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Separating Human Mesenchymal Stem Cells from Cardiomyocytes After Co-incubation

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1. Cardiomyocyte
2. hMSCs
3. Separation

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Abstract

The purpose of this project was to develop a method for isolating cardiomyocytes (CMC) from human mesenchymal stem cells (hMSCs) after co-culturing. This was addressed by developing a device that achieved a viable homogenous CMC population. This would allow the CMCs to be implanted into the patient, without the risk of stem cells differentiating into various lineages other than cardiac cells. Research has shown that CMCs proliferate when co-cultured with hMSCs. Fluid leakage testing, a cell viability assay, immunohistochemistry staining, cell counts, and observation of morphological characteristics through visual microscopy were conducted. Design development resulted in an efficient cell culture method to maintain cell population separation, and improved CMC health.

Introduction

Cardiovascular disease (CVD) is the most fatal disease to affect individuals in the United States. In 2005 alone, 35.3% of all deaths in the United States were the result of cardiovascular disease. Nearly 80 million Americans have one or more types of CVD; these diseases include high blood pressure, coronary heart disease, and heart failure, all of which result from or can result in the death of cardiac muscle cells (cardiomyocytes).[1] This loss of cardiomyocytes is most notable after a myocardial infarction (MI), most commonly referred to as a heart attack. The decrease in functioning cell mass results in decreased performance of the heart, making it necessary to find methods of improving cardiac function.

When there is an insufficient amount of blood supply to the heart muscle, two things may result: ischemia or infarction. Myocardial ischemia results from a lack of blood supply to a region of the heart, resulting in an imbalance in oxygen requirements and the amount of oxygen being supplied[2]. A myocardial infarction refers to the death of myocardial cells and is usually the result of atherosclerosis[3]. For purposes of this report, the primary focus will be on myocardial infarction.

Since a myocardial infarction results in necrosis of cardiomyocytes, the methods for improving heart function are limited. The only current method of restoring the same number of heart cells (and cardiac function) as was present prior to the MI is a heart transplant. There are many concerns involving a heart transplant as patients often have severe immune reactions to the foreign heart, which sometimes results in rejection; thus it is necessary to take copious amounts of immunosuppressants and steroids. In addition, there is a very limited number of donor hearts available. There are approximately 100,000 people awaiting a heart transplant, while only 10,000 hearts are available. Therefore, every year thousands of people die while waiting for a heart[4].

One way to bypass these concerns is the use of stem cells to create cardiomyocytes for implantation into the patient to replace the lost cells. It is possible to isolate human mesenchymal stem cells (hMSCs) from the patient; these cells have the ability to differentiate into osteoblasts, chondrocytes, cardiomyocytes, adipocytes, and endothelial cells[5]. The hMSCs have also been shown to release specific growth factors which encourage cardiomyocyte proliferation.[6] The use of hMSCs eliminates the concern of generating an immune response from the patient, as well as the stigma associated with the use of embryonic stem cells.

While it has been shown that it is possible to co-culture hMSCs and cardiomyocytes to induce cardiomyocyte proliferation, it is necessary to separate the two prior to implanting the cardiac cells into the patient's heart. Failure to eliminate all stem cells may have a detrimental effect inside the patient as the hMSCs may differentiate into undesirable types of cells within the heart, such as fat, bone, or tendon. Also, there is the risk that the stem cells will proliferate uncontrollably, resulting in the formation of a teratoma.

There are currently a variety of methods used to separate hMSCs from cardiomyocytes. These methods include physical separation, various sedimentation/centrifugation methods, fluorescent activated cell sorting, magnetic activated cell sorting, and media activated cell sorting.[7] These methods, however, require the use of expensive and non-standard laboratory equipment. This study will focus on

developing a novel and cost-effective method for efficient isolation the cardiomyocytes by prioritizing various objectives for the method and the functions that must be realized.

The method developed in this project will be one to maintain separate cultures of cardiomyocytes and hMSCs, while still allowing for the stimulation of cardiomyocyte proliferation by the growth factors released by the hMSCs. A media overflow method will be used to transport the growth factor from the hMSC wells to the cardiomyocyte wells. The boundary will be a ring that can be adhered to a standard sized cell culture dish, which is readily available in any lab. Adhesion will be performed by a dip method using petroleum jelly and can be facilitated by using a specially designed stand.

To ensure that the device is functioning properly, quantum dots will be used to track the migration of stem cells. A variety of assays will be performed to monitor and determine the health and proliferation of the cardiomyocyte populations. These assays include cell counting, LIVE/DEAD assays, Immunohistochemistry, and Western blotting.

It is imperative that better treatment options be made available for these debilitating and life threatening conditions. It would be ideal to develop a treatment that is dependent on the patient's own cells, as opposed to dangerous and ineffective procedures.

Background

Structure of the Heart

The heart is approximately the size of a fist and is enclosed in the mediastinum and located in the center of the thoracic cavity, midway between the sternum and the vertebrae. The heart has a broad base at the top and is angled in such a way that the pointed bottom, or the apex, is located to the left[8]. The heart wall is comprised of three layers: the epicardium, the myocardium, and the endocardium (See Figure 1). The epicardium is a thin external wall which provides protection. The myocardium is composed of the cardiac muscle and, therefore, accounts for the majority of the mass of the heart. It is also the layer that causes the heart to contract. The final layer, the endocardium, is composed of squamous epithelium cells and is a thin sheet that lines the interior of the heart as well as the blood vessels leaving and entering the heart.[9]
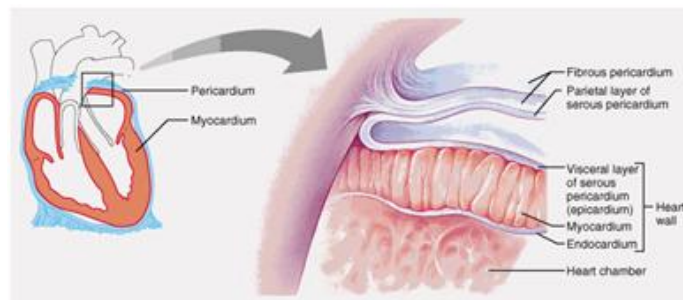
Figure 1: Layers of the Heart Wall[10]

The heart is divided into four main chambers: two atria and two ventricles (See Figure 2). The atria are responsible for receiving venous blood while the ventricles are responsible for ejecting blood into the arteries. The left and right halves of the heart are separated by a muscular wall which is called the septum. The left ventricle is the most muscular region of the heart, as it generates the highest pressures and is responsible for providing systemic circulation.[11]
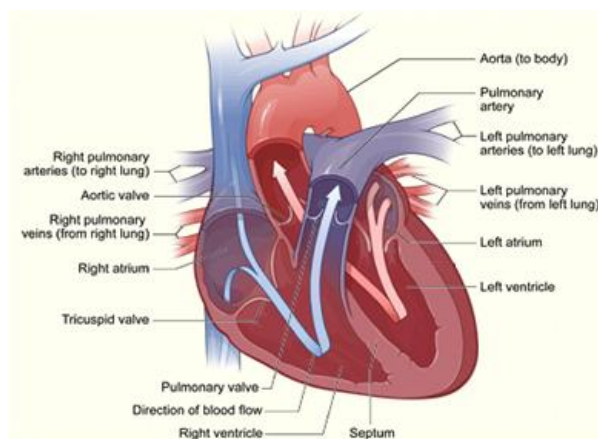
Figure 2: The Interior of the Heart[12]

Blood Flow in the Heart

The right side of the heart is responsible for supplying the blood to the lungs, and is therefore referred to as the "pulmonary circuit pump". The blood returning from the body is pumped into the right atria, through the tricuspid valve and into the right ventricle, then going through this circuit to release carbon dioxide into the lungs and pick up oxygen. This blood then flows into the left side of the heart via the pulmonary veins, which is the "systemic circuit pump". The blood flows into the left atrium and then proceeds to pass into the left ventricle. The blood then passes into the aorta, which branches into smaller vessels, supplying blood to the other organs in the body.[9] If the blood is not circulated properly, cardiomyocytes may die. This results in decreased heart function, affecting blood flow to the other organs.

Diseases of the Heart

80,000,000 Americans suffer from at least one type of cardiovascular disease (CVD)[13], making heart disease the leading cause of death in the United States according to the Center of Disease Control's (CDC) 2005 Chronic Disease Overview[14]. There are many conditions that can result in damaged myocardial tissue. Two of the most common ones are myocardial infarction (MI) and congestive heart failure (CHF).

Myocardial Infarction

An estimated 7,900,000 Americans experience a myocardial infarction (MI), commonly known as a heart attack, every year[13]. An MI is the necrosis of cardiomyocytes and is often caused by an underlying condition, such as atherosclerotic heart disease or other occlusion[11,15]. Atherosclerotic heart disease is the hardening of the arteries, which restricts the blood flow to the heart[16]. The result of an MI is an area of dead (infarcted) cells, which is surrounded by an area of damaged tissue. This area is then surrounded by a region of ischemia, which is the result of oxygen deprivation (See Figure 3). The infarction is generally seen in the left ventricle, but occasionally appears in the right ventricle as well. The area of dead cardiomyocytes forms scar tissue because the heart is not able to regenerate enough cells to replace dead tissue mass. The scar tissue that is left at the site of infarction provides mechanical stability, prohibiting the blood from leaking out of the heart. However, the scar tissue also results in a loss of contractile mass of the heart, leading to less blood being pumped systemically. An acute MI can result in the death of 1 billion cardiomyocytes, which is approximately 25% of myocytes. This results in severely decreased heart function, leading to heart failure (HF).[17,18]
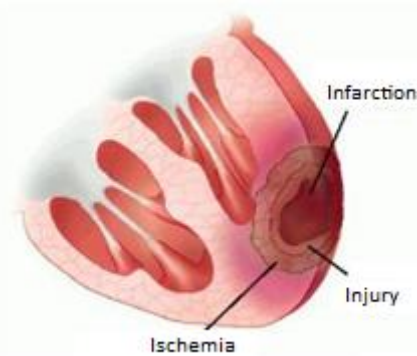
Currently, following an MI a major focus in treatment is the prevention of another occurrence. This is commonly done through the use of thrombolytic drugs, which break up clots, thereby preventing a lack of blood supply through blockage. Aspirin has been shown to be equally effective, though the benefits are not solely due to its blood-thinning properties. Another treatment is the use of angiotensin converting enzyme (ACE) inhibitors, which reduce vasoconstriction by blocking the conversion of angiotensin I to angiotensin II[20]. Also, beta-adrenergic receptor blocking agents (beta-blockers) are used to reduce the heart rate, thereby reducing the workload of the heart[21]. These treatments are somewhat effective and improve the outcome as they avoid further damage; however, in some cases, it is necessary to perform a surgical bypass. This is done by using a piece of healthy vessel from either the chest wall or the leg and creating a detour around the infarcted region for blood flow[11.] This treatment option is effective in alleviating the effects of the ischemia; however, the only truly effective treatment for an MI is a heart transplant. Due to the limited availability of donors, and the high demand, this method is not feasible for many patients. There are over 100,000 patients on the waiting list who need a heart transplant and only about 10,000 organs available. Additionally, the patient is required to remain on immunosuppressant medication in order to prevent an immune reaction and rejection of the implanted organ.[4]

Congestive Heart Failure and Cardiomyopathy

5,700,000 Americans suffer from congestive heart failure (CHF)[13], resulting in a yearly financial burden of between $35 billion and $60 billion in the United States alone[22]. CHF is a condition in which the heart is not able to supply enough blood to the other organs in the body. The ventricle undergoes "cardiac remodeling" in an attempt to compensate for the inability of the heart to efficiently pump the blood. There are several possible causes for the disorder, such as narrow arteries or a past MI. Current treatments of CHF include ACE inhibitors, beta-blockers, diuretics, and vasodilators[23]. An alternative treatment for the aforementioned condition which is employed by VesCell™, focuses on the regeneration of heart muscle and vessels by adult stem cell therapy, using cells obtained from the patient[24]. Due to the inefficiency of the current treatments, however, there is only a 50% survival rate 5 years after diagnosis. [25]

Cell Biology

Cardiomyocytes

The myocardium consists of the muscles that surround the heart and are responsible for the contraction and relaxation that characterizes its behavior as a pump. The cardiac tissue is composed of cells known as cardiomyocytes. Myocardium is one of three muscle types found in the body: skeletal, smooth, and cardiac. Because of its structure, appearance, metabolism, and its excitation-coupling mechanism of contraction, it is often viewed as an intermediate between skeletal and smooth muscle. Cardiac muscle contracts and has an appearance similar to skeletal muscle, while it is controlled involuntarily, similar to smooth muscle.

The structural characteristics of cardiac muscle include the striation, large transverse, and intercalated discs (IDs) that it exhibits. Striation results from an alignment of alternating thick and thin protein filament sections within the myocardium. Cardiac muscle is much like skeletal muscle in terms of its structure and composition as it is composed of the structural proteins actin and myosin. Compared to skeletal muscle, cardiac muscle is often branched rather than strictly linear. It also has thicker myosin and thinner actin filaments, which subsequently results in a more prominent A-band structure and a less prominent I-band structure in this tissue. Transverse tubules are inward folds in the sarcolemma, or cell membranes of muscle cells. The transverse tubules in heart muscle are relatively thick, but are less frequent than those occurring in skeletal muscle. These tubules function in the previously described excitation-contraction coupling involved in cardiac pump functionality. Intercalated discs are complex structures that consist of an undulated double membrane that serves to connect adjacent cells in cardiac muscle fibers and allow for the rapid progression of action potentials that are responsible for the synchronization of muscle function in the heart. The IDs in myocardial tissue occur as thin lines that occur between adjacent cardiomyocytes viewed under light microscopy.

Stem Cells

During early development, rapid proliferation of embryonic cells take place, followed by the differentiation of these cells to form adult tissues. The rate of proliferation however decreases with the differentiation and specialization of cells. The majority of cells in adult animals are unable to proliferate and are arrested in the post-mitotic G0 phase of the cell cycle. Stem cells are a subpopulation of less differentiated self-renewing cells that are found in the majority of adult tissues within the body. As natural cell death occurs, the cells in adult tissue and organs are replaced by the proliferation of less differentiated cells that are generated by self-renewing stem cells. Stem cells divide into two daughter cells; one that remains a stem cell, and one that undergoes further division and differentiation. Despite this cycle of cell renewal, not all tissues contain stem cells with the same potential to replace cells that are list during an organism's lifetime.

Due to the potential stem cells have to differentiate into various cell types, they have become of great interest in medical science. Initially, omnipotent embryonic stem cells were of interest in tissue regeneration because of their ability to differentiate in to potentially all cell types. Despite considerable

success seen with the manipulation of these cells, due to their human embryo sources ethical debate and the lack of government funding has discouraged embryonic stem cell research. Human adult stem cells provide an alternative to embryonic stem cells. Though they are more constricted in terms of differentiation and regeneration, they can be taken and derived from adult tissues without the sacrifice of human life. Mesenchymal stem cells (MS) are pluripotent cells found in the bone marrow; capable of differentiating into osteoblasts (involved in bone formation), chondrocytes (cartilage formation), endothelial cells (lining of blood vessels and lymphatics), and potentially neuron-like cells. MS make up one of the first types of stem cells to be introduced clinically, but represent 0.001% to 0.01% of nucleated cells within the bone marrow[26].

Some of the characteristics of these cells that make them good candidates for clinical use include their ease of isolation from the body, diverse differentiation potential, high in-vitro expansion potential, and their genetic stability. Potential clinical applications include their use in systemic transplantation for systemic disease, local implantation for local tissue defects, the generation transplantable tissues and organs in tissue-engineering protocols, and as a vehicle for genes in gene therapy protocols[49].

The isolation of specific homogenous cell populations from the bone marrow and the task of expanding cell cultures ex vivo without affecting their differentiation potential, however present a challenge. Additional challenges with use of clinical applications include the need to develop efficient methods for quality control of any cellular-based products and the uncertainty if they can be used to change aging processes in addition to alleviating existing disease conditions.

Adult human mesenchymal stem cells (hMSCs) have been applied to efforts in myocardial regeneration. Studies thus far have shown that hMSCs can be used allogeneically without immune suppression, can induce increased angiogenesis, and can differentiate into a cardiomyocyte-like phenotype within the myocardium. Furthermore, they have demonstrated the release of growth factors that may function to encourage repair native myocardium repair mechanisms.[27,28]
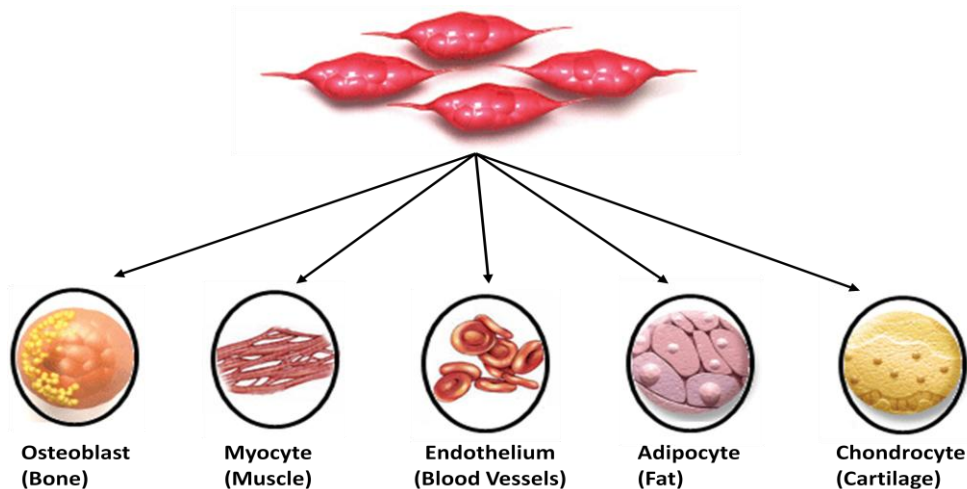
Figure 4: Human Mesenchymal Stem cells have the ability to differentiate into osteoblasts, myocytes, endothelium, adipocytes, and chondrocytes.[29,30]

Co-Culturing hMSCs with Cardiomyocytes

When human mesenchymal stem cells are cultured with cardiomyocytes, it has been shown to have an effect on the amount of proliferation compared with the culturing the cardiomyocytes alone. In one study, it was found that different concentrations of marrow stromal cell conditioned medium (MSC-CM) had varied effects on the cardiomyocytes proliferation. When the cardiomyocytes were cultured with a low concentration (50%) of MSC-CM, the amount of proliferation increased by nearly 60% compared with controls, while a high concentration (200%) of MSC-CM, decreased the cardiomyocytes proliferation by approximately 41%.[31] The images of the effect the different concentrations of MSC-CM had on the cells are shown below:
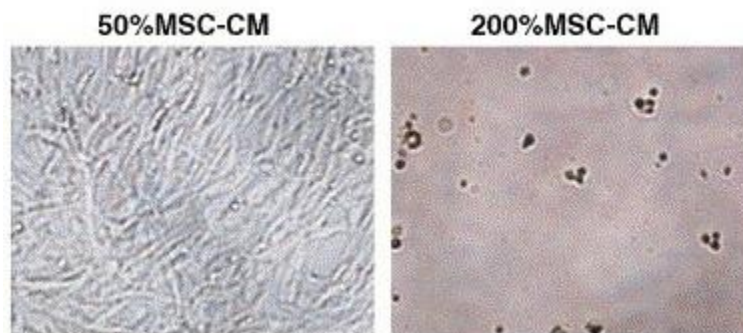
Figure 5: Effect of MSC-CM on cardiomyocyte proliferation[31]

The study found that after 4 weeks, the bone marrow derived MSC-CM had significantly large amounts of vascular endothelial growth factor (VEGF), monocyte chemoattractant protein1 (MCP-1), and hepatocyte growth factor (HGF) present and a minimal amount of insulin-like growth factor-1 (IGF-1) present. The VEGF induced proliferation of the cardiomyocytes depending on the amount present, but once it exceeded a certain level, it inhibited the growth. Overall, the study found that cardiomyocytes proliferate significantly in the presence of MSC-CM, but that cell-to-cell contact between the cells may not be necessary for this proliferation. It is in fact the growth factors VEGF, basic fibroblast growth factor (FGF), IGF-1, and granulocyte colony-stimulating factor which promote cardiomyocyte proliferation.[31]

Cell Tracking

Quantum Dots

Quantum dots (QDs) are fluorescent nanoparticles that can be used to track cells. QDs have been used for various applications since the 1970's, but have only been used as biomarkers since 1998[32]. QDs can be synthesized by the combination of various elements. For biological purposes, QDs are made to be water soluble by replacing their hydrophobic surface with an amphiphylic one[33], and are 13-15 nm in diameter.[34] Their small size allows them to enter into the cells and it has been concluded that they eventually are endocytosed by intracellular vesicles or in a perinuclear region of the cell compatible with endosomal/lysosomal localization. Commonly, they are composed of a combination of atoms from the II-VI groups, including CdS, CdSe, and CdTe. Due to the release of cadmium (Cd) and Selenium (Se), the quantum dots could potentially be toxic after exposure at high concentrations. However, at optimal concentrations for tracking purposes, QDs have been shown to not affect cell viability, morphology, or function over a period of several days.[33] Additionally, Invitrogen produces Qtracker® quantum dots which have a PEG surface coating, which provides protection to the cell from the byproducts of the quantum dots and also reduces any immune response that could be encountered.[35] QDs can be tracked using confocal microscopy, internal reflection microscopy, or basic wide-field epifluorscence microscopy. QDs are advantageous over other cell tracking methods due to the fact that they are able to maintain their fluorescence over long periods of time and also because, upon cell division, the QDs are distributed between the daughter cells[32,33].

