that will come in contact with the agarose.
6. Press the mold into the agarose while being careful to not reach the bottom of the well.
7. Leave to solidify for about 5 minutes and remove the mold by lifting slowly straight upwards.
8. Following the passaging procedure outlined in Appendix F, resuspend the cells to a final concentration of 5.5 million cells per mL.
9. Pipette 55µL into each well. This will result in 300,000 cells per ring.
10. Allow four days for the rings to form. They can then be removed from the wells for analysis.

Appendix C

Cell Integration Protocol

Purpose:
To monitor how different cell types interact when they become in contact with each other.

Materials:
- Petri Dishes (60mm x 15mm)
- PDMS
- DMEM with 10% FBS
- Hoechst Stain
- MB-MDA-231 Cells (previously transfected with GFP)
- CRL-1301 Breast Tissue Cells

Protocol:
1. Create PDMS slabs according to PDMS protocol in Appendix G.
2. Incubate Tissue Cells with Hoechst at 1µL stain per 1mL of cell suspension for 10min at 37℃.
    a. MDA-MB-231 cells were transfected with GFP
3. Rinse 2X with sterile PBS.
4. Resuspend in cell culture medium to obtain the desired cell density of 250,000 cells/mL.
5. Seed 1mL of MDA-MB-231 cells and 1mL of CRL-1301 cells on opposite sides of the PDMS in DMEM with 10% FBS.
    a. Seed cells at 250,000 cells on each side.
6. Add 1 ml of media to bring up to a final volume of 2 ml on each side of the PDMS slab.
7. Label different petri dishes with desired time points up to 5 days
    a. Fluorescent imaging may harm cell DNA so a different petri dish is needed for each time point.
8. Incubate cells for 24 hours at 37°C and 5.0% CO_2 to allow the cells to completely adhere.
9. Remove PDMS from each petri dish used.
10. Using Fluorescent Microscope, image petri dishes.
    a. DAPI for Hoechst
    b. FITC for GFP
11. Take images at the site where PDMS was in the petri dish to see how the cells have proliferated and migrated.
12. Observe how the two cell types interact.

Appendix D

BrdU Experimental Design

Objective: Determine how long it takes for cells to exit the cell cycle and stop proliferating when exposed to reduced serum media.

Materials:

- 5 Four well plates
- DMEM Supplemented with 10% FBS
- Reduced Serum Media (Appendix H)
- MB-MDA-231 Cells
- CRL-1301 Cells
- Methanol (stored in the -20C freezer)

Experimental Design:

Each four-well plate will have two wells for the MB-MDA-231 cells and two wells with CRL-1301 cells. Cells will be seeded at a density of 20,000 cells/well in 0.5 mL of DMEM with 10% FBS. The cells will be given 24 hours to adhere to the wells.

Day 1: 24 hours after seeding

The cells in plate 1 will act as the control and will remain in media with 10% FBS. The media in plates 2-5 will be aspirated off and replaced with the reduced serum media. BrdU will be added to the control plate and plate 2 at a ratio of 1µL:1mL and the plates will be returned to the incubator.

Day 2: 48 hours after seeding

The cells in plates 1 and 2 will be fixed in methanol and stored in the 4C refrigerator. BrdU will be added to plate 3 at a ratio of 1µL:1mL and the plates will be returned to the incubator.

Day 3: 72 hours after seeding

The cells in plate 3 will be fixed in methanol and stored in the 4C refrigerator. BrdU will be added to plate 4 at a ratio of 1µL:1mL and the plates will be returned to the incubator.

Day 4: 96 hours after seeding

The cells in plate 4 will be fixed in methanol and stored in the 4C refrigerator. BrdU will be added to plate 5 at a ratio of 1µL:1mL and the plates will be returned to the incubator.

Day 5: 120 hours after seeding

The cells in plate 5 will be fixed in methanol. All plates will be analyzed following the protocol outlined in Appendix I. The plates will then be imaged using fluorescent microscopy. After imaging the ratio of proliferative to non-proliferative cells can also be quantified.

Appendix E

Ring Formation Experimental Design

Objective: Confirm that the ring mold can create the agarose wells within a 24-well plate. Confirm that cells seeded into the wells self-assemble into rings. Compare ring formation between cells in reduced serum to cells in standard culture conditions.

Materials:

- MB-MDA-231 Cells
- CRL-1301 Cells
- Reduced Serum Media (Appendix H)

Experimental Design:

There will be four flasks for each cell type: two with DMEM plus 10% FBS and two with the reduced serum DMEM plus 5% adult horse serum (AHS). Follow steps 1-7 from the tissue ring protocol (Appendix B) to create the wells. Following the passaging protocol (Appendix F) resuspend each to 5.5 million cells per mL. In another conical tube combine 1mL of the MB-MDA-231 cells with 1mL of the CRL-1301 cells to create a homogenous mixture of cells. Do this for the cells in FBS and AHS.

Following the remainder of the tissue ring protocol, pipette the cell suspensions into their respective wells in the plate. Let the rings form for four days. Monitor the rings for the following weeks and observe any changes in the tissue. Quantitative data such as percent of successful ring formation and ring thickness can also be quantified.

Appendix F

Protocol for Routine Sub-culturing and Passaging of Cells

Adapted from Professor Ambady at Worcester Polytechnic Institute

Purpose:

Prolong the life of the existing cells or expand the number of cells in culture.

Materials:

- Cells at ~85% confluency
- DPBS (-)
- Pasteur Pipettes
- Serological Pipettes
- 0.25% Trypsin-EDTA
- DMEM with supplements
- Microscope
- Conical Tubes
- Centrifuge
- Hemocytometer (if cell count is required)
- New cell culture flask or petri dish
- Media suitable for cell type

Procedure:

1. Before bringing the culture plate into the hood, check it under the microscope to:
    a. Check the health and degree of confluency of the cells.
    b. Ensure that cells are not contaminated.
2. Bring the plate inside of the hood.
3. Attach a sterile Pasteur pipette to the tube attached to the vacuum pump.
    a. Carefully aspirate the medium.
4. Using a serological pipette, gently add **5 mL DPBS (-)** along the side of the plate.
    a. This is to rinse cells and help detach cells from the plate.
    b. There is no Mg++ or Ca++ in the DPBS(-).
5. Attach a sterile Pasteur pipette to the tube attached to the vacuum pump.
    a. Carefully aspirate DPBS (-).
6. Using a serological pipette, gently add **3 mL of 0.25% Trypsin-EDTA** solution along the side of the plate.
7. This is also to help detach the cells from the plate.
    a. Close the lid.

8. Incubate the plate on the slide warmer at **37 degrees Celsius for 5-10 minutes.**
    a. After incubation, check the cells under the microscope to make sure that cells are detached and loose.
    b. Bring the plate inside of the hood.
9. Using a fresh serological pipette, add **2 mL** of complete medium to the plate.
    a. This neutralizes and inhibits the trypsin.
    b. The total volume is now 5 mL.
10. Using the same serological pipette, disperse cells by repeated pipetting.
    a. Avoid air bubbles.
11. Transfer cell suspension to a fresh, sterile 15 mL conical tube.
    a. Repeat pipetting to break up cell clumps.
    b. If you plan on doing a cell count, you can use a sample of cell suspension at this point.
12. Spin the tube at **200G** for **5-10 minutes** in the centrifuge.
    a. After centrifugation, look for the cell pellet at the bottom of the tube.
13. Using the vacuum set up, carefully aspirate the medium using a fresh sterile Pasteur pipette, **leaving about 0.5 mL fluid in the tube**.
14. Resuspend cells in an appropriate amount of complete media, depending on the ratio of sub-culturing (1:3, 1:4, 1:5, 1:10, etc.) or plate a specific amount of cells as needed for the experiment.
    a. A good rule of thumb (if you have counted the cells) is to resuspend the cells at 1 million cells/mL.
    b. Resuspension was typically in 3 mL of complete media, a ratio of 1:3 sub-culturing.
15. Plate enough cells into a fresh plate. **Make up the total volume of medium to 10 mL.**
    a. If you are plating 1 mL cells, add 9 mL complete media (e.g. 1 mL + 9 mL, 2 mL + 8 mL, 3 mL + 7 mL, etc.).
    b. Check the cells under a microscope. You will see some cells that are settled at the bottom and some in suspension.
16. Transfer the plate to the incubator for continued incubation for the desired duration.

Appendix G

Protocol for Making Polydimethylsiloxane (PDMS) Slabs

Adapted From Professor Reidinger at Worcester Polytechnic Institute

Purpose:
Create PDMS for cell culture applications.

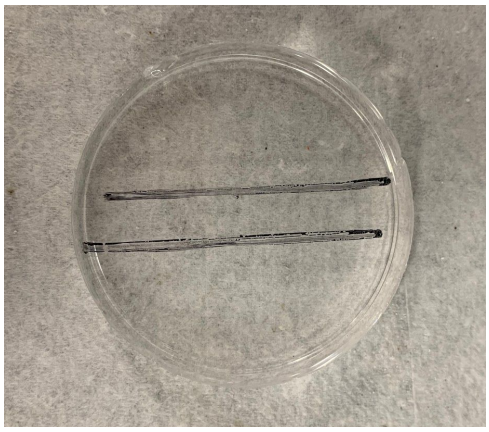
Materials:

- Sylgard Silicone Elastomer base (Ellsworth Adhesive #184 SYL ELAST)
- Sylgard Silicone Elastomer curing agent (Ellsworth Adhesive #184 SYL ELAST)
- Gloves (The elastomer reagents are sticky and may be difficult to wash off)
- Razor Blade
- 7 (60 mm diameter, 15 mm height) Petri Dishes
- Ruler
- Permanent Marker
- 70% Ethanol
- DPBS
- Vacuum Chamber
- Vacuum source
- Scale (~grams)
- Weigh boats
- Oven for curing

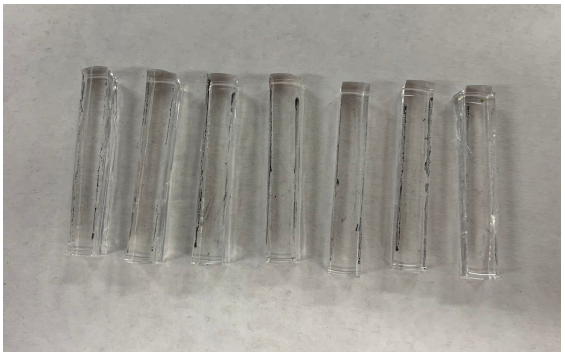
Procedure:

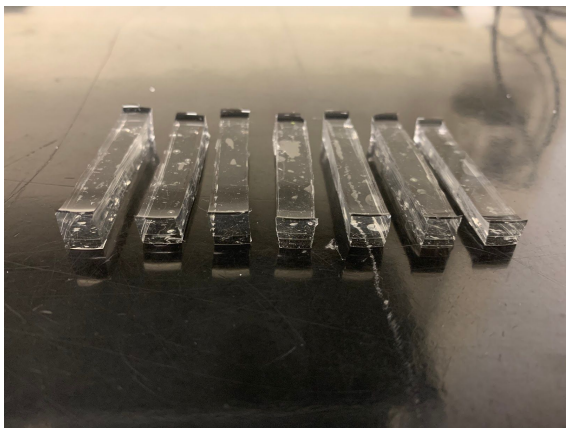
1. Weigh 10 parts Sylgard silicone elastomer base and 1 part Sylgard silicone elastomer curing agent. Note: DO NOT MIX THE STOCK SOLUTIONS!!! Use separate weighing materials for each reagent.
2. Pour reagents together and thoroughly mix the elastomer base and curing agent.
3. Pour the well mixed solution into your mold (Petri Dishes).
4. Degas the PDMS by putting it into a vacuum chamber for at least 1 hour (larger/thicker volumes of PDMS may require more time).
5. After degassing, visually inspect the PDMS to ensure that there are no more bubbles. If there are, repeat steps 4 and 5.
6. Cure the PDMS by placing the mold into an oven set for 60 °C for at least 1 hour (larger samples may require more time).

7. Using a ruler and permanent marker, make two lines in the center of the PDMS to mark a 0.5 cm gap. This can be shown below.



8. Take the entire PDMS block out of the mold and use a razor blade to carefully cut off excess PDMS, leaving only the 1 cm slab. Do this for each mold. These slabs are shown below.





9. Soak slabs in 70% ethanol for approximately one minute, and then rinse in DPBS for approximately one minute, two times each in order to sterilize before use in cell culture.

Note:
One important thing to keep in mind is that the uncured reagents are very tacky and can make a big mess of anything they contact (the degassing chamber, the scale used to weigh reagents). Students should wear gloves when handling PDMS, be careful not to spill, and make sure they clean up the space and equipment they use when preparing PDMS.

Appendix H

Reduced Serum Media

Adapted From Professor Page at Worcester Polytechnic Institute

Purpose:
This media is intended to remove the cells from the cell cycle in order to remove their ability to proliferate.

Materials:

- DMEM (non-supplemented)
- Adult Horse Serum (AHS)
- L- Ascorbic Acid (CAS 84309-23-9)
- ITS (25-800-CR)
- Syringe
- 0.2µm Sterile Syringe Filter (Part No. 431219)

Procedure:

1. Add ascorbic acid at 50 µg/mL into DMEM.
    a. Vortex until dissolved and evenly mixed.
    b. Sterile filter.
2. Add AHS so that it is 5% of the desired volume.
3. Add ITS so that it has a 1X concentration in the final volume.

Appendix I

Standard Operating Procedure
Title: Cell Proliferation Assay using Bromo-deoxyuridine (BrdU)

1.0 **Purpose**

To describe the procedure for assaying cells for proliferation by incorporation of BrdU into DNA.

2.0 **Scope**

This procedure applies to cultured animal cells fixed in methanol such that intracellular and surface antigens can be probed.