Light Microscopy

Light microscopy is a technique that can be used to observe and image living cells. By using light microscopy, the size, shape, position, and function of the cell can be evaluated. There is an upright light microscope and an inverted light microscope. For the upright scope, the lens is facing downwards towards the sample, while the lens on the inverted faces upwards to the bottom of the sample. Since many of the cells that will be observed will be cultured in plates, it will be beneficial to use the inverted scope. The advantage lies in the fact that with the upright scope, there is a large gap from above the plate to the cells. However, with the inverted scope, the lens can be close to the cells, which results in less obstruction and noise. Therefore, the images of the cells will be clearer. The inverted scope shows what is occurring at the top surface of the plate, while the lower levels will be slightly blurred.

Light microscopy also allows for the use of fluorescence imaging. Fluorescence generally involves staining the cells for specific markers and proteins in order to highlight specific regions of a cell. This staining can be used to distinguish cell types when they are co-cultured by staining for cell specific proteins.

Immunohistochemistry

Immunohistochemistry is a method used to detect the presence and location of specific proteins in cells or tissue samples. This is especially useful since different cell types exhibit different proteins and in many cases, different proteins exist during various stages of the cell's life cycle. Thus, protein detection allows for the identification of cells as well as the recognition of cell activity such as proliferation or death within the sample.[36]

First, it involves using a primary antibody to bind to the protein of interest. A fluorescent marked secondary antibody is then applied to attach to the primary antibody, thus allowing the sample to be analyzed using fluorescent microscopy. This secondary antibody has been conjugated with an enzyme that forms colored products in the presence of the specific substrate. The conjugation between the enzyme and secondary antibody ensure that the colored products are deposited specifically where antibody-antigen binding occurs.[37]

Ki-67 is a protein that can be used to determine if a cell has recently been involved in the cell cycle. It is present in the cell throughout all phases of the cell cycle, with the exception of the G_0 phase. Therefore, detection of Ki-67 indicates that the cell is undergoing mitosis.[38] Another protein that can be used to determine the cell activity is cyclin-D1, which is present during the G_1 phase of the cell cycle[39]. Alpha-actinin is a protein commonly used to distinguish cardiomyocytes through immunohistochemistry staining[40]. Since alpha-actinin is not expressed in hMSCs, probing for the protein reveals if the cardiomyocytes are proliferating. Another example of a cardiomyocyte specific protein is titin[41]. Immunohistochemistry can also allow for confirmation of complete cell separation since it allows for identification of any cells remaining in the culture that do not express alpha-actinin or other cardiomyocyte specific proteins.

Western Blotting

Western blotting is a technique that utilizes gel electrophoresis to separate proteins based on their molecular weights. The cells are "lysed", or broken down, to obtain a fluid containing the cellular content, which is referred to as the lysate. After isolating the lysate, or protein, from the cells or tissue of interest, it is necessary to determine what the protein concentration is in the lysate. To do this, a Bradford assay can be used. A Bradford assay is a spectroscopic procedure in which the absorbance of the Coomassie dye changes color from red/brown to blue by binding with protein in the sample. The absorbance of the protein sample can be found by using the information obtained from a standard curve. The standard curve is developed by measuring and plotting the absorbance of a known protein sample (such as bovine serum albumin) versus the known protein concentration.[42]

Based on the protein concentration, a specific amount of protein (20 μg) can be loaded into a polyacrylamide gel. The density of the gel can be altered by varying the ratios of the components of the gel. The density of the gel controls how quickly a sample is able to separate and pass through the gel, as the components of the lysate are separated based on molecular weight. The proteins can then be transferred from the gel to a polyvinylidene difluoride (PVDF) membrane. The membrane is "probed" for specific antibodies. A wide variety of information about the cell and protein can be obtained by using western blotting. As the sample is separated by molecular weight, the size of the protein can be determined. Proteins can be identified based on their ability to bind to the antibodies used as "probes". As with immunohistochemistry, alpha-actinin and Ki-67 can be used as antibodies to determine if the cells being investigated are functional cardiomyocytes and if they are undergoing proliferation.

Based on the location of the protein of interest within the cell, the results can be optimized by using various lysis buffers. Alpha-actinin, for example, is a cytoskeletal bound cytoplasmic protein. Therefore, a tris-triton lysis buffer should provide the best results.[43,44]

Acetone Precipitation

If the lysate is found to have a concentration too low to run a western gel after performing the Bradford assay, there is a method to concentrate the sample that is called acetone precipitation. In this method, the protein is precipitated and interfering substances are removed from the lysate. The protein can then be resuspended in a lesser volume to produce a more concentrated lysate sample that can be used for western blotting.[45]

LIVE/DEAD Assay

A LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) can be used in order to identify the live and the dead cells in a cell population utilizing dyes that can be observed using fluorescent microscopy. The procedure used can identify live cells versus dead cells using two fluorescent dyes: calcein AM and ethidium homodimer-1. The probes function by recognizing specific characteristics of live and dead cells. In particular, the probes identify the intracellular esterase activity and the integrity of the cell's plasma membrane. Live cells exhibit intracellular esterase activity, which results in the conversion of non-fluorescent Calcein AM to fluorescent anion calcein through hydrolysis, which appears bright green under observation. Calcein is retained by the cytoplasm of live cells, and can therefore be used to detect which cells are alive[46]. The Ethidium homodimer-1 (EthD-1) is a nucleic acid stain that will only be taken up by cells with a compromised cell membrane. . The EthD-1 becomes fluorescent when it is bound to nucleic acids, which are present in the nucleus of the damaged cells[47]. Under observation, the EthD-1 fluoresces red. In order to view the fluorescent stains, a 635-700 nm filter must be used for the Eth-D1 stain and a 520-570 nm filter must be used for the Calcein AM stain. Bandpass filters can be used to view the Calcein AM and EthD-1 individually, while a longpass and dual-emission filter can be used to view both simultaneously.[48]

Cell Culture and Plating Densities

Cells must be cultured under specific conditions mimicking physiologic conditions to maintain their health. In particular, they must be maintained at 37˚C, at a pH level of 7.4, and at a 5% CO_2 level.

When plating cells, it is important to monitor the number of cells within a specific area to ensure an even cell distribution and to avoid over-population, which would result in cell death. In addition, the cell density must not be too low in order to allow for adequate cell-cell communication. For both the human mesenchymal stem cells and the cardiomyocytes, it is recommended that cells be plated at a density of approximately 5000 cells/cm^2.[49] When plating cells, counting can be done using a hemocytometer to determine the number of cells in a cell suspension. To use the hemocytometer to determine the number of live cells in the suspension, trypan blue dye is used. The trypan blue is able to penetrate dead cells due to their compromised cell membranes. Based on the amount of trypan blue and cell suspension used, the dilution factor can be determined (e.g. 10 μl trypan + 10 μl cell suspension = dilution factor of 2, 90 μl trypan + 10 μl cell suspension = dilution factor of 10). The number of live cells in the nine main boxes is counted. Based on this, the total number of cells can be determined using the equation displayed below (Equation 1), where 10^4 is the volume that the hemocytometer can hold.

$$\frac{Total\ cells\ counted}{Number\ of\ boxes\ counted\ (9)} * 10^4 * (dilution\ factor)$$

When plating cells for co-culture in particular, it is necessary to consider the difference in proliferation rates of the cell types being cultured. This ensures the health of both cell populations, as over-confluence of a cell type will result in nutrient deprivation. hMSCs have a doubling time of approximately 1-4 days[50]. While the doubling time for cardiomyocytes is not fully established, it is significantly lower than that of the hMSCs.

Cell Separation Techniques

Cell characterization can be done by the following methods: light microscopy, which is usually the first technique used to identify cell types; phase-contrast microscopy; electron microscopy; flow cytometry, magnetic beads; immunohistochemistry; and metabolic characteristics.

Fluid Stream

Fluid stream cell separation is a size-based technique in which the cell-containing fluid is passed through obstacles which isolate the cells based on size. This method is considered to be advantageous because it does not rely on cell markers, which allows for the separation of cells or particles that do not have known markers. Another advantage of this method is that the micro-scale geometry results in laminar fluid flow ($Re \approx 10^{-3}$) fluid flow, thereby producing predictable patterns of cell movement. The obstacles are arranged in such a manner that the lane size around the obstacles gradually decreases. Therefore, as the fluid stream travels through the sets of obstacles cells become trapped within on region of the device when they are no longer able to pass through the lanes. The system is also beneficial because takes less time and costs less than other methods, including FACs and MACs. Nonetheless, there are also disadvantages of this system. First, the system requires that the fluid flow be laminar in order to work properly. The other disadvantage is that there is the possibility of the cells or particles diffusing into adjacent lanes, which could result in an impure cell population.[51]
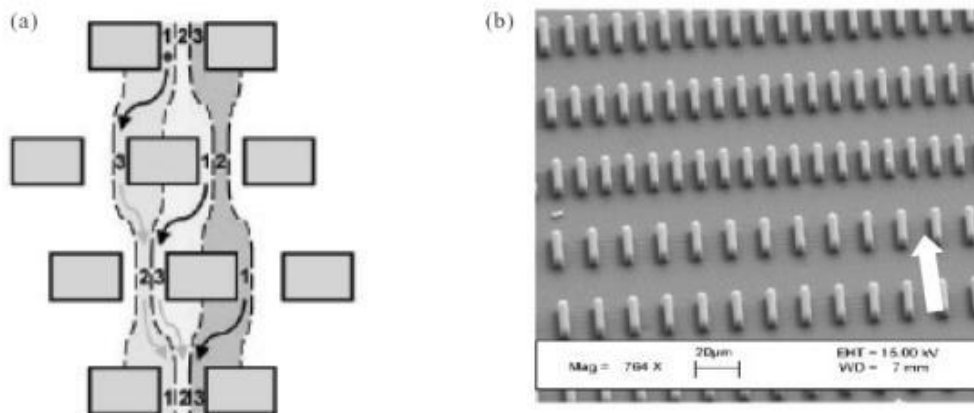
Figure 6: Fluid Stream Cell Sorting[51]

Counterflow Centrifugation Elutriation (CCE)

A non-invasive method that facilitates the separation of proliferating cells. It separates the cells by increasing size and mass. Can accommodate more than 2×10^9 cells, which is approximately 4 liters of cell culture[52]. In general this method utilizes Stoke's law which defines the theoretical basis for separating cells of varying diameters and densities. The separation chamber subjects the cells to two forces: centrifugal field generated by the spinning rotor, and the viscous drag elutriation buffer flowing in the opposite direction.

The cells fist enter the chamber at a given buffer flow rate and rotor speed, the cells subpopulations begin to separate according to size and mass with the smallest cells getting out first. This happens when the buffer flow rate is increased. This is done a second time until the small cells reach the elutriation boundary and can be collected[53].

In a study conducted at the Bristol-Myers Squibb Pharmaceutical Research Institute, the researchers separated T-cells and B0cells into progressive stages of the cell cycle. In this study cells were not loaded through the chamber instead they were loaded using a syringe into the media upstream of the pump. They did this because using the general way caused the dilution of cells to increase in load. They used a Beckman chamber, as a bypass position to trap cells. In this study the rotor speed was held constant at 1800rpm, 2×10^8 cells in 10ml of elutriation media were loaded into the chamber at a pump speed of 13ml/min. They conducted many fractions to separate the cells. The first fraction had the pump speed increased to 15ml/min by 0.1increaments. This was used to collect 125ml of media. The rest of the fractions were loaded and the pump speed was increased by 3ml/min and collected the same amount of media. The fractioning stopped after the speed had reached 48ml/min, any cells remaining were removed. The rotor speed is dependent on the size and amount of cells loaded into the chamber[52].

The buffer used in the elutriation method depends on the cells being used, routinely though Kreb-Henseleit solution can be used. This is made with 95% oxygen, 5% CO_2, supplemented with 0.1% bovine serum albumin and 0.1% glucose immediately prior to use. The number of cells loaded into the chambers also depends on the cells being used, but as a general reference, up to 10^9 cells can be loaded into the small chambers, and up to 10^{10} cells can be added to large chambers. If the chambers are overloaded, the cells will clump together. Another aspect of this method is temperature, the process should be run at a constant temperature otherwise the cells could be susceptible to shear-forces, which might result in the damage of the cell and plasma membrane at low temperatures, at high temperatures clumping occurs.

The advantage of this method are that its choice of medium is adaptable, produced high viability of cells, the method is sterile, and can be performed at any temperature as long as it remains constant through the procedure, and lastly it does not expose cells to high forces. Other advantages include that it can handle large numbers of cells rapidly, separation can sometimes be completed in 15-20min, can be used to remove dead or dying cells since they generally have a lower density. Some disadvantages are that it requires specialized equipment, and it is reliant upon having a high quality single cell suspension[53].

Magnetic Activated Cell Sorting (MACS)

This process uses magnetism to isolate cells in order to purify cell, cell organelles and biologically active compounds, proteins, among others taken from samples. There are several different methods in this magnetic cell sorting. This technique was one of the first ways in which cells were sorted; it has advanced by developing newer techniques in isolating cells. Magnetic separation has many advantages which other techniques lack. The way it works is by separating cells from low magnetic technique samples. This technique is done directly on samples of stool, blood, flood, soil, etc. The magnetic technique is fast and simple. It can also be considered a very productive method for chromatographic and electromigratory analytic procedures. Other procedures can have the same results in the isolation of cells, however many other procedures and instruments are required to be combined in order to thoroughly separate a cell. Nevertheless, magnetic separation is a procedure that is delicate, which makes it easier to quickly handle the cells in their raw habitat.

Principles of magnetic separation techniques

There are two types of magnetic separation. The first type pertains to separated cells that show enough central magnetic moment in order that the magnetic separations done having no changes. The second types, includes one or more non-magnetic factors of a mix have to be labeled by a magnetic device in order to require the contrast in magnetic susceptibility for the cell and the medium. The separation of cells requires a process in order to obtain the specific cells, by using magnetic labels and magnetic separators as well as following three specific steps in order to accomplish the separation. The first step consists of mixing the sample of cells with the magnetic labels. The label cells attached to the sample cells in a period of about 30 to 60minutes in the incubator. After the magnetic formation is then partitioned by using a distinguished magnetic divider and the excesses are thrown away or saved for another process. The second step pertains to rinsing the magnetic complex in order to remove excessive contaminants. In the third step, the magnetic label needs to be taken away from the divided cells.

Magnetic labeling is done by different components such as magnetic and superparamagnetic particles, magnetic colloids and magnetoliposomes. There are issues with using small (1µm) or large (50-200nm) magnetic particles for labeling, particle size is key because it determine the physical behavior and various types of manipulations necessary for a specific particle[54].

Sedimentation Methods

There are two types, velocity sedimentation and gradient centrifugation. Velocity sedimentation refers to stopping sedimentation before cells arrive at the same densities but different sizes. Two types of methods use velocity sedimentation; these are sedimentation at gravity and sedimentation using rate-zonal centrifugation[55].

Centrifugation is a common method to isolate and purify cells. Cells may be separated by size using simple differential pelleting procedures or separated based on density using gradient centrifugation.

Differential pelleting involves having cells that have a tenfold difference in size. Cells can be divided into two major types- those with a rigid cell wall and those without. Cells without a rigid cell

wall, such as animal cells and protoplasts, are difficult to fractionate since any discrepancy in the medium will change the volume of the cell. In this method cells migrate against the direction of the centrifugal force (moving to the top of the centrifuge tube) when the density of the medium exceeds that of the cell.

Velocity sedimentation

Separation of cells at unit gravity

Sedimentation is allowed to proceed under the influence of earth's gravity in a shallow gradient, the cells in a medium of low density sediment principally on the basis of difference in size. The sample is loaded as a narrow band on top of a continuous gradient of a medium.

The density of the gradient increases down the tube of the chamber but is such that it does not exceed the buoyant densities of the particles in the sample. The cells continue to sediment ahead of the smaller ones, forming zones that contain particles of similar sizes. The advantage of this technique is that it avoids the stress imposed on the cells of centrifugation which disrupts the normal functioning of cells. A disadvantage of this method is that the need to prepare a concentration suspension of cells and the subsequent interaction of cell in the sample layer. Since the density of the cells is greater than the density at any point in the gradient, if left for too long, all the cells will form a pellet. Another is that the process is longer.

Separation of cells using rate-zonal centrifugation

This method is almost the same as unit gravity separation, except it uses centrifugation to decrease the time required to achieve the separation. The cells are centrifuged through a gradient, the maximum density of which is less than that of the cells so that cells can continue to sediment until they reach the bottom of the tube, given sufficient time and speed of centrifugation. An advantage of using isokinetic Ficoll gradients is that cells sediment down at a constant rate. The main problem to be aware of is that there is a tendency for cell clumping particularly when loading gradients with concentrated cell suspensions. Isopycnic centrifugation partitions cells according to their buoyant densities and uses centrifugal force and time to deposit cells to locations where the gradient and cells have equal densities. A disadvantage is that most cells can be severely injured or killed at large forces[55].

Separation of cells on the basis of densities

This method uses discontinuous and continuous gradients. The latter formed should cover the range of densities of the cells to be separated. The density at the bottom of the gradient is greater than the density of the densest cells to be separated and so however long the cells are centrifuged, the cells will never sediment to the bottom of the tube. Cells may be loaded anywhere in the gradient.

Centrifugal elutriation

Elutriation is separation by washing and straining or by decanting. Centrifugation provides a means of finely regulating this process. This method is a rapid, non-invasive procedure for the preparation of specific subpopulations of cells from mixed cell type utilizing a balance of two opposing forces; centrifugal forces and fluid counterflow. The main advantage of this technique is that large numbers of cells can be handled rapidly and gently, also the medium for elutriation is adaptable, this

method can be performed at any required temperature, the process gently handles the cells and does not expose them to high g forces, and lastly it handles extremely large numbers of cells relatively rapidly. A note to keep in mind is that dead or dying cells are generally of much lower densities that their fully viable counterparts. One of the only disadvantages in this technique is that this technology is costly and requires specialized techniques to handle it.

Separation by partitioning in aqueous two-phase systems

Another method to separate cells is by aqueous two phase partitioning. In this method cells are added to the aqueous two-phase systems that are formed when aqueous solutions of certain polymers are mixed above the critical concentrations. The cells distribute between the top and bottom phase, and the bulk interphase. The partitioning can be selected to be dominated by different cell surface properties, such as cell surface charge, non-charge surface properties and surface antigen status.

Cells will not show sufficient marked differences in partitioning behavior in a single step. Multiple partitions are therefore required and carried out by countercurrent distribution (CCD). Information of heterogeneity of cell populations is provided by the CCD, the change in the partitioning coefficient marks the heterogeneity of the cell surface, while a constant coefficient indicates that the cell population is homogenous in its surface properties.

Phase systems when mixes break down to very small droplets of both bottom and top phases, similar to an emulsion. When mixing has been completed larger droplets form due to gravity between similar droplets, the cells when present in partition associate with these droplets and move with them as phase separation proceeds. Cells are found in all the phases, they are distributed according to the strength of attachment they have to the droplets. An example of this is when weakly attached cells will break off rapidly moving droplets to smaller slow moving droplets[56].

Flow Cytometry

Flow cytometry uses a flow separation to deliver cells in single file past a point of measurement, this method uses light and focuses it at a point where it collects and measures the fluorescence and light from cells. This method can be used to define and enumerate a subpopulation accurately. Some advantages of this method are purity in the cells, small subpopulations can be selected and several parameters can be used to select the cells for sorting. The major disadvantage is that it has a low rate of cells that can be passed through the light at a given time; clumps of cells will block the flow chamber orifice and disrupt the sorting. Basic flow cytometry consists of a light source, a flow chamber, an optical component, electronics to amplify and process the signals and a computer. Flow cytometry only analyzes and sorts any cell that can be prepared as a suspension of single cells.

Fluorescence Activated Cell Sorting (FACS)

Fluorescence activated cell sorting (FACS) is normally used in immunology, although much attention has been directed in using FACS in molecular biology. The way this works is that cells in a raw sample go through a sensing region where the cells are illuminated by a laser beam. In other words the cells give off signals which are collected from the fluorescent light. The signals are analyzed through a computer system. FACS works by creating what's known as quantitative multiparameter measurement,

on an essentially large sample of cells. Cell sorting happens at many thousand cells per second. Due to the large amount of sorting this method is great at selecting isolating cells in a short amount of time and therefore obtain analytical results at an efficient pace. Essentially cells can be isolated and identified using multiparameter analyses, which are identified by results from the analysis. The analysis is required for physical observation among other testing.

FACS method works efficiently when particles are 1 to 30µm in size. In the early 1970's, FACS was developed because of the increasing need for specific cell separation methods. During the light scatter analysis a cell flows through a laser beam, the laser light is distributed unevenly. As the light scatters in distinct angles, the light contains all sorts of impatient information as of the size, internal structure and shape of cell. There is a difference in the measurements of the distinct angles of the distribution of light. This information is important because angle ranges reveal the types of cells or the different states where cells are found. The first step of the light scatter measurement is cell detection, in this step light is scattered , at an angle of $90°$, which provides an efficient outlook of any cell-sized particle. The second step is cell size evaluation; light is distributed in very small angles , which are less than $2°$. Size is important, however at this specific range signals are not found in most instruments, its difficult measuring closely to the uneven scattered laser beams. The third step was to evaluate the cell structure, in this step granularity laser light beams are scattered to same sized cells. The last step is to classify the cells into live or dead, this means that it differentiates the dead cells from the live ones. Dead cells appear to have ragged edges and are grainy compared to live cells[57].

Microscale Impedance Spectroscopic Differentiation

Microscale spectroscopic differentiation is a method of separating cells which employs micro-machining technologies. For this particular method, electrodes are placed on the bottom of the channel to cause non-uniform current distribution. The electrodes are able to measure the impedance change in the channel based on two consecutive electrodes. Based on this information, the system can then determine the cell type and size. Placing the electrodes on the interior of the channel allows for the tracking of a single cell, which can then be classified and separated out. This system, however, is not fully developed, and the exact specifications must be optimized before it can be used. It is considerably slower than FACS, with rates of over 100 samples s^{-1} compared to FACS, which can sort at a rate of 10^4 samples s^{-1}. There are also advantages of this system. Unlike in FACS, the cells do not have to be modified by labels and antibodies. In addition, it would be more cost efficient than FACS, which requires an expensive system and extensive training.[58]

Cell sorting by droplet deflection

Most common method that uses electrostatic deflection of charged droplets. A conductive sheath fluid is used such as buffered saline. The flow chamber us vibrated vertically causing the fluid emerging from the exit nuzzle to break up into droplets. The flow chamber is charged at the moment a cell of interest is inside the droplet currently being formed. The stream of droplets passes through a pair of charged plates so that droplets which are charged are deflected and collected together with the cell. The stability of the sorter can be affected by a change in temperature, a draught, and a shift in the flow chamber orifice.

Free flow Electrophoresis (FFE)

Free flow electrophoresis (FFE) is an efficient method to separate cells, organelles and any suspension of biological materials. The amounts of cells that can be loaded into the device have to start from 2 to 5 x 10^7 cells/ml, although loading fewer cells has worked before. Free flow electrophoresis separates cells from one another by maximizing differences in the charge of cell surface molecules, by using the net surface charge of cells at physiological pH levels. This method is used in particular for cells that differ only slightly in size and or density. FFE can also be used to monitor charges in the electrophoretic mobility of a single population of cells which have been treated in some way (surface labeled). Some problems with this method are bacterial contamination, temperature fluctuations, and leaks in locations where the tubes leave and enter the separation chamber[59].

The advantages of this method are that the separation is performed continuously and produces pure substances per hour of hundreds of milligrams or grams; it also handles the cells gently and preserves enzymatic activity of the separators. This method separates charged particles by first injecting them through a thin buffer film carrier flowing between two plates. The charged particles deflect due to the applied electric field that is perpendicular to the flow direction. Through one end of the chamber are injected the electrolyte used for separation and the sample, and through the other end are collected, the fractionate samples and electrolyte[60]. There are several aspects that need to be kept in mind when preparing cell samples before FFE. Cells should be kept at a temperature that does not cause them to go into thermal shock of activation. For example, tissue culture cells should be maintained at constant high temperatures before, during and after FFE[59].

Mechanical Separation

Mechanical separation refers to separating the cardiomyocytes from the human mesenchymal stem cells by a physical barrier. There are various types of mechanical separators that can be used to separate materials, such as a liquid from a liquid, a solid from a liquid, a gas from a liquid, etc. Since this experiment is interested in separating cardiomyocytes from hMSCs, this section will focus on solid-from-solid mechanical separators.

Mechanical separators can divide a solid from another solid based on either size or density. Screens, air and wet classifiers, and centrifugal classifiers separate materials based on size. Air and wet classifiers, centrifugal classifiers, magnetic separators, and electrostatic separators are also used to separate one solid from another.

Classifiers

Air classifier sorts materials using size, shape, and density. It injects a stream of air and separates particles by size.[61]

Wet classifier is a "device for the separation of solid particles in a mixture of solids and liquid into fractions, according to particle size or density by methods other than screening; operates by the difference in the settling rate between coarse and fine or heavy and light particles in a tank-confined liquid."[62] Centrifugal classifier separates particles into size groups by centrifugal force.[63]

A magnetic separator is "a machine for separating magnetic from less magnetic or nonmagnetic materials by using strong magnetic fields; used for example, in tramp iron removal, or concentration and purification."[64]

An electrostatic separator is "a separator in which a finely pulverized mixture falls through a powerful electric field between two electrodes; materials having different specific inductive capacitances are deflected by varying amounts and fall into different sorting chutes."[65]

Measuring Cell Viability

Viability counting refers to whether a cell or particular cell population is performing the many processes indicating normal function. There are several ways of accomplishing this, by fluorescent dyes (which attach to intracellular components and produce fluorescent stains), trypan blue dye solution, assessment of metabolic functions by protein synthesis, and cell proliferation measured by enzyme activity.

Electrospinning

Electrospinning is a technique used to produce polymer fibers with diameters in the nanometer range. These fibers can then be used to create mesh structures with varying pore sizes. The process of electrospinning involves three main components: a voltage supply, a needle or capillary tube which holds the polymer solution, and a metal collecting screen or plate. The voltage is used to create an electrically charged polymer jet which flows out of the needle. The intensity of the voltage controls the rate at which the polymer solution is ejected from the needle. The polymer solution evaporates or solidifies while being directed   at the collecting plate. The solidified solution forms polymer nanofibers that can form a web on the collecting plate.[66] The diameter of the fibers produced can be controlled by altering the molecular weight of the polymer used[67]. When using electrospinning, the correct parameters must be used to ensure that fibers are obtained as opposed to polymer beads. This structure is referred to as electrospraying. The variables affecting the outcome could include the polymer molecular weight, concentration, and the voltage applied[67]

The technique can be used with various polymers, including poly(vinyl alcohol), poly(caprolactone), P(LLA-CL), PHBV, and polystyrene (PS). As this is a preliminary investigation into using this method of cell separation, polystyrene will be the focus as to minimize the variables. Studies have used polystyrene with molecular weights ranging from 19,300 to 1,877,000 g/mol. For polystyrene, fibers were found to form when using a polymer of a moderate molecular weight of 393,400 g/mol, a concentration of 21.2 wt%, and the application of a 30 kV voltage. The fiber diameter is also dependent on the molecular weight and polymer concentration used. Using 393,400 g/mol polystyrene, the fiber diameters can be made to range from 0.5-10 μm, depending on the solvent used to create the polymer solution.[67] The fibers can be manufactured to have porous surfaces in order to increase the surface area of the fibers and these pores are typically in the submicron range.[68]

Project Strategy

In order to determine the best designs to pursue, it was necessary to clarify the objectives and constraints of the project. For the design to be successful, the constraints must be adhered to. Ideally, all of the objective will be met by the final design.

Gantt Chart

To be sure the project would be completed within the allotted time frame, the team created a Gantt chart, which acted as a timeline for the project. All deadlines for the development and testing of prototypes were highlighted, as well as deadlines for writing the final report. This timeline is displayed in Appendix 1.

Client Statement

Original Client Statement: Design a system to isolate cardiomyocytes that have been co-cultured with mesenchymal stem cells allowing the cardiomyocytes to be delivered to a patient.

Revised Client Statement: Design an inexpensive system to separate cardiomyocytes from human mesenchymal stem cells after co-culturing. As the purpose for co-culturing is to induce the proliferation of cardiomyocytes, the separation method involved should not hinder viable cardiomyocyte production. The system should also be efficient, aseptic, and result in an adequate number of isolated cardiomyocytes to be delivered to patients for cardiac regeneration. The method should confirm proliferation and isolation of viable cardiomyocytes. The device or method should consider the ease of implementation in standard laboratory environments, specifically concerning cell inspection, delivery after culture, and user variables. The final cardiomyocyte cultures must meet standard regulation requirements so that the cells to be implanted in the patient are not contaminated with co-cultured cells.

Constraints

The project was limited by specific constraints on both the team and the design. The first constraint to limit the team was the timeline since the project had to be completed within one academic year. The entire process, including development, manufacturing, and testing, had to be completed within this year. Another significant constraint taken into consideration was the budget- the team was given a budget of $156 per member, or $624 in total. Available resources and project member laboratory experience were also taken into account.

Cell culture barriers were vital to ensure cell survival. The cells had to be free of contaminants from the environment; and had to be maintained at a constant temperature (37°C), pH (7.4), and carbon dioxide level (5%). The device/method also had to induce cell proliferation within the shelf life of the cells (about 7-14 days).

The final category of constraints on the project was design limitations. The device had to be small enough in size to make it easily applicable in a standard laboratory setting. The device also had to be sterile/sterilizable in order to prevent cell contamination. Finally, the device had to be easy to manufacture to allow for inexpensive production.

Objectives

Identifying Objectives

In order to identify the objectives of the project, several design tools such as an Objectives Tree and a Pairwise Comparison Chart were used. These tools allowed us to identify which objectives were most important in developing a successful separation method.

Objectives Tree

After performing the necessary preliminary research, we were able to determine objectives for our device. The main objective of this MQP was to develop a method to separate human mesenchymal stem cells after they have been co-cultured. Cell processing, ease of implementation, cell viability, and clinical applicability were the four immediate main objectives necessary to developing the separation method. These primary objectives were then divided into secondary objectives, and then tertiary objectives. An objectives tree was created to illustrate the relationships between the objectives and is shown in Figure 7.
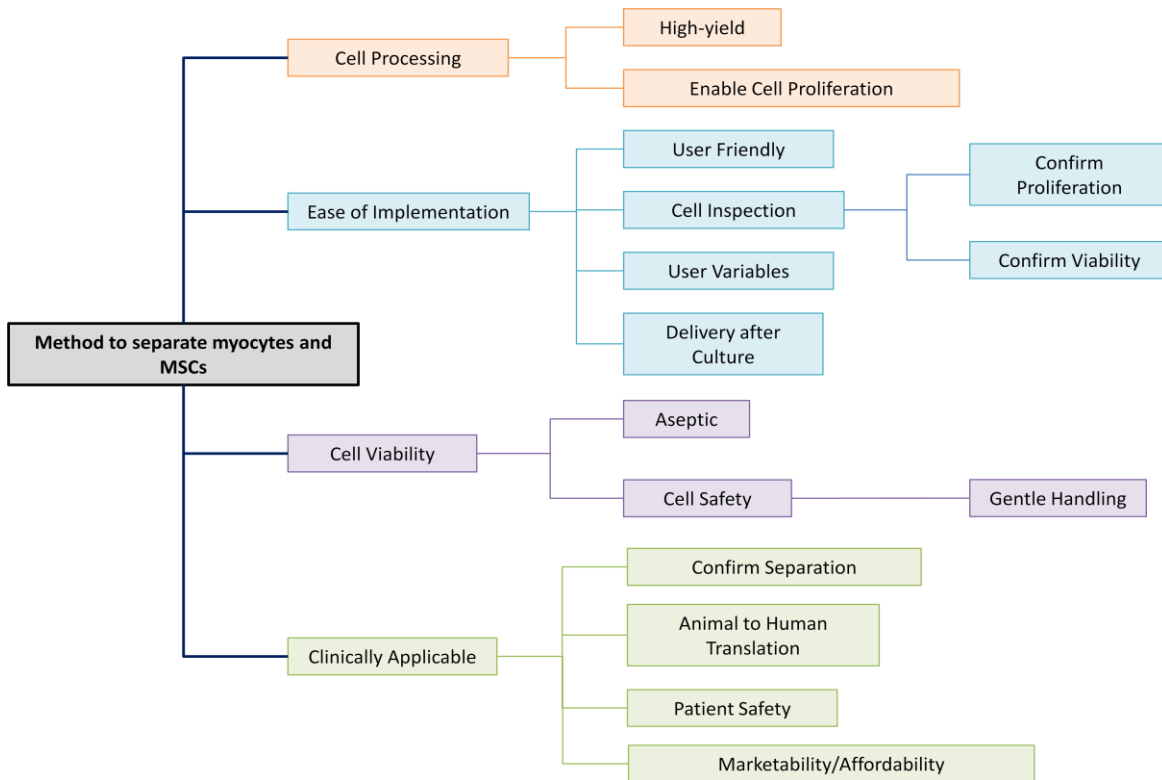
Figure 7: Objectives Tree

Functions-Means Table

Functions-Means tables were used to match the different functions necessary for the method with possible means for accomplishing them. This tool was useful in developing design alternatives, and is shown in Table 1.

Table 1: Functions-Means Table

FUNCTION	MEANS							
Separating hMSC's from Myocytes	Magnetic	Fluorescence	Density Gradient	Mechanical Dissection	Media Cell Sorting	Different Level Sorting	Myocyte Attractant	Smart Polymers
Tracking	Quantum Dots	Smart polymers	Fluorescence					
Identification	Morphology	Cell Markers	Light Microscopy	Molecular Weight/Density	Spectroscopy			
Culturing (Proliferation)	Growth factors	Antibiotics	External Stimuli					
Culturing (Survival)	Incubator & Media	Controlling Cell Population	Hanging drop	Suspended media solution				
Quantifying Cells	Cell-count							
Verifying Cell function (unique/periodic)	Observed Contraction	Metabolism monitoring						
Identifying functional myocytes	Contractile Elements	Striation						

Functions-Means Tree

A functions-means tree was also created as they allow an illustration of not just the different functions and possible means, but also secondary and tertiary functions and the different means to achieve these. This tree can be seen in Appendix 2.

Pairwise Comparison Charts

This section contains the pairwise comparison charts for the primary objectives and the secondary objectives. These charts allowed for prioritizing the primary objectives against each other and rank in order of highest importance to lowest. We also took the secondary objectives (those branching off of Ease of Implementation and Clinically Applicable) and ranked them against each other. The pairwise comparison charts were also given to the Client to ensure that their needs were taken into consideration.

Table 2: Primary Objectives

Primary Objectives							
Goals	Cell Processing	Ease of Implementation	Cell Viability	Clinically Applicable	Score (Team)	Score (Client – Glenn)	Score (Client – Jacques)
Cell Processing	****	1	0	1	2	2	1
Ease of Implementation	0	****	0	0	0	0	0
Cell Viability	1	1	****	1	3	3	2
Clinically Applicable	0	1	0	****	1	1	2

Table 3: Secondary Objectives - Ease of Implementation

Ease of Implementation							
Goals	User Friendly	Cell Inspection	User Variables	Delivery After Culture	Score	Score (Client – Glenn)	Score (Client – Jacques)
User Friendly	****	1	1	1	3	3	1
Cell Inspection	0	****	0	1	1	1	1
User Variables	0	1	****	1	2	2	0
Delivery After Culture	0	0	0	****	0	0	3

Table 4: Secondary Objectives - Clinically Applicable

Clinically Applicable							
Goals	Confirm Separation	Animal to Human Trans.	Patient Safety	Marketability/ Affordability	Score	Score (Client – Glenn)	Score (Client – Jacques)
Confirm Separation	****	1	0	1	2	2	2
Animal to Human Trans.	0	****	0	1	1	1	1
Patient Safety	1	1	****	1	3	3	2
Marketability/ Affordability	0	0	0	****	0	0	0

Alternative Designs

To develop conceptual designs, the team brainstormed independently and with the Client. Sixteen possible methods were identified. After additional research on each of these potential methods was performed, this list was then shortened to ten possible design alternatives:

- Different Media
- Erosion of Media
- Physical Gap
- Apoptosis
- Initial separation
- Media exchange
- Cardiomyocyte Attractant
- Velcro
- Suspension

These methods were chosen based on their ability to separate effectively and efficiently, while taking into account constraints such as budget and time. The next step in the design process is to develop conceptual designs.

Different Media Separation

One method that can be employed for cell separation in this project is the use of different media upon which the respective cardiomyocyte and hMSC cell types are grown. A degree of separation can either be maintained on the basis that each cell type is grown on a type of media that either does not support the growth of the other cell type or that is less favorable to this other cell type. Separation may also be possible by growing each cell type on a different media level. Figure 8 shows an exploration of orientations that can be incorporated into this different media design.
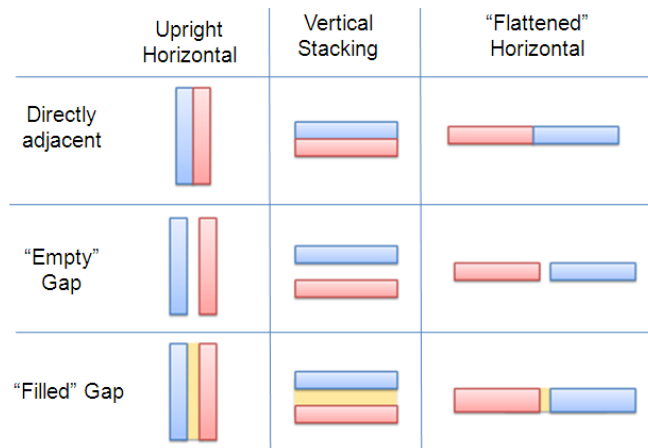
Figure 8: Different Media and Different Level Sorting Orientations

Same-level Media Culture

The flattened horizontal orientation places both the hMSC and the cardiomyocyte cell cultures adjacent to one another on the same level within a culture. This technique would employ the use of two

different media types for the respective cell cultures. Figure 9 presents a variety of two-dimensional media pattern geometries that can be used for the layout of each culture type on the incubation plate. Factors that must be considered in the selection of the media pattern to be used include the amount of surface area between each media type and the ratio of cardiomyocytes to hMSCs in each culture dish. While an optimization of surface area over which each culture is exposed to the other may be desirable for increased cell-to-cell communication, the optimal ratio of cardiomyocytes to hMSCs that allows for optimal cardiomyocyte proliferation can also be explored and incorporated into our project design.
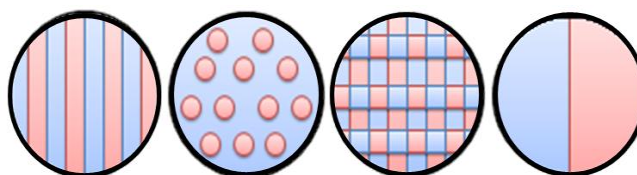
Figure 9: Potential Geometries of Same-level media Culture

Multi-level Media Culture

The vertical stacking orientation illustrated in Figure 8 incorporated a multi-level media design culture where one culture media is spread across the culture plate and the other media is located directly above it. This type of media culture design may be favorable in the instance where the cell densities and anchorage propensities of each cell type differ significantly. Another multi-level media culture that can be explored is the upright horizontal orientation that also appears in Figure 8. This design is essentially the vertical stacking orientation design, rotated by 90 degrees. It is also similar to the flattened horizontal design, but significantly increases the vertical depth of the culture and maximizes the exposed surface area between the two media types by the increasing of this depth dimension in the z-plane of the culture plate. This orientation may be favorable in the event where we see cell populations migrating or falling to the lower level media (regardless of cell type) as the result of gravitational and sedimentation techniques.

Spatial Gap Separation

The incorporation of a spatial gap to maintain separation can also be employed in our cell culture designs. In considering this factor, the first option we present is the instance where there is no spatial gap and the different media types are cultured directly adjacent to one another with immediate contact. An "empty" gap was considered to provide spatial separation of the media types to prevent the transfer mixing and contamination of cell types between the two media. Is such a gap is to be employed, the dimensions of the gap must be optimized to allow for cell-to-cell communication between the two cell populations, while retaining concurrent physical separation. A final "filled" gap option was explored to enhance cell-to-cell communication as well as alleviate potential constitutive forces such as gravity that has the potential to negatively affect separated cell growth. This feature incorporates the use of a fluid media that is injected between the two media types. The fluid may allow for the migration of growth factors passed between the cell types while the physical gap may prevent the mixing of the cell populations. Additionally, when incorporated into the upright horizontal design, such a fluid may introduce buoyant forces that may prevent the migration of cells, indifferent of their cell-type identities, to the bottom of the plate as a result the influence gravity. Such migration would be

undesirable under the circumstances where it causes crowding at the bottom of the plate and sparse populations at the top of the culture dish. Meanwhile crowding in such instances may also be accompanied by undesired cell population mixing, which circumvents initial and continuous separation efforts.