3.0 **Responsibility**

All personnel working in research facilities at Worcester Polytechnic Institute.

4.0 **Definitions**

PBS – Phosphate Buffered Saline
Alexa – Alexafluore fluorochrome label
Hoechst 33342
AB - Antibody

5.0 **Materials**

- Culture medium appropriate for cells being analyzed
- BrdU stock solution (1000X, 1.0 mM in DMSO, stored at 4C)
- PBS with 0.05% Tween-20 (PBS-Tween)
- DPBS
- Alexafluor-488 conjugated anti-BrdU antibody
- Ice cold methanol (stored at -20C)
- Tween-20 (10% Solution)
- Cells grown in 24- or 48-well plates.
- 1.5 N HCl
- Hoechst 33342 (1 mg/ml stock), use at 0.5 μg/ml), stored at -20C

6.0 **Procedure**

1. Add 1.0 μl of BrdU stock solution per ml of culture medium to cells being assayed and incubate for 4 hours or the time required by the experimental protocol.
2. Aspirate culture medium and wash cells in DPBS for 10 min.
3. Aspirate DPBS and add ice cold methanol (0.5 ml/well for 48-well or 1.0 ml/well for 24-well plate). Incubate for 10 min at -20C

4. Aspirate methanol and wash with 1.0 ml PBS for 10 min (plates can be stored at 4C if analysis is not to be done right away.
5. Aspirate PBS and hydrolyze by adding 1.5 N HCl (0.5 ml/well for 24-well or 0.25 ml/well for 48-well plate) and incubate at 37C for 10 min.
6. Aspirate HCl and wash 3X with 1.0 ml PBS-Tween, 5 min each
7. Dilute Alexafluor-488 conjugated anti-BrdU antibody 1:200 in PBS-Tween.
8. Add antibody solution at 100 µl/well for 24-well plate or 50 µl/well for 48-well plate and incubate at RT for 30 min.
9. Aspirate antibody solution and wash 2X with PBS for 5 min each.
10. Counterstain with Hoechst at 0.5 µg/ml (stock is 1 mg/ml) in PBS. Use 100 µl/well for 24-well plate or 50 µl/well for 48-well plate incubate for 10 min at RT.
11. Aspirate Hoechst solution and wash 1X with 1.0 ml PBS.
12. Add 80:20 v/v glycerol:PBS/Azide (1.0 ml/well for 24-well or 0.5 ml/well for 48-well plate).
13. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light.

Appendix J

Thawing Cells

Adapted from Professor Ambady at Worcester Polytechnic Institute

Purpose:
Take cells from liquid nitrogen and freeze for use in cell culture.

Materials:

- Plastic Beaker
- Applicable cell media
- 15 mL conical tube
- Desired plate (petri dish, T75 flask, etc.)

Procedure:

1. Fill a plastic beaker with 30 mL of lukewarm water.
2. Hold cryovial in water until the contents thaw.
3. Put 1 mL of cell mixture (in vial) into 9 mL of applicable media in a 15 mL conical tube.
4. Centrifuge at 1,000 rpm for 5-7 minutes.
5. Aspirate the supernatant, leaving a cell pellet.
6. Resuspend cells in 5 mL of media.
7. Plate cells and add culture media as necessary.
8. Place cells into the incubator.

Appendix K

Freezing Mammalian Cells

Adapted from Professor Ambady at Worcester Polytechnic Institute

Purpose:

Freeze mammalian cells for storage and future use. To ensure cells are healthy, remain actively dividing, and successfully thaw and recover, freeze cells at about 70-80% confluency. Avoid freezing cells when their density is very low or very high. **A successful freezing technique should result in greater than 85% cell recovery after thawing.**

Materials:

- Cryovials
- Applicable cell media
- DMSO
- Freezing container (ex: Mr. Frosty)

Preparation:

- Write the following details on the cryovials:
- Name of cell line or cell type
- Passage number
- Cell count per vial (500k is typical)
- Today's date
- Your initials or full name (on vial and on vial cap)

Procedure:

1. Trypsinize cells and continue passaging as per the "routine sub-culturing and passaging" protocol
2. After you receive cell count, resuspend cells in media. The amount of media depends on the cell density that you wish to freeze.
3. Transfer 500 µL of this cell-media mixture to cryovial.
4. Add 100 µL of DMSO.
5. Add 400 µL of fresh media to cryovial (making a total of 1 mL in vial) and shake very lightly.
6. Place vials in storage (such as "Mr. Frosty") which ensures that the vial will freeze at 1 degree Celsius/hour. Place the container with vials into the -80 (degrees Celsius) freezer overnight.
7. The next day, transfer vials to liquid nitrogen for long term storage.