The Multi-level Media Velcro Model

A more sophisticated multi-level gel media Velcro model was explored to maximize the exposure and communication between the cell types to enable cardiomyocyte proliferation. This model is illustrated in Figure 10. In this design, the cell types are grown on different media levels that employ interlocking geometries, much like Velcro. A closed, interlocked position is maintained during cell culture. After culture, one later is then subsequently "peeled off." In the consideration of this design, stresses exerted during and fragility of the cells must be examined to prevent the damage of cells during this layer removal process. To prevent the damage of the cultured cardiomyocytes, it was proposed that the hMSC cell culture layer should be that which is actively "peeled off" and removed from the culture dish. Similar to the design options previously explored, the orientation of this Velcro model can be manipulated to improve the cell culture yield. Similarly, a fluid may be injected between the two media layers to allow both a physical gap for separation and vector for growth factor migration and cell communication. The final design feature of a mesh barrier in close proximity to the surface of each media later was incorporated and can be seen in the bottom right illustration in Figure 9. Such a barrier would be designed to be semi-permeable to allow for the exposure of each cell population to the median fluid and for transmission of factors between the two populations, while preventing the migration and cross-contamination of the actual cardiomyocyte and hMSCs.
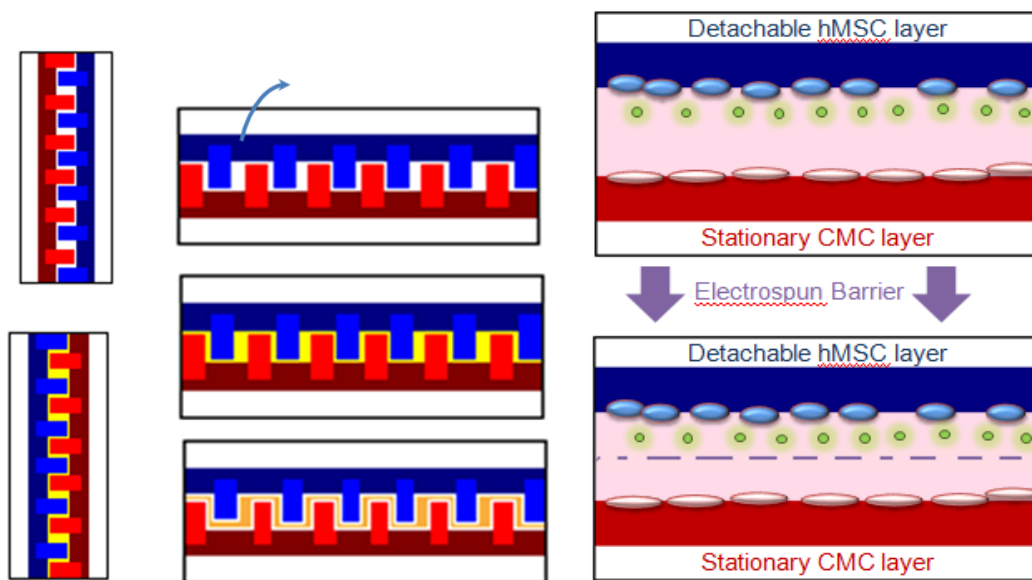
Figure 10: Multi-level Media Velcro Design Illustrations: Showing multiple orientations, absence and presence of fluid media between cell populations, and incorporation of a porous, electrospun barrier.

Separation by the Erosion of Media

Another separation technique uses erosion as a means of separating the two cell cultures. This technique would involve the culturing of each cell type on two different types of gel media. After the

culture and co-incubation of the cells, a substance would be introduced to erode and physically break down the media upon which the hMSCs are grown. Enzymes may be of use for this purpose. Theoretically, the breakdown of the hMSC media will result in the fragmentation of this media and the hMSC culture. A fluid washing agent then can be applied transversely across the culture dish to carry these fragments away so that only the cardiomyocyte culture remains. In this model, the physical structure of the culture dish must be designed to optimize flow and prevent the build-up of fragmented hMSC culture at the flow terminal end of the unit.
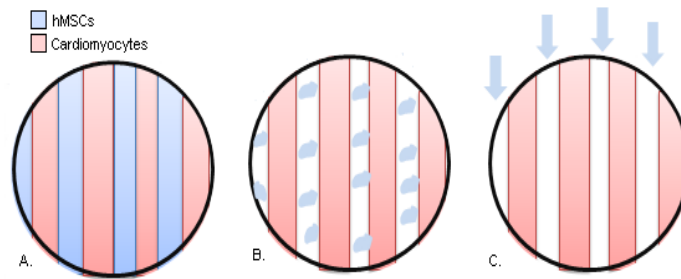
Figure 11: Erosion of Media Technique.

The Overflow Model

An overflow model design incorporates the use of a physical barrier to separate the cells. This barrier is likely to consist of a polymer and can be incorporated as part of the actual culture dish anatomy, as seen in Figure 11 (A and B). Available casting techniques may be used for the independent manufacturing of such culture dishes for the use of this project.   This barrier is intended to create two separate wells in which cardiomyocyte and hMSCs can respectively be independently cultured. Because they are initially independently cultured, the same media can be used to culture each cell type. Meanwhile, different media can also be used. The latter technique may be employed in efforts to individually accommodate for and maximize the cell growth of each cell population type. Once initial culture has enabled the anchorage of each cell type in their respective media wells, a fluid would be added to the hMSC cell culture well. The physical barrier would be designed so that it is considerable shorter than the outer dish walls that serve to contain the cultures. This barrier design would then allow for the overflow of the fluid from the hMSC well into the cardiomyocyte well as fluid is added to the dish. Theoretically this fluid will function as a vector for cell-to-cell communication between the hMSCs and the cardiomyocytes to allow for induced cardiomyocyte proliferation.
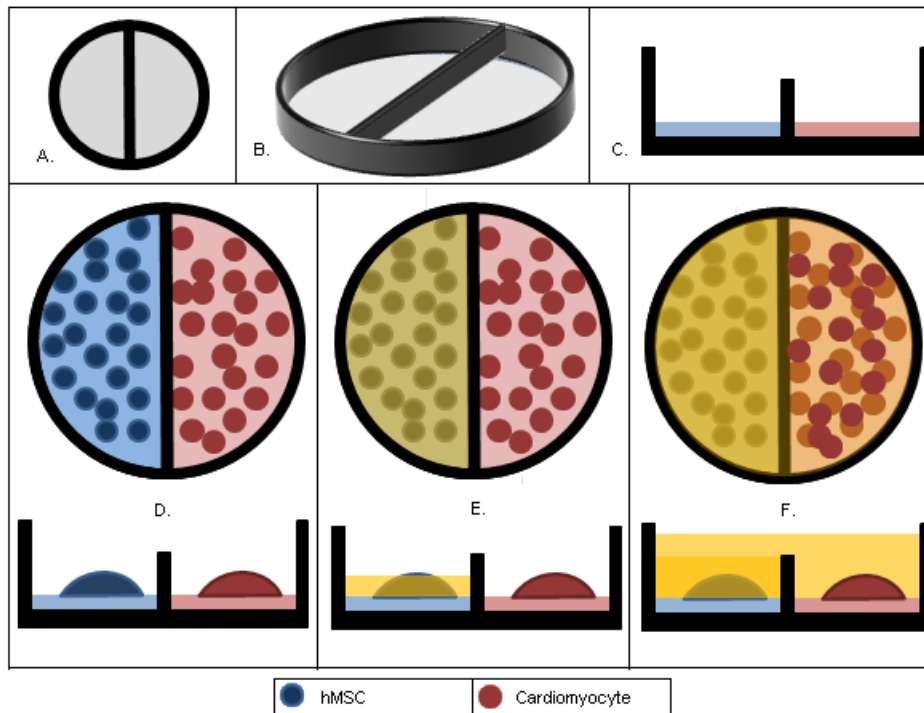
Figure 12: Illustration of the Overflow Model: (A) Aerial view of culture dish, (B) angled view of dish, (C) cross section of dish; (D-F, top) Aerial view of cell culture as fluid overflow is employed, (D-F bottom) Aerial view of cell culture as fluid overflow is employed.

Circular: Overflow Method

The first prototype developed was the circular media overflow ring. The bottom of the plate was removed to allow the user to adhere it to a petri dish using a sterile petroleum jelly. By removing the bottom, it gives the user the flexibility to adhere the ring to a multitude of plates with different surfaces, depending on the needs of the cells that are to be cultured.
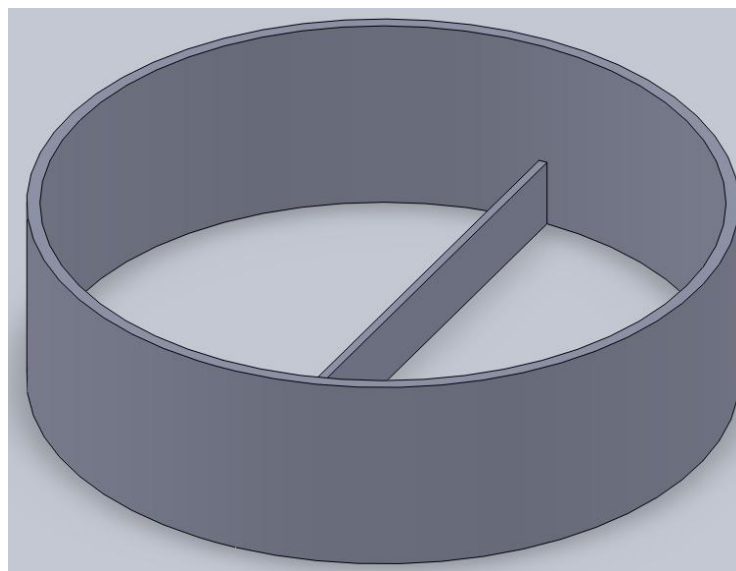
Figure 13: Circular Media Overflow Prototype

Interconnected Rows: Overflow Method

Two different designs were developed for the interconnected rows overflow method. Both prototypes have the same basic design: two sets of rows that are connected, which will allow for easier addition and removal of cell media, making it more user-friendly. By simplifying the method of which media can be removed and added, there is a lower probability that there will be splashing of the media into the rows containing a different cell-type. To further lessen this occurrence, we altered the original interconnected rows design (shown in Figure 14) to allow a slider to pass through which would cover the rows during cell feeding such that it would only allow the user to access one of the cell types (see Figure 15). As in the circular media overflow design, both prototypes lack a bottom to allow the user to adhere the device to the surface of their choice.
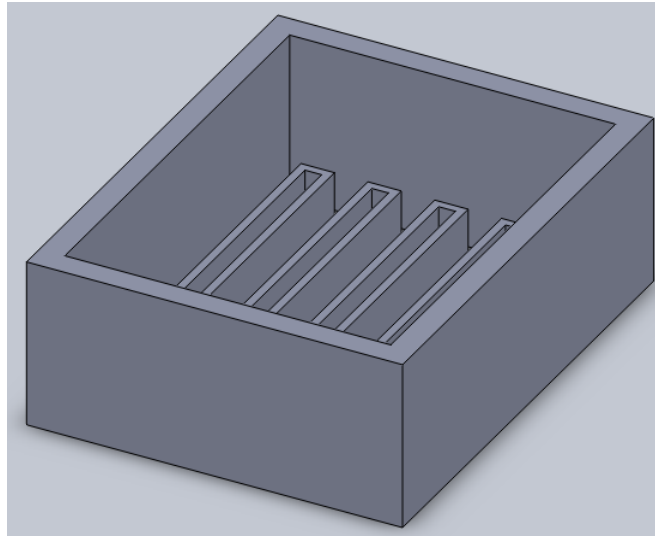
Figure 14: Interconnected Rows
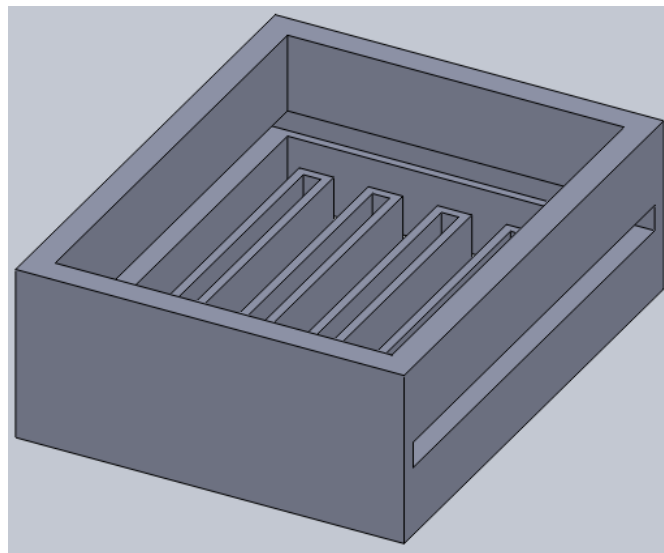
Figure 15: Interconnected Rows with Slider Capability

Velcro Model

The most complicated of the prototypes is the Velcro model. This design consists of three separate sections: the bottom (see Figure 16), the top (see Figure 17), and an electrospun mesh inserted between the two layers. The hMSCs and cardiomyocytes will be cultured individually (cardiomyocytes on the bottom dish, hMSCs on the top) and separated by an electrospun mesh (made of polystyrene or polyvinyl alcohol) with pores sized less than 10 micrometers which will prevent the hMSCs from falling onto the bottom dish and contaminating the cardiomyocytes, while still allowing growth factors to pass through and stimulate proliferation.
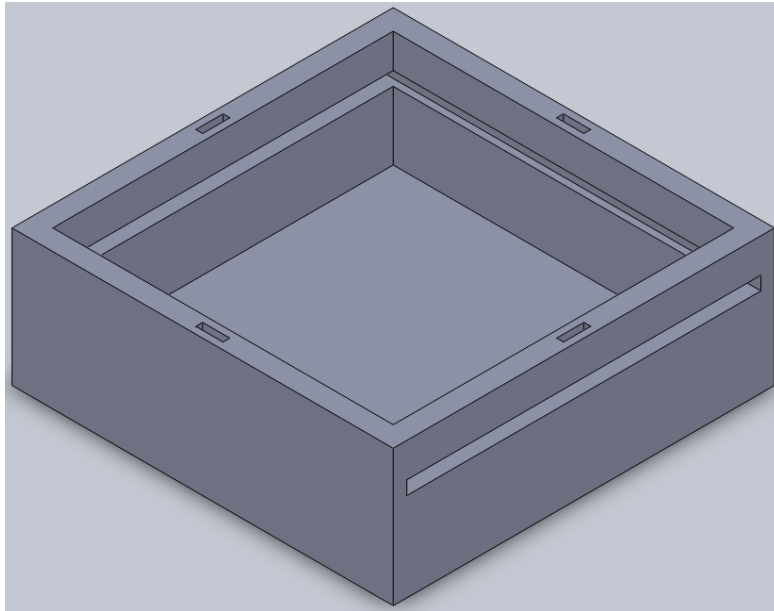
Figure 16: Velcro Model, bottom
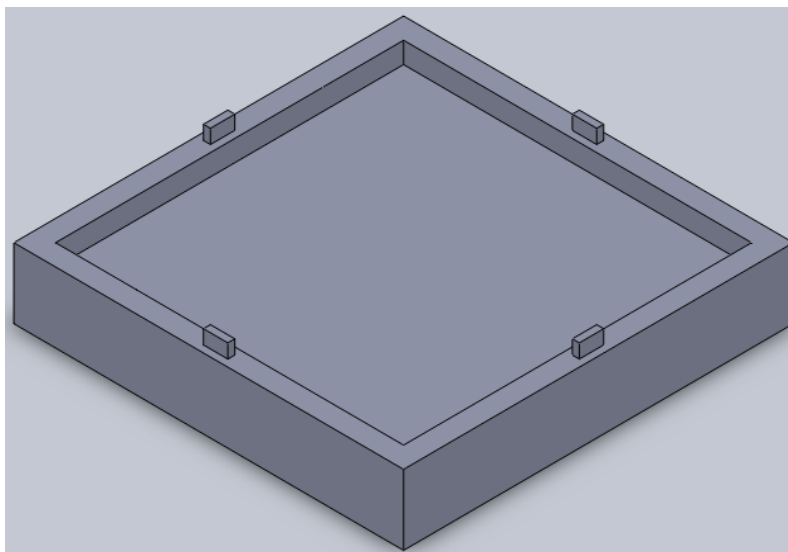
Figure 17: Velcro Model, top

Final Designs

In order to determine which of our conceptual designs to continue forward with testing, it was necessary to rank the different designs based on their difficulty-to-implement, cost, and ability to cater to certain cell variables. Based on our analyses, we decided to proceed with the overflow method and the velcro model. These designs were modified and four initial prototypes were developed. The drawings with the dimensions of each prototype can be found in the Appendices.

Manufacture and Sterilization

All prototypes were created using the Rapid Prototype Machine in Higgins Labs at WPI. The plastic used was Acrylonitrile Butadiene Styrene. This plastic has a melting temperature of $80^{o}C$, and is therefore not autoclavable.[69] Since we could not autoclave the prototype, we decided to use an alternative method of sterilizing the prototypes. We soaked the prototypes in an ethanol bath to kill any bacteria residing on the surface, but we found that the prototypes became disfigured during this process as the barriers separated, which could prove to allow the cells to pass through to the other chambers. To eliminate the possibility of altering the prototypes, we decided to use ethylene oxide sterilization, which does not require a high temperature and since it is a gas, it can access all surfaces without damaging the prototype.

Evolution of Prototypes

To develop an effective final design, we evaluated the positive and negative characteristics of all of our prototype designs. Using pairwise comparison charts, the objectives tree, and functions-means charts, we determined that the design that most adequately addressed all of the necessary objectives was the media overflow model. The media overflow model was the most inexpensive, most sterile, and eliminated the possibility of hMSCs contaminating the cardiomyocyte population.

There were two prototypes developed within this design: the simple model (Figure 18) and the interconnected row model (Figure 19). The simple model and interconnected row model were based on the same principle: plate the cardiomyocytes in one chamber, and hMSCs in the other, then overflow the media to allow the growth factor to induce proliferation of the cardiomyocytes.
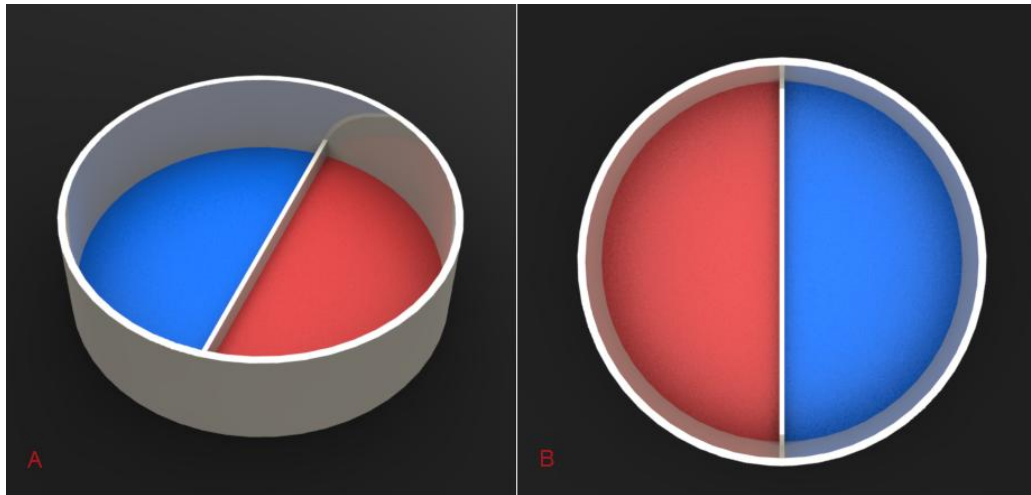
Figure 18: Simple Model; A: Isometric View, B: Top View
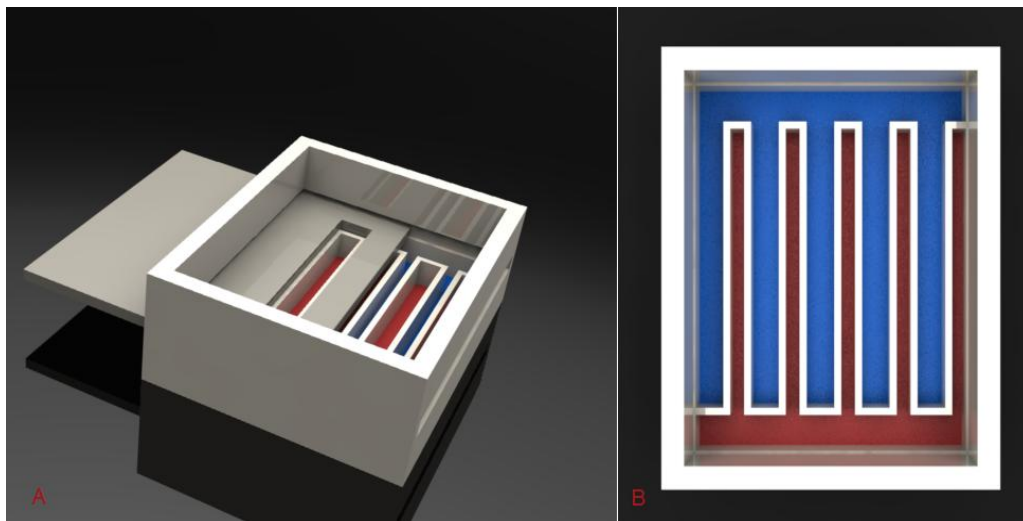
Figure 19: Interconnected Row Model; A: Isometric View, B: Top View

The interconnected rows model was superior to the simple model in that the design increased the amount of contact area for the growth factor to affect the cardiomyocytes. However, the design was difficult to handle and there was a high possibility of contamination during feeding and plating due to the closeness of the rows. We also found that adhesion of the prototype to the petri dish was difficult due to the large perimeter of the rows. A higher perimeter leads to a higher possibility of not applying the petroleum jelly efficiently and this would result in the hMSC migration to the cardiomyocyte chamber.

We incorporated the benefit of a higher contact area and took into account that the perimeter of the chambers must be as large as possible, yet not be in a complex design which would hinder the application of the petroleum gel. This resulted in a radial well design, shown below in Figure 20.
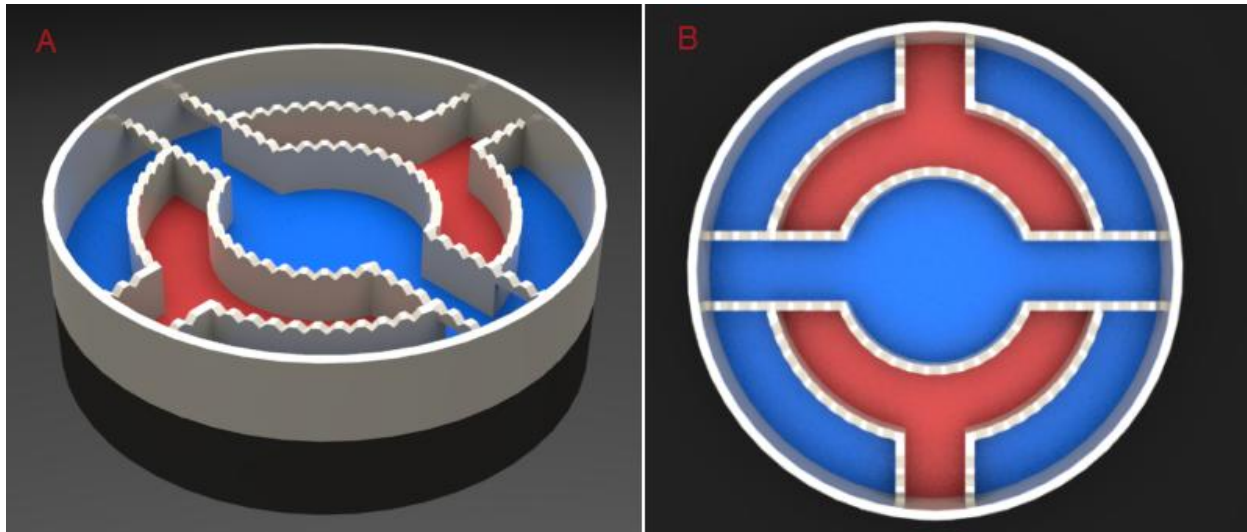
Figure 20: Radial Well Model; A: Isometric View, B: Top View

The most beneficial aspect of the interconnected rows was the high contact area between the hMSC population and the cardiomyocyte chamber. However, the small area allotted for hMSC growth did not take into account the higher proliferation rate of the hMSC. The simple model did allow a large space for the hMSCs and cardiomyocytes to grow, but had a very small contact area. We incorporated these deficiencies and strengths to create the radial well design, which has a reasonably high contact area, and is tailored to address the different proliferation rates of the two cell types. The dimensions for each design are displayed below in Table 5.

Table 5: Dimensions of Design Prototypes

Design	Contact Area (mm)	hMSC Area (mm^2)	Cardiomyocyte Area (mm^2)
Interconnected Rows	399	945	595
Simple	77	2290	2290
Radial Well	239	2860	1350

Methodology

Herein, the methods for developing, testing, and verifying the design are described in detail. This is inclusive of classifying prototypes, obtaining and culturing cells, and the procedures for tests and assays used to confirm the identity and healthy of the cells.

Prototype Classification

After determining the final prototypes to build, we rated each of them based on the previously discussed objectives and constraints to determine which design to pursue. Based on this rating, it was found that the prototype that would best meet the objectives and constraints of the design is the media overflow model. There were three points possible per criteria, with overflow leading in both the objectives and the constraints. These tables are shown below (Table 6 and

Table 7).

Constraint	Overflow	Velcro
Cell Constraints	9	9
Temperature	√√√	√√√
pH	√√√	√√√
Shelf life	√√√	√√√
Resources	12	8
Budget	√√√	√
Lab Equipment	√√√	√√√
Duration of Project	√√√	√√√
Lab Training	√√√	√
Design Constraints	5	4
Size	√√√	√√√
Contaminant Free	√√	√
Total	26	21

Objective	Overflow	Velcro
Cell Processing	5	5
High-yield	√√√	√√
Enable Proliferation	√√	√√√
Ease of Implementation	8	3
User Friendly	√√√	√
Cell Inspection	√√√	√
User Variables	√√	√
Cell Delivery	-	-
Cell Viability	6	5
Aseptic	√√√	√√√
Cell safety	√√√	√√
Clinically Applicable	-	-
Confirm Separation	-	-
Animal -> Human	-	-
Patient Safety	-	-
Marketing	TBD	TBD
Total	19	13

Prototype Adhesion

One of the obstacles necessary to overcome was developing a method for adhering the bottom-less dish to a standard petri-dish. By consulting with the client and research, we found that a method of adhering the prototype to the dish would be through the use of silicone gel. We explored various methods of applying the gel to the barriers.

Development of Adhesion methods

Various methods were examined to facilitate the adhesion of the prototype and device rings to the standard culture dishes. Dow Corning 976V silicone grease and Vaseline® Petroleum Jelly were the primary materials explored as adhesion media used between the prototype rings and the culture dish surfaces. Pasting methods using sterile spatulas, cotton swabs, and transfer pipettes were explored, as was a dip method similar to the method used in the production of dip candles. The dip method was focused upon since, once optimized, it allowed for the easier, faster, and more efficient adhesion. A device stand and a device pressing applicator were designed and produced from polyethylene using rapid prototyping to expedite the process and increase its ease of use.

Adhesion Materials: Silicone Grease

Dow Corning 976V silicone high-vacuum grease was first investigated to be used in adhesion, as it has been used for similar applications such as the isolation of clonal populations using stainless steel or glass cloning rings to microscope slides. A pasting method was developed to apply the silicone grease to the prototype device rings.

The Pasting Method

Using this pasting method, a spatula was used to apply a layer of gel along the bottom face of the ring barriers, which then be exposed to the surface of the culture dish in which the cell cultures would be cultivated. The silicone grease was administered to the bottom of the prototype rings using a manual pasting method with metal spatulas and forceps to hold the ring. The silicone grease and the metal utensils used in handling the device were sterilized using a steam autoclave. The application of the silicone grease was conducted within a culture hood to ensure the sterility of the device and culture environment. After the application of the silicone grease to the prototype rings, the prototype ring was pressed down onto the culture dish surface using a pair of sterilized metal forceps.

It was found that the pasting method yielded poor control in respect to the accuracy of the application. This limited control with respect to the accuracy of application resulted in uneven layers of silicone grease administered to the prototypes. Additionally it contributed to a thicker application of grease to the device rings, which often hindered the design productivity. This was particularly evident in the case of the interconnected rows prototype, where layers of grease bridged multiple rows. Sterile cotton swabs were used to clean excess silicone grease from the device rings.

Adhesion Materials: Petroleum Jelly

Vaseline® Petroleum Jelly was explored as a secondary alternative adhesion material, since its' use had been reported in literature as a substitute to silicone grease for the securing of cloning rings to microscope slides. Vaseline proved to have advantages over silicone grease due to its' lower cost and material properties. The Vaseline® petroleum jelly was softer and slightly less viscous than the silicone grease, making it easier to apply to the device when using the pasting method. Vaseline® Petroleum Jelly had a melting temperature of approximately 55° C. The silicone grease was found to have a melting temperature significantly higher than that of the autoclave, making Vaseline® a better alternative. The melting temperature also allowed for the development of additional adhesion methods including a revised pasting method (seen in Figure 21) using transfer pipettes, and a dipping method (seen in Figure 22) similar to that used in the creation of dip candles. While having a lower viscosity and melting temperature, the Vaseline® still maintained the desired characteristics of the silicone grease of being hydrophobic and maintaining a solid form at incubation temperatures of approximately 37° C.

The Revised Pasting Method

We developed a revised pasting method using Vaseline® petroleum jelly, which involved the sterilization of the jelly via heat in an autoclave. The liquid petroleum jelly was loaded into plastic disposable transfer pipettes within sterile cell culture hood. The petroleum jelly was then allowed to cool slightly above room temperature. After 5 to 15 minutes of cooling, the loaded transfer pipettes were squeezed to provide an output stream of the semisolid petroleum jelly along the barriers of the device. Sterile cotton swabs were used to clean areas where excess jelly had been applied, and the device was pressed to the culture dish while the final applied layer petroleum jelly cooled. Although this revised dipping method appeared to be much more effective than the previous method of using spatulas and silicone grease, it still required a large amount of dexterity and often resulted in the application of excess adhesion material and an uneven layer. Additionally, the application of material

along the perimeter of the ring device barrier was tedious, particularly when more details were incorporated into the device design.
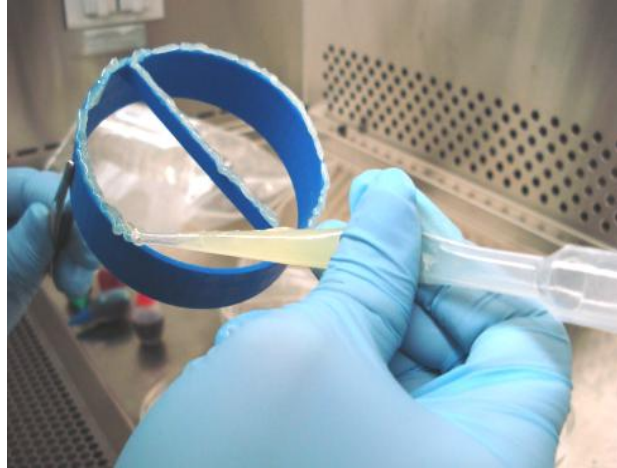
The Dip Method

A dip method of adhesion material application was developed in an effort to expedite and improve the ease of obtaining a uniform layer of the jelly. First, we heated the sterilized petroleum jelly to 55° C on a hotplate and water-bath within a cell culture hood. The heated petroleum jelly was then removed from the heat source and the ring device was held, suspended in petroleum with a pair of forceps as the gel cooled. To account for heating and cooling times, two heating containers with areas larger than that of the device ring areas were used.
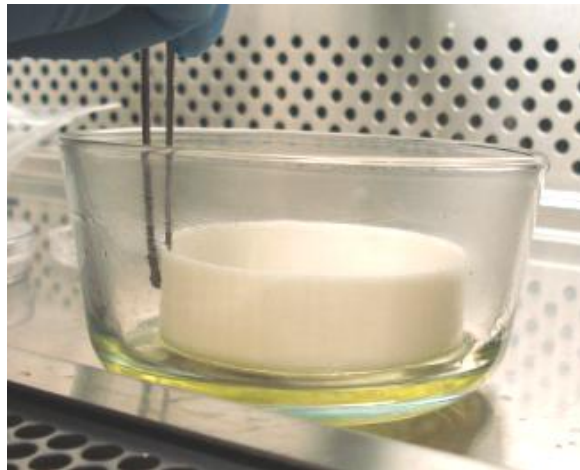
Figure 22: Dip method using heated petroleum jelly.

To reduce the time required for adhesion using this method, a stand was developed to suspend the plate. This suspension allowed application of an even layer of gel along the barriers, in addition to simplifying the process. A CAD model, actual model, and its actual application are shown in Figure 23, Figure 24, and Figure 25. The development of this stand was coupled with the development of the

overhang beam features within the final radial media well ring design, which both increased the support of the device and allowed for the suspension.
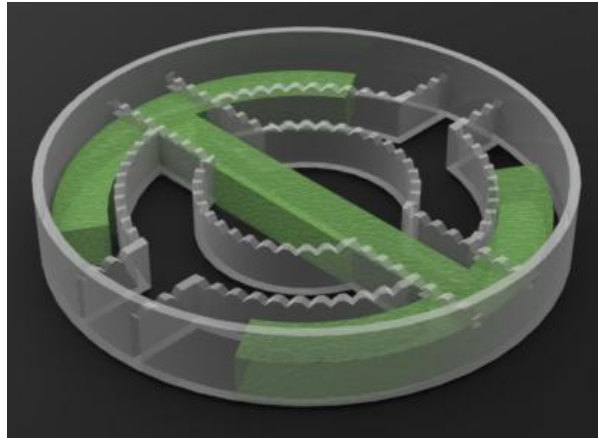
Figure 23: CAD model, plate and stand

Figure 24: Actual Model, plate and stand

Figure 25: Plate suspended by stand in gel

Final Adhesion to the Culture Dish

The final adhesion method involved the use of forceps to maneuver the device rings. To obtain uniformity, two pairs of sterile forceps were used and placed at opposite ends of the device ring. A cylindrical stamping device was designed to allow for the uniform pressing of the device rings to the culture dish. The stamp also maintained a uniform adhesion layer by applying uniform pressure onto the device when was placed in the dish.

Design Verification and Prototype Testing

Once the prototypes were produced, it was necessary to develop a method to test whether the design met the criteria established by the client statement. The design properties were established through tests that allowed for the proliferation of cardiomyocytes. Cross-well leakage tests were conducted to verify that adhesion and anchorage methods of the device rings to their culture dishes were implemented. Upon the development of a final adhesion method that exhibited no leakage between wells, a series of tests were then conducted to monitor cell growth within the devices. The tests also compared cardiomyocyte growth under overflow conductions with that of contemporary lone cardiomyocyte cultures, and cardiomyocyte cultures co-cultured with hMSCs.

Device Anchorage to Culture Dish

The first test examined whether or not the adhesion methods used to anchor the device rings to their respective culture dishes were adequate and satisfactory. Prior to examining the performance of the developed ring devices with respect to cell culture, a cross-well leakage test was developed and implemented. The test involved using aqueous media stained with specified dilutions of colored media within the multiple wells of the device, incubating the device over a 7-day period at standard cell culture conditions, and making optical observations of the wells within 24 hour time intervals. The dye ratios implemented were 2 drops of dye per 300mL of aqueous fluid (See Figure 26). Distilled water, PBS, and cardiomyocyte media were used as testing fluids. A blue dye was administered to wells of the dish to be used for hMSC cultures and a red dye was administered to the wells of the dish to be used for Cardiomyocyte cultures. Negative controls of the color dilutions of each dye were used, while subsequent positive controls simulating various levels of mixing were also used to calibrate these tests. The tests were conducted on the following prototypes: adhered simple, interconnected rows, and subsequent radial-well models of the overflow ring device (See Figure 27).
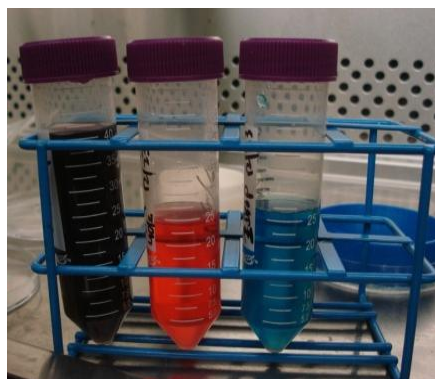
Figure 26: Three sample dye dilution controls.

Figure 27: Fluid-filled wells illustrating the different well sections within the radial-well ring device

Sterility of Prototypes and Device Strength

To ensure that the device was aseptic, the prototypes were sterilized using two methods. The initial method was to submerge the device ring within sterile ethanol. Sterile cotton swabs were used to wash regions of the prototypes with ethanol mechanically to allow for improved cleaning of the device, particularly after reuse. The prototypes were observed during handling to examine strength and material integrity after sterilization. The second method was to sterilize using ethylene oxide gas. The latter was chosen because ethanol sterilization appeared to compromise the prototype material.

Cell Culture

Both human mesenchymal stem cells and cardiomyocyte cell populations were required to conduct this project in cell culture, co-incubation, and cell population isolation. The hMSCs were obtained from additional flask passages that resulted from graduate study projects within the Gaudette Laboratory at Worcester Polytechnic Institute's Gateway Park facilities. These hMSCs were cultured in 75-mm^2 culture flasks at 37°C and standard %O2 and %CO2 settings. They were fed every other day with 15mL of a fluid media solution [10% Fetal Bovine Serum (FBS), 89% Dulbecco's Modified Eagle Medium (DMEM), 1% Penicillin: streptomycin (Pen-Strep)], with washes of PBS between feedings. Passage numbers 5 through 9 of hMSCs were within this study. All cardiomyocytes used in this study were obtained from Lewis mice or Sprague Dawley rats that had initially been used for other independent laboratory uses such as educational surgery and anatomical exercises. A fluid media solution containing 5% FBS, 94% DMEM, and 1% Pen-Strep was used to feed cardiomyocyte populations every 48 hours during their culture within standard cells treated 6-well culture plates.

Cardiomyocyte Isolation

The first step in the process in obtaining cardiomyocyte populations was to extract the cardiomyocytes from mice. Once isolated, these cells were plated and co-cultured with hMSCs. Therefore, it was important that the procedure generated a high healthy cell count to permit a superior yield of proliferated cardiomyocytes. The procedure used to extract cardiomyocytes from mice (and subsequently rat) hearts is described below.

Preparation

In advance
1. Make Tyrode's Solution
2. Make KB Solution
3. Store 30mL of DMEM
4. Make 5% FBS DMEM (with 1% P/S)

Preparation

Day of procedure

1. Spray ethanol on tubing connected to oxygen tank to sterilize it, place it in the biosafety hood.
2. Attach a sterile syringe filter to the tubing and use parafilm to connect to a 5mL pipette.
3. Place the sterile pipette tip in the Tyrode's Solution and allow oxygen to bubble in for about 7 minutes.
4. Replace the 5mL pipette and repeat for KB Solution.
5. While bubbling the solutions, fill the water bath and set it to 38C, connecting the inlet and outlet to the water chamber apparatus. Be sure to fill the water bath fully to allow the pump to work.
6. As the water bath begins to flow, sterilize water using a syringe and a syringe filter into the jacket of the apparatus. Fill the apparatus to about the 200mL mark.
7. Aliquot ~20mL of Tyrode's solution into a 50mL conical tube to collect the heart(s).
8. Add gentamicin (1:1000 dilution) to the prepared Tyrode's solution in the 50mL conical tube.
9. Aliquot 7mL of Tyrode's solution into 2 15mL conical tubes and add gentamicin at the same dilution.
10. Get a supply of sterile petri dishes (2), glass pipettes, 15mL conical tubes (2), 50mL conical tubes, a conical tube storage rack, and three 8 well slides and place in biosafety hood.
11. Remove one aliquot of blendzyme from the -20C and place in biosafety hood to thaw.

Isolation

1. Isolate hearts from sacrificed mice- place in prepared 50mL conical tube of Tyrode's solution.
2. Bring hearts back to hood (spray conical tube with ethanol beforehand)
3. Empty contents of tube into an unused half of a petri dish.
4. Gently, using forceps, squeeze hearts to remove blood.
5. Put hearts in one 15mL conical tube and invert several times gently to rinse heart
6. Repeat steps 3-5.
7. Repeat steps 3 and 4.
8. With the hearts in the third petri dish, cut of the top third of the heart off and rinse the ventricles, removing as much blood as possible.
9. Transfer the ventricles into the fourth petri dish and add 5-10mL of Tyrode's solution. Mince the tissue with a scalpel and scissors as much as possible.
10. Mix the aliquot of Blendzyme (200uL) into 20mL of Tyrode's solution in a 50mL conical tube
11. Using a 25mL pipette, transfer the fluid as well as the minced tissue from the petri dish to the conical tube.

12. Putting the conical tube in the jacket, triturate for 7 minutes, being careful to not let the tissue clog the pipette.
13. Allow the tissue to settle and carefully pipette the solution into 2 15mL conical tubes, being careful to not pipette any tissue.
14. Cap and label these tubes as "H-1" and spin in the centrifuge at 300-400 rpm for 5 minutes
15. Add 10mL of KB solution to the minced tissue (suspended in a bit of Tyrode's solution with the Blendzyme) and triturate for an additional 7 minutes, with the tube remaining in the water jacket.
16. Repeat steps 14 and 15, label these tubes "H-2" instead of "H-1".
17. (Optional) Repeat steps 15 and 16 for "H-3" cells
18. Aspirate the media from both "H-1" and "H-2" (and "H-3") tubes, being careful to not disturb the cell pellet.
19. Resuspend the cells in 5mL of KB solution and spin them again at 500rpm for 5 minutes.

Here the user will resuspend the cells in a volume relevant to their experiments.

Cell Growth Prototype Testing

Cell Growth in Different Media Types:
The following outlines the experimental set-up implemented to the effect of media composition on cell growth of the hMSCs and cardiomyocytes. The compositions of media tested varied primarily in the percentages of FBS contained within the media. Traditional cardiomyocyte media is composed of 5% FBS, 94% DMEM, 1% Pen-Strep. Meanwhile hMSC media is composed of 10% FBS, 89% DMEM, 1% Pen-Strep. A "median media" composition was developed by averaging the amounts of %FBS and %DMEM found within the two original media types (which resulted in a composition of 7.5% FBS, 91.5% DMEM, 1% Pen-Strep. Each media was used in cell culture tests if cardiomyocytes and hMSCs. The following is an outline of the experimental set-up used to implement this cell growth test.

Materials:
- Cardiomyocyte media (5% FBS, 94% DMEM, 1% Pen-Strep)
- hMSC media (10% FBS, 89% DMEM, 1% Pen-Strep)
- Median media (7.5% FBS, 91.5% DMEM, 1% Pen-Strep)

    I.)Testing hMSC growth in cardiomyocyte media
        o   Control: hMSC growth in hMSC media
        o   Variable: hMSC growth in cardiomyocyte media

    II.)Testing cardiomyocyte growth in hMSC media
        o   Control: cardiomyocyte growth in cardiomyocyte media
        o   Variable: cardiomyocyte growth in hMSC media

III.)Testing hMSC growth in "median" media - can be run parallel to Test I
- o   Control: hMSC growth in hMSC media
- o   Variable: hMSC growth in median media

VI.) Testing cardiomyocyte growth in "median" media - can be run parallel to Test II
- o   Control: cardiomyocyte growth in cardiomyocyte media
- o   Variable: cardiomyocyte growth in median media

Usability Testing

Prior to long term cell culture tests, preliminary usability tests were conducted on each of the device prototypes. Feedback from these tests enabled the project team to implement design modifications to optimize the functionality and appeal of the device. Usability tests included device loading and handling, cell monitoring, and eventual cell passaging tests. Such tests were conducted qualitatively by having the operator note comments regarding the ease of use of each prototype when implementing activities such as loading the prototypes with fluid media, moving the wells from the incubator to the culture hood, observing the wells via microscopy, and aspirating media from the wells. Meanwhile a passaging test was conducted on the final radial-well ring device to ensure that both cardiomyocytes and hMSC populations could be passaged to and from the culture device.

Testing for Migration of hMSCs and the Proliferation of Cardiomyocytes

One of the purposes of co-culturing hMSC and cardiomyocytes was to have the cardiomyocytes benefit from the various cytokines, growth factors, and other cell-signaling molecules released by hMSCs. However, for the purposes of this project it was a primary objective to maintain an isolated, homogenous population of cardiomyocytes. One of the goals of this project was to simulate a co-culture environment while maintaining isolated cell populations this project included tests for both the migration of hMSCs and for the proliferation and or increased viability of cardiomyocytes.

Monitoring of Relocation and Migration of hMSCs

The relocation and or migration of hMSCs were conducted via optical and fluorescent microscopy. The morphological characteristics of hMSCs were studied and identified in cultures grown during the early stages of this project. Meanwhile, all hMSCs implemented within the standard prototype ring and final device ring cultures were tagged with quantum dots. Tagging was conducted per 75-mm^2 flask of hMSCs by adding 11 micrometers of quantum dots (QD) into 10 mL of hMSC media that would be fed to the cells, as the QDs would then be endocytosed within the cells. After 24 to 48 hours of incubation, the QD-loaded hMSCs would then be washed with PBS and passaged into new culture wells (either for direct co-culture or overflow culture within the prototype or final device ring dishes.

Monitoring Proliferation and Viability of Cardiomyocytes

The proliferation and viability of cardiomyocytes was monitored through the observation of the appearance and morphological characteristics of the cells, using optical culture counts, and using a cell viability assay, both of which are described in further detail in their own respective sections of this report.

Experimental Set-up of Long-Term Cell Culture Tests

Although four major device prototype models (the simple, interconnected rows, sliding cover, and radial-well models) were developed as an exploration of the media overflow design alternative, only the final radial well model was implemented for the final long-term cell culture tests used to examine cell relocation and changes in cardiomyocyte presence and viability. The following outlines the experimental set-u implemented to execute these tests:

Experimental Controls for Cell Cultures used to test for hMSC migration and cardiomyocyte viability:

- Contemporary lone cardiomyocyte cultures in sample prototypes and 6-well plates.
- Contemporary hMSC and cardiomyocytes direct co-cultures in 6-well plates
- Fresh (within 24-48 hours of cardiomyocyte isolation) lone cardiomyocyte cultures in 6-well plates
- Fresh (within 24-48 hours of cardiomyocyte isolation) hMSCs and cardiomyocytes directly co-culture in 6-well plates

Initial Overflow Model Tests:

1. Variable conditions tested:

Culturing QD-loaded hMSCs on one side of the overflow plate

A. No cardiomyocytes present in second well, no media in cardiomyocyte well

B. No cardiomyocytes present in second well, media present in cardiomyocyte well

C. Cardiomyocytes present, selected media for overflow

2. Overview of Procedure:

A. Adhere prototype "ring" to 900mm diameter PS petri dish plate

B. Passage hMSCs into designated section of plate (ideally, hMSCs will be QD-loaded)

- Simple model: hMSC media, 9 mL cardiomyocyte media,
- Rows & sliding cover model: 4.5 mL hMSC media, 3.8 mL cardiomyocyte media
- Radial-well model: 3.5 mL cardiomyocyte media (per well), 5mL hMSC media

C. Passage cardiomyocytes onto designated section of plate (when applicable)

D. Allow for cell attachment and culture for 48 hours

E. Examine dish for undesired migration of hMSCs

F. Administer overflow media to dishes, approaching from cardiomyocyte culture section

- 26 mL of overflow media for simple model
- 12 mL of overflow media for row models
- 25 – 30 mL overflow media for radial-well model

G. Incubate cells in "overflow" conditions for 8 days:

-Change media every 48 hours

-Initial aspiration of media should be conducted on hMSC culture section

H. Evaluate cell populations from each section of the culture dishes

Refinement of Cell–culture Conditions

Based on the preliminary cell culture tests, various parameters were decided upon for the second phase of culture testing. At this stage, other methodologies such as microscopy techniques and cardiomyocyte isolation had also been refined to ensure more accurate results of this second phase of cell culture. Such parameters that were determined included duration of incubation, the composition of the media to be used during overflow, order of cell loading, direction of overflow, and direction of

aspiration. Controls of lone cardiomyocyte populations, direct co-cultures of hMSCs and cardiomyocytes, and overflow cultures within the designed culture device were observed over a 7-day and 14-day incubation period, with day 0 being the day that co-culture was implemented (which was kept to a target 48 hours after the cardiomyocyte isolation). Contemporary lone-cultures and direct- co-cultures were conducted for each set of overflow cultures grown in the devices. As initial testing showed that cardiomyocytes appeared to be sensitive to increased concentrations of FBS, traditional cardiomyocyte media was used to implement the overflow. Similarly, cardiomyocyte media was used to the feed direct co-cultures controls.

After preliminary tests to optimize cell culture environment and monitor the location of hMSCs, as no hMSC migration was observed, the second testing phase implementing cell culture was executed. This phase was conducted using only radial-well models of the final ring device, adhered to culture dishes . Testing using the simple, interconnected row, and sliding models was discontinued given the respective flaws of these models and the conclusion that the radial well model had been an improvement to each design. During preliminary testing, a pre-conditioning of culture plates using 1% gelatin was examined to test whether or not this increased cardiomyocyte attachment to the culture dish. As no significant difference in cell attachment was observed, this 1% gelatin treatment was not continued in the second phase of cell culture tests.

During the second phase of testing, sixteen radial-well device samples were produced. The source of the cardiomyocytes taken for this phase of testing included four cardiomyocyte isolations from adult Sprague-Dawley rats. Device prototypes were divided into groups of four. Each group contained cells from the same isolation of cells. All isolations were conducted using tissue from three to four rat hearts. Each isolation yielded two 6-well plates of cardiomyocyte cells. Six of these 12 wells were then co-cultured so that contemporary lone cardiomyocyte cultures and direct co-cultures could be obtained for each set of prototypes.

Overflow was carried out from the cardiomyocyte well to the hMSC well as a measure to prevent the relocation of hMSCs as the result of forces and currents created by fluid flow. Similarly, aspiration was conducted initially from the hMSC well, then using new utensils from the respective cardiomyocyte well so that all the media could be aspirated.

The first two sets of prototypes were cultured using conditions identical to initial cell culture phase experiments. hMSCs were first passaged to the central well, followed 24 hours later by the addition of freshly isolated cardiomyocytes within the cardiomyocyte wells. Overflow was then conducted 24 hours after cardiomyocyte attachment. The second two sets of prototypes were cultured by first plating the cardiomyocytes within their respective wells. An additional layer of petroleum jelly was then applied to the hMSC well along the radial well boarder to prevent hMSC relocation or seepage through across the wells, and QD-loaded hMSCs were passaged onto the hMSC wells. Overflow was conducted upon the confirmation of cell attachment.

Cell Identification and Cell Viability

Cell Counting

A specific method was developed for counting cardiomyocytes in order to monitor their health and proliferation. The counts were conducted using optical microscopy at a 400X magnification. The method was applied to lone cardiomyocyte cultures, direct co-cultures (cardiomyocytes and hMSCs), and cardiomyocytes cultured in the prototypes following overflow.
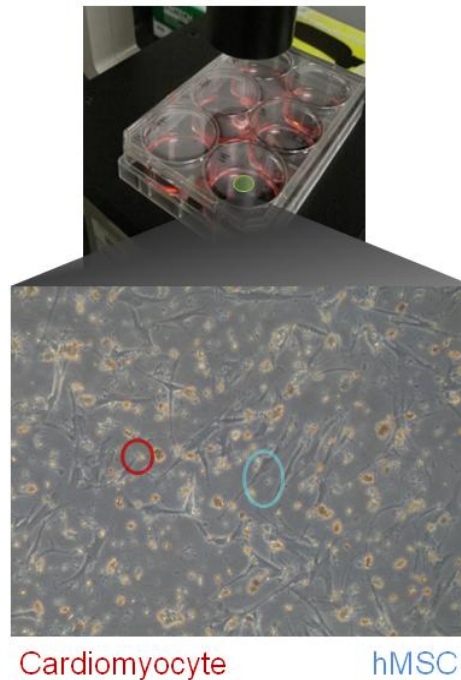
Figure 28: Illustration of 100X FOV magnification of hMSC and cardiomyocyte co-culture.

Counts were performed on days 0, 1, 3, 7, and 10 after plating and following overflow in the prototypes. In this method, ten fields of view within the well were chosen at random and the number of healthy cardiomyocytes and the number of unhealthy cardiomyocytes were counted and recorded. In addition, cultures were monitored for cell "islands". Cardiomyocytes were classified as healthy and unhealthy based on observable morphological characteristics, where healthy cells were characterized as being elongated with distinguishable striations and unhealthy cells were characterized as being "blebbed", or round in shape (See Figure 29). Islands were defined as clusters of approximately three or more cells in a small area (See Figure 30).
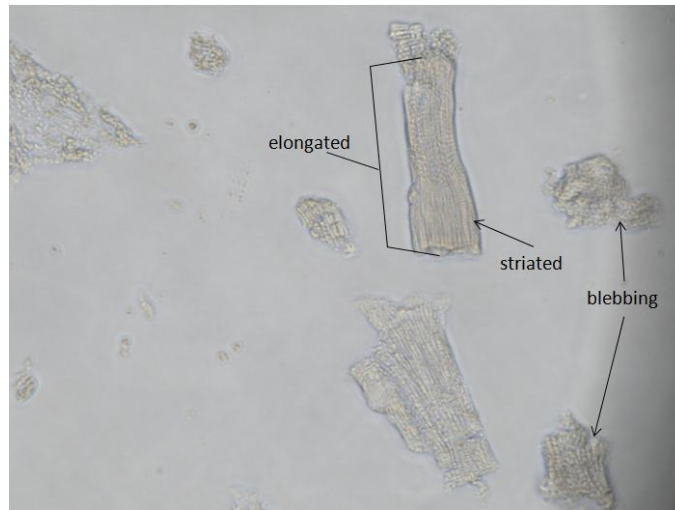
Figure 29: Optical microscopy (400X) image of healthy (elongated, striated) and unhealthy (round, blebbing) cardiomyocytes from adult rats
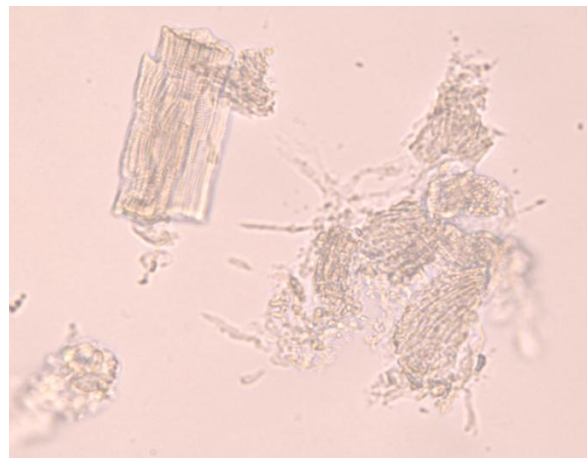
Figure 30: Cardiomyocyte Island, optical microscopy 400X.

Immunohistochemistry

Immunohistochemistry (IHC) staining was conducted on cell samples collected from lone, co-culture, and overflow cultures grown within the radial-well devices. IHC staining provided a means for cellular identification. The protocol outlining the steps involved in this staining, the dehydration, and coverslip preparation of the slides is provided below. Specifically, this staining procedure was geared towards the identification of cardiomyocytes, but also incorporated a nuclear stain to assist in cell counting.

Monoclonal anti α-actinin was used as a primary antibody because it binds to α-actinin, a protein characteristic of cardiac muscle cells. A *rabbit anti-mouse IgG* antibody tagged with the reactive fluorescent green dye *Alexa Fluor 488* was used as a second antibody derived from a rabbit that reacts to mouse protein. Because the blocking agent is intended to prevent the secondary antibody from nonspecific binding to the sample cells or tissue, it is important that the blocking serum used during IHC is a serum from the same host species used to derive the secondary antibody. Thus, normal rabbit

serum was used as a blocking serum. Finally, a Hoescht counterstain was used since it is a fluorescent stain that actively transports out of cells through ATP-binding proteins. Because certain cell types differ in transport activity levels than others, this stain was valuable in distinguishing the different populations of cells from one another in mixed samples.

The staining procedure was performed on a control of cardiomyocytes that were fixed within 12 hours of isolation and on a direct co-culture that was fixed 24 hours after plating the hMSCs, both of which were plated directly into the chamber slides for IHC. Staining was also performed on cells passaged from a cardiomyocyte lone culture, a direct co-culture of hMSCs and cardiomyocytes, and from the cardiomyocyte wells within the overflow prototypes. The lone cardiomyocytes and direct co-cultures were cultured for seven days prior to IHC and the cardiomyocytes were passaged from the prototypes after being in the overflow condition for seven days.

Upon the preparation of slides that were fixed and stained using immunohistochemistry, the slides were examined under fluorescent microscopy and cell counts were obtained to be compared to optical counts conducted in the absence of staining. This was conducted to ensure the accuracy of such optical counts and to ensure for the proper identification of cardiomyocyte and hMSC cell types.

Materials for IHC staining:

- Primary antibody: Sigma A – 7811, Monoclonal alpha-actinin (1 vial makes 2mL)
- Secondary antibody: Invitrogen A11059 , Rabbit anti-mouse
- Alexa Fluor 488 stain
- Hoeschst (light-sensitive counterstain)
- Q dots 655
- 4% Parafromaldehyde
- Tritons-100
- 4 microLiters 48% Alexa
- 1600 microLiters 1.5 NRS
- PBS, PBS w/0.005 % Tween
- 2.5 microLiters Alexa
- 1000 microLiters 1.5% NRS w/ 0.5% tween
- Normal Rabbit Serum (NRS)
- Light-blocking hydration chamber
- Aspiration tubing and glass pipettes
- Coverslips
- Cytoseal-60

Notes:
- All PBS w/0.05% Tween
- Al PBS rinses (after chambers removed) in coplin jar.

IHC Protocol:
1. Wash slides with PBS 1X 5 minutes.
2. Cover slides with 4% Paraformaldehyde and let sit for 15 minutes.
3. Wash slides with PBS 2X 5 minutes.
4. Apply triton-100 to the slides and wait about 20min.
5. Block for 30 min at room temperature, using Alexa Fluor 488 w/ 1.5% NRS.
6. Add primary antibody 1:400 w/NS (NRS), O/N at 4˚C, overnight
7. Wash slides with PBS 3X 5min
8. Spin in micro-centrifuge for 10min at 10 kRPM
9. Apply second antibody and leave for 45min at room temperature in the dark
10. Wash with PBS 3X for 5min.
11. Counterstain with 0.5µL Hoecsht in 3mL DI H2O for 5min
12. Rinse slides with PBS 1X for 5min
13. Dehydrate

Dehydration Protocol:
1. Dip 20 times for approximately 1min in 95% alcohol
2. Repeat step 1
3. Immerse in 100% alcohol for 1min
4. Repeat step 3 for 2min
5. Repeat step 3 for 3min
6. Immerse in Xylene IV for 2min
7. Immerse in Xylene V for 2min
8. Immerse in Xylene VI for 3min
9. Put coverslip on using Cytoseal 60

LIVE/DEAD Assay

A LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Lot # 705637) was obtained from Invitrogen and stored at -20˚C in a dark, dry environment. The Eth-D1 stain is obtained at an initial stock concentration of 2 mM in a 1:4 volume/volume ratio of DMSO/H_2O. The stock concentration of the Calcein AM is 4 mM in anhydrous DMSO. These stains must be diluted to the appropriate concentration, which varies based on the cell type. As the calcein AM may hydrolyze when exposed to moisture, it is necessary to dilute it immediately before use. Based on initial experiments, it was determined that the optimal concentrations for use with cardiomyocytes are 1 µM for the Eth-D1 and 2 µM for the calcein AM stain. The stains were diluted using sterile PBS. A total volume of 2.5 mL was prepared to be used in one 9.6 cm^2 well (1 well in a 6 well plate). The following procedure was used to prepare the required 2.5 mL of the solution:

1. Add 2.5 mL PBS to a 15 mL conical tube.
2. Briefly centrifuge both the Eth-D1 and the Calcein AM.
3. Add 1.25 µL Eth-D1 stock
4. Vortex thoroughly

5. Add 1.25 µL calcein AM stock
6. Vortex thoroughly
7. Add to cells immediately
8. Incubate for 15 – 30 minutes
9. Observe using appropriate fluorescent filters

To confirm that the concentrations were appropriate, a negative control was used in which the cells were exposed to 70% methanol for a period of 15 minutes to obtain a dead cell population prior to performing the assay.

At incubation day 7, a live/dead assay using the aforementioned stains was conducted on six 9.6 cm^2 wells containing a lone culture of cardiomyocytes plated at a concentration of 5500 cells per square centimeter. 2.5 mL of ethanol was added to three of these wells to create a "dead cardiomyocyte" control. A "live cardiomyocyte control" was conducted on cardiomyocytes within 24 hours after incubation and plating.

At incubation day 7, an identical live/dead assay was performed on both co-culture cells and cells grown in the radial-well media overflow devices. A dead co-culture control was similarly prepared and observed.

Western Blotting

Lysate Preparation

Based on the location of the protein of interest within the cell, the results can be optimized by using various lysis buffers. In our experiments, the protein of interest was alpha-actinin, which is a cytoskeletal-bound cytoplasmic protein. Therefore, the lysis buffer that was used was a Tris-Triton buffer.

Tris-Triton buffer: (Cytoskeletal proteins)

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate

The buffer can be stored at 4˚C for several weeks or for up to a year at -20˚C.

From Tissue:

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round-bottom microfuge tubes or Eppendorf tubes and immerse in liquid nitrogen to "snap freeze". Store samples at -80°C for later use or keep on ice for immediate homogenization.
3. For a ~5 mg piece of tissue, add ~300 µl lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2x300 µl lysis buffer
4. Maintain constant agitation for 2 hours at 4°C (e.g. place on an orbital shaker in the fridge). Volumes of lysis buffer must be determined in relation to the amount of tissue present (protein extract should not be too diluted to avoid loss of protein and large volumes of samples to be loaded onto gels. The minimum concentration is 0.1 mg/ml, optimal concentration is 1-5 mg/ml).
5. Centrifuge for 20 min at 12000 rpm at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice; discard the pellet.

From Cell Cultures:

1. Place the cell culture dish in ice and wash the cells with ice-cold PBS (a minimum of 5×10^5 cardiomyocytes should be used).
2. Drain the PBS, then add ice-cold lysis buffer (1 ml per 107 cells/100 mm dish/150 cm^2 flask; 0.5ml per 5x106 cells/60 mm dish/75 cm^2 flask).

3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microfuge tube.
4. Maintain constant agitation for 30 minutes at 4°C.
5. Centrifuge in a microcentrifuge at 4°C. You may have to vary the centrifugation force and time depending on the cell type; a guideline is 20 minutes at 12,000 rpm but this must be determined by the end-user (e.g. leukocytes need a very light centrifugation).
6. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

Bradford Assay

A standard curve was developed using Bovine Serum Albumin (BSA) at varying dilutions. The stock BSA was diluted with DI water at appropriate volumes of protein and diluents (see Table 8). A 25 µl sample of the protein can then be combined with 200 µl of the Coomassie Blue dye and incubated at 37˚ C for 15 minutes. The absorbance of the undiluted lysate sample can be determined by adding 200 µl of the Coomassie Blue dye to 25 µl of the lysate sample. This should be performed when the standard curve is developed to ensure a consistent incubation period. The absorbance is then measured at a wavelength of 562 nm. Using the equation of the standard curve, the concentration of protein in the lysate sample can be determined. For samples with lower protein concentrations (less than 250 µg/ml), a different set of dilutions was used to determine the protein concentration of the lysate sample (See Table 9).[70]

Table 8: Diluted BSA preparation for 20-2000 µg/ml protein sample

Vial	Diluent Volume	BSA Volume and Source	Final BSA Concentration
A	0	300 µl Stock	2000 µg/ml
B	125 µl	375 µl Stock	1500 µg/ml
C	325 µl	325 µl Stock	1000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	325 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

Table 9: Diluted BSA preparation for 5-250 µg/ml protein sample

Vial	Diluent Volume	BSA Volume and Source	Final BSA Concentration
A	700 µl	100 µl Stock	250 µg/ml
B	400 µl	400 µl of vial A dilution	125 µg/ml
C	450 µl	300 µl of vial B dilution	50 µg/ml
D	400 µl	400 µl of vial C dilution	25 µg/ml
E	400 µl	100 µl of vial D dilution	5 µg/ml
F	400 µl	0	0 µg/ml = Blank

Acetone Precipitation

In acetone precipitation, a lysate sample can be concentrated by precipitating the protein using acetone. The following is the procedure to be followed when performing acetone precipitation.

1. Add cold acetone (-20˚ C) to the lysate sample at a volume four times greater than the sample volume.
2. Vortex and incubate for one hour at -20˚ C.
3. Centrifuge at 14000 x g for 10 minutes.
4. Remove supernatant, without disturbing protein pellet.
5. Expose to air at room temperature for 30 minutes to allow acetone to evaporate.
6. Add appropriate volume of buffer and vortex to dissolve the protein pellet.

The protein concentration in the obtained lysate can be determined through a Bradford assay.

Gel Preparation

Polyacrylamide gels are prepared in glass plates following a recipe that can be altered based on the desired density of the gel. The density is changed based on the molecular weight of the protein of interest, as the density will control how quickly the sample can pass through the gel. For alpha-actinin, which is expected to appear at a molecular weight of 105 kDa, a 12% gel will suffice.

Table 10: Recipe for Western Blot polyacrylamide running gel (above) and stacking gel (below)

Running Gel (12%)	
dH2O	3.3
30% Polyacrylamide	4
1.5M Tris (pH8.8)	2.5
SDS (10%)	0.1
10% Ammonium persulfate (25%)	0.1 (.04)
TEMED	0.004
Total volume	10 mL

Stacking Gel (5%)	
dH2O	3.4
30% Polyacrylamide	0.83
1.5M Tris (pH8.8)	0.63
SDS (10%)	0.05
10% Ammonium persulfate (25%)	0.05 (.02)
TEMED	0.005
Total volume	5 mL

The ammonium persulfate and TEMED should be added last in order to prevent copolymerization from beginning before the gel is cast into the plates. The gels can be stored for a period of approximately 4 days at 4°C in a humid environment.

Sample Preparation

1. Based on the protein extraction yield, calculate what volume of sample needs to be added to each lane to equal 20 µg of protein.
2. With a 10 well mini gel, a volume (including sample, buffer, beta mercaptoethanol) of no more than 50 µl is recommended for each lane.
3. Add 10-15 µl of Laemmli buffer to a microtube.
4. Add beta mercaptoethanol. (5% of total volume).
5. Add a protein sample volume that will contain 100 – 150 µg of protein to the tube with the other reagents.
6. Heat the microtubes at 70° Celsius for 2 minutes, then place on ice

Loading the Gel

1. Pour 1X SDS-PAGE Buffer into the Western Blot tank.
2. Position the acrylamide gels in the gel holder assembly and immerse into the tank.
3. Fill the inner compartment (between the two gels) with SDS-PAGE Buffer.
4. Carefully load the samples in the wells (using a fine-tipped pipette).
5. Place the lid on the tank and plug it into the power source. (Note: this procedure uses electric current to separate proteins – use caution when working with tank electrophoresis to avoid injury).
6. Run the apparatus at 100V until the samples have passed the stacking gel.
7. Turn the voltage up to 160V and allow the samples time to separate; use a pre-stained molecular weight marker to determine the end-point of the electrophoresis.

Results

Cross-well Leakage Test Results

Initial cross-well leakage exhibiting dye mixing as a result of spilling that took place between the two wells present in the simple well prototypes. Corrections were made by increasing the height barrier. An increased height barrier, increased caution taken while handling the ring devices and culture dishes, and improved handling techniques prevented such spilling in subsequent prototypes and during subsequent tests. Initial pasting experiments showed inconsistent test results, but were positive to dye mixing. This appeared to be the result of improper and non-uniform adhesion methods and anchorage. Pasting methods using petroleum jelly and transfer pipettes proved to prevent leakage during 90% of testing. Upon their refinement the final dipping methods that employed petroleum gel, the device stand, and the stamping device feature exhibited no cross-well leakage during the 7-day period of incubation and observation.

Testing Cell Growth in Different Media Types

Cardiomyocyte cell growth appeared to be the most sensitive to variation in media types. Optimal cardiomyocyte growth was exhibited in the traditional cardiomyocyte media, while increased levels of FBS appeared to be unfavorable based on documented microscopic observation of confluence and morphological characteristics. No significant difference in hMSC growth was observed among the variations in media composition, although it was initially predicted that hMSC cultures would grow best in traditional hMSC media.

Usability Testing – Device Loading, Cell Monitoring, and Cell Passaging:

Initial usability tests showed that the inter-connected row and sliding barrier models were insufficient as cell loading, aspiration, and observation of the multiple wells via microscopy were extremely difficult and tedious actions to carry out. The final radial-well model improved upon all areas of usability testing substantially while allowing for an greater difference in surface area ratio and a greater contact perimeter between the hMSC and cardiomyocyte wells, as compared to the simple model. Cell passaging for both hMSC and cardiomyocyte populations were successful both to and from the final radial-well culture device.

Optical Counts

Fluctuations in the total number of cardiomyocytes observed among all three culture types (lone cardiomyocyte cultures, direct hMSC and cardiomyocyte co-cultures, and overflow cultures) were observed. However, morphological characteristics appeared to indicate healthier populations within the overflow culture as compared to both culture controls. The number of healthy cells observed in island cultures in particular appeared to increase over both the 7-day and 14-day culture periods within the overflow cultures conducted using the radial-well device rings. Data from the optical counts conducted is summarized in figures 31, 32, and 33.
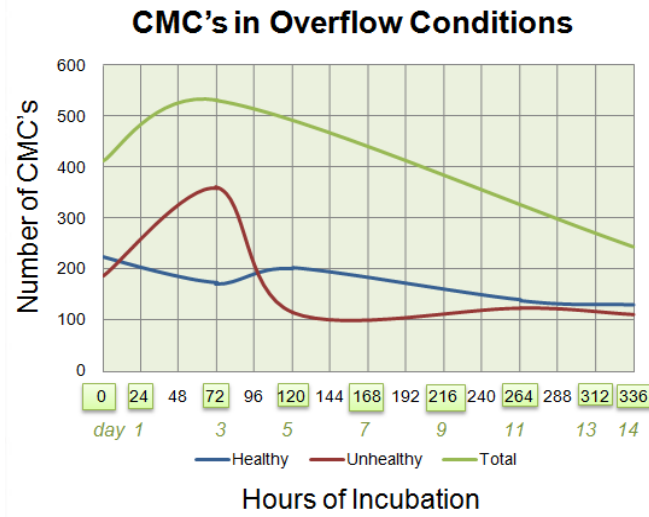
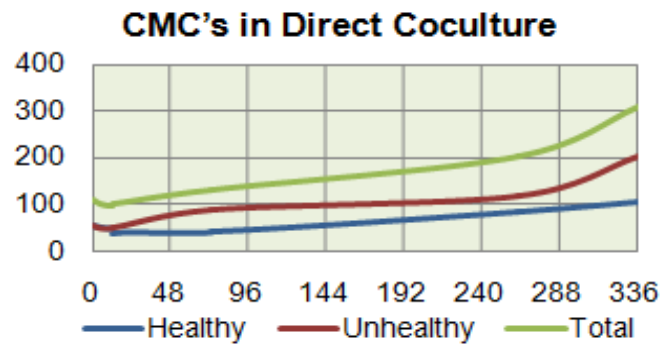**Figure 31: Summary of Optical Count Averages in Overflow Culture**

Figure 32: Summary of Optical Count Averages in Direct Co-Culture
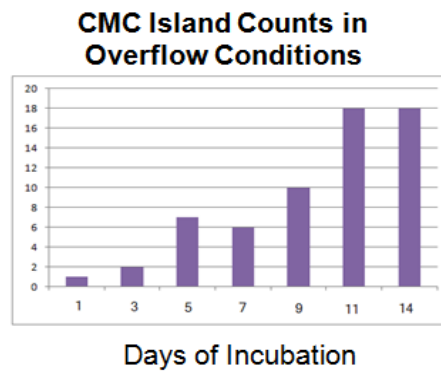

Figure 33: cardiomyocyte Island Counts in Overflow Culture

Cell Migration and Relocation

With the exception of one prototype that was incorrectly adhered to its culture dish and disturbed during handling, cell migration of hMSCs was not observed over the course of the initial 7-day incubation periods of the overflow-cultures. Relocation of cardiomyocytes was observed in all tested prototypes that involved the plating of cardiomyocytes after the plating of the hMSCs. Cardiomyocyte relocation was only observed in one of the prototypes in which an the cardiomyocytes were plated first, before the hMSCs, and a secondary layer of adhesion applied along the barrier within the hMSC well. With the exception of this prototype, hMSC migration appeared to be prevented within the set of prototypes that included a second layer of petroleum jelly adhesion material, applied via transfer pipette. The remaining prototypes that did not receive this additional layer of petroleum jelly gradually exhibited initial migration starting at days 9,10, and 11 of overflow incubation. Migration and relocation however was perceived as minimal: remaining along the device barriers rather than being fully implemented or sporadically located within the cardiomyocyte wells.

LIVE/DEAD Assay

The Calcein-AM dye appeared to yield positive results among the hMSCs in the fresh co-culture wells that were observed. As seen in Figure 34, the hMSCs appeared to express the dye and fluoresce a bright green. The assay appeared however to be time-dependent as the brightness expressed gradually decreased over time. Positive results and the fluorescence of the cardiomyocytes within these co-culture wells could not be determined as any possible green fluorescence of these cells was masked by that observed within the hMSCs.
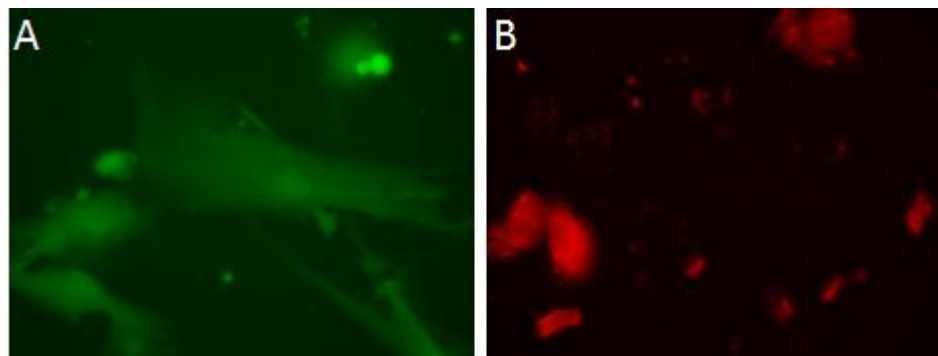
Figure 34: LIVE/DEAD Assay on direct co-culture A. Live stain B. Dead stain

Auto-fluorescence experienced with Calcein-AM appeared to skew the live-stain results with the remainder of the cell cultures examined. The fresh lone cardiomyocyte cultures (see Figure 35), 7-day co-cultures, and overflow cultures (see Figure 36) did not show the same degree of green fluorescence observed within the fresh co-cultures. The cardiomyocytes in said populations did appear to have some fluorescence, however extracellular areas also appeared to exhibit a low level of fluorescence. No apparent difference in fluorescence between extracellular and intracellular areas was observed among the dead culture controls.

All tested cell populations appeared to exhibit some degree of red fluorescence. Both live and dead controls as well as the experimental (lone, overflow, and direct co-incubated) cultures from

exhibited cardiomyocytes that appeared to express low degrees of red light when observed under fluorescent microscopy. The hMSCs observed only appeared to express a similar fluorescence in the dead controls treated with ethanol prior to the implementation of the LIVE/DEAD assay.

A positive red nuclear staining was observed among the "dead culture" controls and to a lesser degree among the lone cardiomyocyte cultures that were examined at incubation day 7.

Viability as measured by the red staining did not appear to correlate with the estimated viability of cells based on their morphological characteristics. Cardiomyocytes that appeared to have healthy, elongated, striated morphologies appeared to exhibit lower levels of red fluorescence in their nuclei. No significant correlation was also observed between the expression of the green live stain and the red dead stain, as the majority of the cardiomyocytes observed (outside of the "dead control" cultures) appeared to express both fluorescent dyes.

Lone CMC Culture, 400X

Optical   635–700 nm Filter   520-570 nm Filter
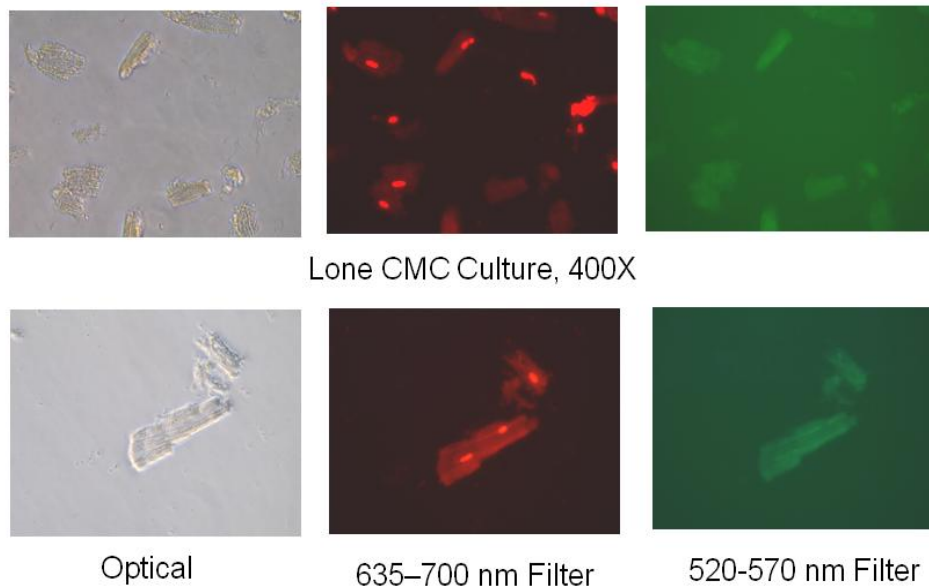
Figure 35: Two fields of view portraying lone cardiomyocyte culture results for Invitrogen LIVE/DEAD mammalian cell assay. Right to left: optical view under total 400 times magnification, identical view showing dead Eth-D1 stain under 635-700 nm filter, identical view showing dead Eth-D1 stain under 635-700 nm filter, identical view showing live calcein, AM stain under 520-570 nm filter.

CMCs from Prototype 15, 400X

Optical   635–700 nm Filter   520-570 nm Filter
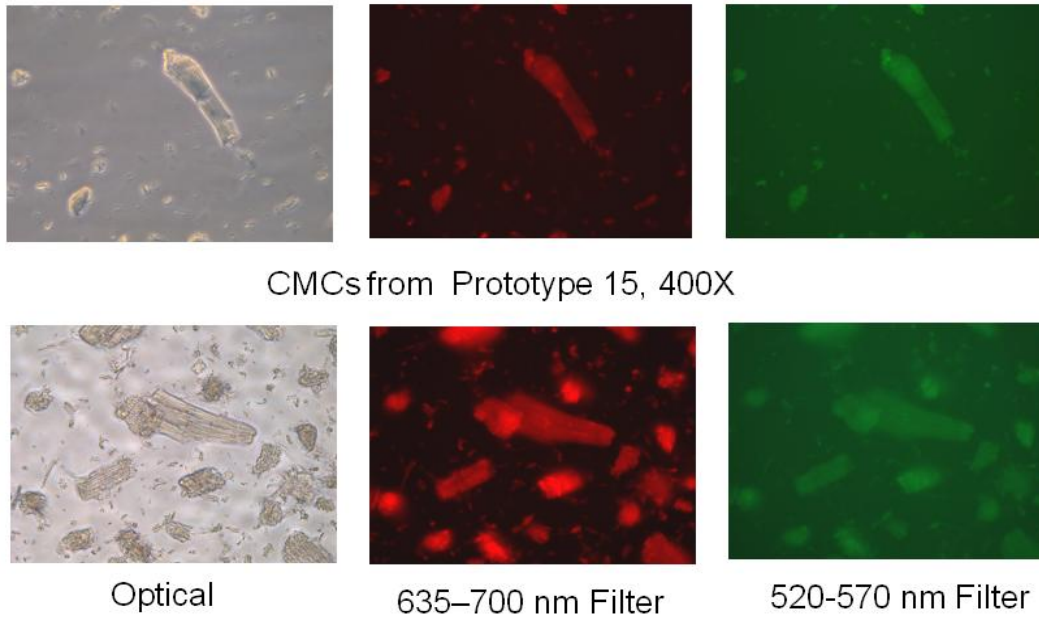
Figure 36: Two fields of view portraying overflow cardiomyocyte culture results for Invitrogen LIVE/DEAD mammalian cell assay. Right to left: optical view under total 400 times magnification, identical view showing dead Eth-D1 stain under 635-700 nm filter, identical view showing dead Eth-D1 stain under 635-700 nm filter, identical view showing live calcein, AM stain under 520-570 nm filter.

Western Blotting

The results from the Bradford assay are displayed below in Table 11. Based on the protein concentrations in these samples, the lysate isolated from the tissue sample and from the direct co-culture is adequate for western blotting analysis. However, the volume of lysate required to be loaded into a gel for the lone cardiomyocytes culture and the prototypes would exceed the maximum capacity of the well.

Table 11: Results of the Bradford Protein Assay

Sample	Protein Concentration (μg/mL)
Tissue Sample	2056
Lone Cardiomyocytes	584
Direct Co-culture	952
Prototypes 3-6	325
Prototypes 11-14	384

Though the lysate samples from the tissue sample and the direct co-culture were adequate, no valuable information regarding the design would be obtained. Therefore, due to time constraints, the results of the western blots are inconclusive, as the assay could not be completed with the protein concentration in the lysate samples obtained.

Immunohistochemistry

The immunohistochemistry (IHC) protocol used during our testing was carried out on the cultures of hMSCs and cardiomyocytes. Monoclonal anti α-actinin was used as the primary antibody because it binds to the protein that is characteristic of cardiac muscle cells. The secondary antibody was a *rabbit anti-mouse IgG* antibody tagged with *Alexa Flour 488* which is derived from a rabbit that reacts to mouse protein. The blocking agent is intended to prevent the secondary antibody from nonspecific binding to the sample cells or tissue, it's important that the blocking serum used during IHC is a serum from the same host species used to derive the secondary antibody. A Hoescht counterstain was also used because it actively transports ATP binding proteins out of cells. This counterstain also is valuable in distinguishing different populations of cells from one another in mixed samples. Figure 3737 shows the Hoescht counterstain of one of our co-cultures. Figure 38Figure 38 shows the actinin stain for one of our co-cultures. The IHC testing was inconclusive due to the ineffective binding of the antibodies.
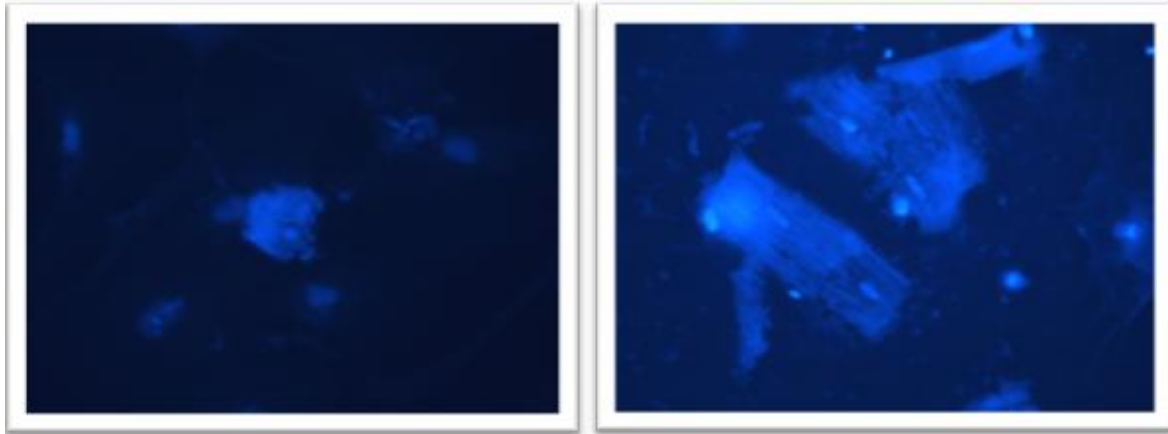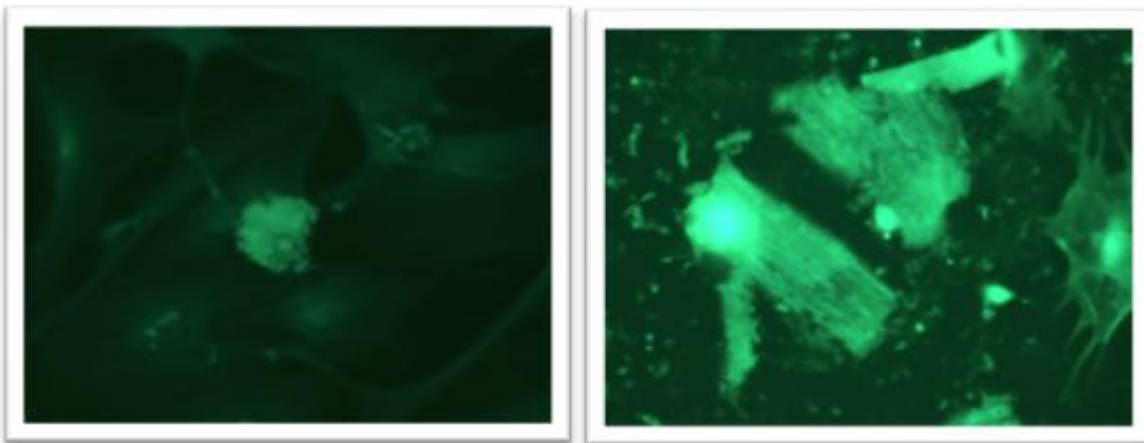

Figure 37: Hoescht Counterstain

Figure 38: Monoclonal α-actinin stain

Discussion

Methods for Adhesion of Device to Culture Dish

Petroleum jelly proved to be a cheaper alternative to silicone grease while also having the advantages of being more maneuverable and applicable to multiple adhesion application methods. The final dip method implementing a separate device stand and stamping part appeared to expedite the adhesion process while allowing for the application of a uniform layer of adhesion material to the device. This method is also favorable compared to the transfer pipette application method in terms of sustainability, as the later method requires the use of disposable products (glass pipettes were tested but failed in application functionality). Although the transfer pipette pasting method requires a certain degree of dexterity from its operator and is somewhat tedious, it may be implemented as an additional measure to secure a second divider layer along the barriers of the designed device.

Cross-well Leakage Test Results

Initial cross-well mixing was observed during the colored dye cross-well leakage tests, however this appeared to result from poor initial dimensions. The final device dimensions and adhesion procedure appeared to be adequate as no cross-well leakage was observed under these final testing conditions.

Cell Growth in Variable Media

Traditional cardiomyocyte media appeared to be the best cellular media candidate for co-culture and overflow culture as cardiomyocytes shows sensitivity to increased levels of FBS, while conditioned media introduces a risk of hMSC relocation into cardiomyocyte populations.

Optical Counts

Optical counts indicated that morphological characteristics observed among the wells indicated a healthier population of cardiomyocytes within overflow conditions compared to co-culture conditions. These cells also appeared to be healthier than direct co-cultured cells based on the number of cells involved in healthy island formation. Apparent inconsistencies and variation in counting, as well as the tedious process involved in obtaining optical counts would suggest the investigation of additional measures of viability to be paired with these counts to validate count findings and to provide a more efficient measure of continued viability assessment.

Cell Re-location and Migration

While the device ring barrier was successful in preventing the migration of hMSCs into cardiomyocyte wells over a 7-day incubation period, hMSC migration across the wells was observed after 7-days of incubation during the 14-day observation period. A 7-day prevention of migration may be sufficient for certain applications. To prevent migration and relocation during longer periods, the application of additional layers of petroleum jelly or adhesion material may be explored, at the time before the loading of the cell populations, and at regular subsequent intervals during the culture period. The inclusion of a porous barrier may also be a means of preventing further relocation of cells between the two cell-type populations.

Passaging of Cells from Culture Device

As passaging cells to and from the developed culture device was successful, the device should prove useful in the end harvesting of cells grown on the device.

Immunohistochemistry

Although the cardiomyocytes did not show staining of the striations within the cells, the immunohistochemistry staining that was conducted was useful in staining for cell nuclei and to validate that optical cell counts of cardiomyocytes correlated with the nuclear staining counts within lone cardiomyocyte populations. IHC observations also confirmed hypotheses that hMSC growth appeared to take place at levels vertically below cardiomyocyte location within the direct co-cultures.

LIVE/DEAD Assay

Due to auto-fluorescence and the tendency for all cardiomyocyte populations to absorb the red Eth-D1 dye, it is recommended that alternative LIVE/DEAD assay stains be used to further assess the viability of the lone, co-incubated, and overflow cardiomyocyte cultures. Characteristics specific to cell type and or the cardiomyocyte isolation processes may influence the cell membranes of the cardiomyocytes to allow for the entrance of the Eth-D1 dye into both live and dead cells. A similar uptake of trypan blue into both dead and apparently healthy cardiomyocytes was observed when conducting optical microscopy counts with a hemocytometer to determine cell plating density.

Because of what appears to be the positive uptake of the Eth-D1 dye regardless of cell viability, the staining of nuclei with this dye appears to yield more accurate viability measures. Increased nuclear staining within the lone cardiomyocyte populations as compared to the overflow cultures may indicate greater viability levels and health among the overflow cultures. No significant staining differences resulted between the cardiomyocytes in the overflow cultures and the cardiomyocytes in the direct co-cultures. Healthier morphological characteristics however were observed among the former, as the cardiomyocytes within the direct co-incubated cultures appeared to be hindered by hMSC growth.

Future Work

In accordance with the initial goals of the project to create a method that was easily reproducible and cost-effective, there are several steps that must be taken to produce the radial-well plate on a large scale. First, it would be necessary for a counter mold to be created that would allow the producer to pour in melted plastic and, once hardened, remove the mold. This mold is shown below in Figure 39.
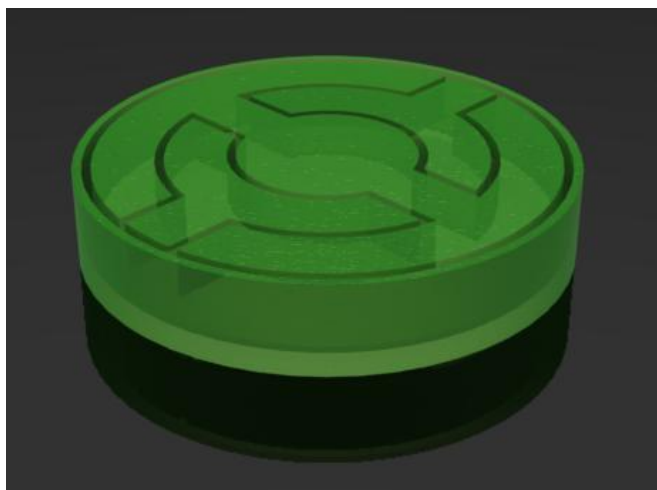
Figure 39: Countermold

The plastic used must be autoclavable to allow the users a cheap method of sterilizing the plates prior to use. We compared the maximum temperature and cost (at time of publication) of different plastics to determine which would be best for mass production of the plate. We found that polypropylene had a maximum temperature of $135^{\circ}C$[71] and costs 60-62 cents per pound[72].

To improve ease of use for the client, we developed a cover to be used during feeding and plating that would eliminate the possibility of contaminating the cardiomyocyte chamber. This is shown below in figure 40.

The implementation of an electrospun porous barrier is also recommended. A pore size of approximately 1,0 to 3.0 microns is recommended given the apparent sizes of the average cardiomyocyte (17.0 +\-4.0 diameter) and hMSC (23 +/- 1.1 microns).
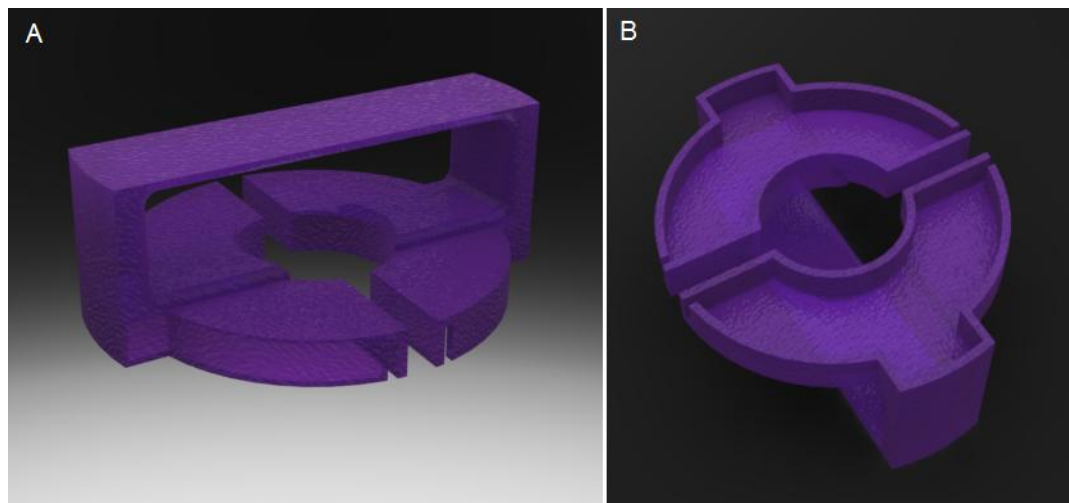
Figure 40: CAD Model of Radial Well Feeding Cover (with picketed surface suppressed around well barriers); A: Top View of Handle, B: Bottom View of Chamber Covers

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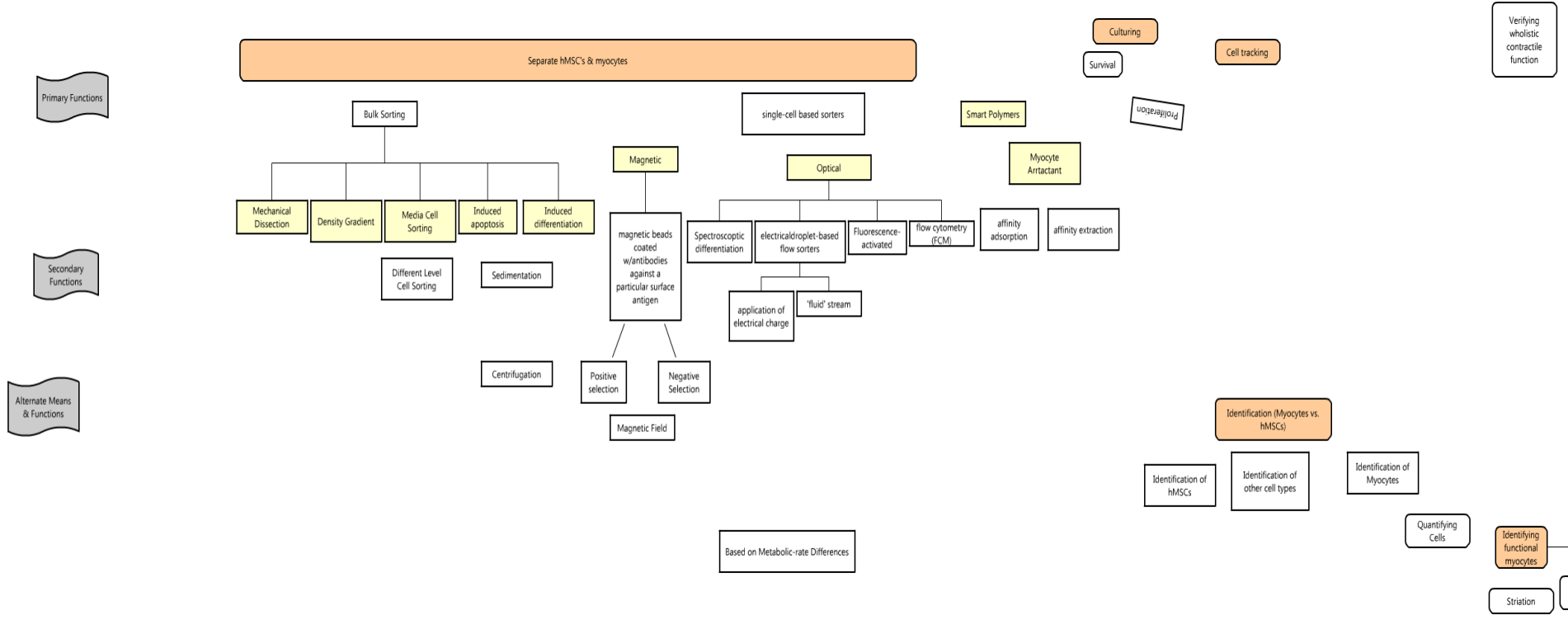
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Appendices

Appendix 1: Gantt Chart

Task #	Task Name	Start Date	Finish Date
1	Background	27-Aug-2009	31-Mar-2010
2	Final A Term Background	27-Aug-2009	16-Oct-2009
3	Final B Term Background	27-Oct-2009	17-Dec-2009
4	Final C Term Background	14-Jan-2010	05-Mar-2010
5	Final Edits	15-Mar-2010	31-Mar-2010
6	Design Alternatives	23-Sep-2009	06-Nov-2009
7	Categorize into Groups	05-Oct-2009	09-Oct-2009
8	Prioritize by Objectives	05-Oct-2009	12-Oct-2009
9	Prioritize by Constraints	05-Oct-2009	17-Dec-2009
10	Select DA to become prototypes	12-Oct-2009	27-Oct-2009
11	Prototypes	12-Oct-2009	04-Dec-2009
12	Descriptions	12-Oct-2009	28-Oct-2009
13	Diagrams	12-Oct-2009	04-Dec-2009
14	Sketches	12-Oct-2009	30-Oct-2009
15	Final (of top 3)	30-Oct-2009	04-Dec-2009
16	Specifications	14-Oct-2009	04-Dec-2009
17	Advantages/Disadvantages	27-Aug-2009	17-Dec-2009
18	Theoretical Comparison	26-Aug-2009	13-Nov-2009
19	Testing	27-Oct-2009	13-Nov-2009
20	Trial 1	27-Oct-2009	20-Jan-2010
21	Trial 2	27-Oct-2009	21-Feb-2010
22	Trial 3	27-Oct-2009	31-Mar-2010
23	Actual Comparison	13-Nov-2009	04-Dec-2009
24	Revision of Prototypes	16-Nov-2009	17-Dec-2009
25	Testing Revised Prototype	01-Feb-2010	31-Mar-2010
26	Review of Revised Prototype	15-Feb-2010	31-Mar-2010
27	Methodology	16-Oct-2009	17-Dec-2009
28	Draft 1 (Prior to lab work)	16-Oct-2009	26-Feb-2010
29	Draft 2 (Prototype Development and Testing)	27-Oct-2009	26-Feb-2010
30	Draft 3	01-Apr-2010	15-Apr-2010
31	Results	15-Apr-2010	08-Apr-2010
32	Final Presentation	22-Apr-2010	22-Apr-2010

Appendix 2: Functions-Means Tree

Primary Functions

Secondary Functions

Alternate Means & Functions

Separate hMSC's & myocytes

Bulk Sorting

single-cell based sorters

Culturing

Survival

Cell tracking

Smart Polymers

Proliferation

Magnetic

Optical

Myocyte Arrtactant

Mechanical Dissection

Density Gradient

Media Cell Sorting

Induced apoptosis

Induced differentiation

Different Level Cell Sorting

Sedimentation

magnetic beads coated w/antibodies against a particular surface antigen

Spectroscoptic differentiation

electricaldroplet-based flow sorters

Fluorescence-activated

flow cytometry (FCM)

affinity adsorption

affinity extraction

application of electrical charge

'fluid' stream

Centrifugation

Positive selection

Negative Selection

Magnetic Field

Identification (Myocytes vs. hMSCs)

Identification of hMSCs

Identification of other cell types

Identification of Myocytes

Quantifying Cells

Identifying functional myocytes

Based on Metabolic-rate Differences

Striation

Verifying wholistic contractile function

Appendix 3: hMSC Culture

Making Media for hMSCs

The media for hMSCs is 89% DMEM + 10% FBS + 1% Pen-Strep.

Materials:
- Media (Cellgro, DMEM 1X, Cat. # 10-013-CV)
- Penicillin-Streptomycin Antibiotic Cocktail (Cellgro, Pennicillin-Streptomycin Solution, Cat. # 30-001-CI)
- Fetal Bovine Serum (PAA Laboratories, FBS, Cat. # A15-201)
- 500 mL Filter Unit (Nalgene)
- Pipettor
- Aspirator

Procedure (to make 500 mL hMSC media):
1. Add 445 mL DMEM, 50 mL FBS, and 5 mL Pen-Strep to top of filter unit.
2. Connect aspirator to the filter unit and turn on, allowing media to flow into the bottom portion of the filter unit.
3. When all of media is in the bottom, turn off and detach the aspirator.
4. Detach the top portion and dispose in biohazard.
5. Label media and store at 4° C.

Feeding hMSCs

Materials:
- hMSC media
- Sterile phosphate buffered saline (PBS, Cellgro, Cat. # 21-030-CM)
- T-75 flask
- Serological pipettes
- Pipettor
- Sterile glass Pasteur pipettes and aspirator
- Quantum dots (QD655 ITK carboxyl quantum dots, Invitrogen, Cat # Q21321MP)

Procedure:
1. Place media and PBS in water bath to warm.
2. Wearing gloves, place all materials in hood that will be needed for the procedure.
3. Remove cells from incubator and place in biosafety cabinet
4. Aspirate old media using Pasteur pipette
5. Use pipettor to add 5 mL sterile PBS to flask. Gently tilt the flask to expose the entire bottom to PBS. Aspirate PBS.
6. Use pipettor to add 10 mL fresh media to the flask. Check the flask with the microscope to be sure cells appear healthy.
7. Return flask to incubator. Repeat every other day.
8. Clean the biosafety cabinet with 70% ethanol.

9. In order to load quantum dots into hMSCs, it is necessary to feed the cells with medium containing quantum dots. For this, 10 µL of Quantum Dot 655 were added to 10 mL medium, and the medium was fed to the cells following the steps above. Medium was removed after 24 hours and replaced with fresh media.

Passaging hMSCs

Materials:
- 5ml, 0.25% Trypsin aliquot (Cellgro, Trypsin 1X, Cat. # 25-050-CI)
- Media
- 15 ml conical tube
- 2 T-75 flasks
- Serological pipettes
- Pipettor
- Hemocytometer
- Micropipettor and tips
- Trypan blue

Procedure:
1. Place trypsin, media, and PBS in water bath.
2. Remove the desired T-75 flask (with cells) from the incubator and place in cell culture hood.
3. Place trypsin, media, and PBS in cell culture hood.
4. Aspirate off the media using a sterile glass Pasteur pipette.
5. Using a 10 mL pipette, add 5 mL of PBS to the flask to wash the cells.
6. Aspirate the PBS from the flask.
7. Using a pipette, remove 5 mL of trypsin from aliquot and add to the T-75 flask.
8. Place the T-75 back in the incubator for 5-10 minutes. Check the flask under microscope to make sure that the majority of cells have lifted.
9. Add 5 mL of media to the flask.
10. Using a pipette, remove the entire cell suspension and place in a new 15ml conical tube.
11. Centrifuge the cells (be sure to use a counter balance). Centrifuge at 800 rpm for 5 minutes.
12. Aspirate the supernatant (media and trypsin) using a sterile glass Pasteur pipette. Be sure not to disturb the cell pellet.
13. Re-suspend the cell pellet with 2ml of media.
14. Remove 10 µL of the cell suspension, combine in an eppendorf tube with 10µl of trypan blue stain (5 µL trypan blue + 5 µL PBS).
15. Mix the cells / trypan blue well using the micropipette.
16. Remove 10 µL of the cells / trypan blue and place in the hemocytometer.
17. Count the cells using the hemocytometer.
      Cells/ml = (#cells/#squares) x 2 (dilution) x 104
18. Plate the cells with the desired concentration in a new T-75 flask.
19. Add fresh media to the T-75 to achieve the desired final amount of media (10 mL).
20. Write necessary information on the new T-75 (cell type, passage number, date, initials, cell plating concentration), place in incubator.
21. Check for remaining cells. If over 50% confluence, continue to culture flask.
22. Place media and PBS back in refrigerator, clean the cell culture hood with 70% ethanol, turn on UV assuming no other experiments are in hood.

Appendix 4: Cardiomyocyte Culture

Making Media for cardiomyocytes

The media for cardiomyocytes is 94% DMEM + 5% FBS + 1% Pen-Strep.

Materials:
- Media (Cellgro, DMEM 1X, Cat. # 10-013-CV)
- Penicillin-Streptomycin Antibiotic Cocktail (Cellgro, Pennicillin-Streptomycin Solution, Cat. # 30-001-CI)
- Fetal Bovine Serum (PAA Laboratories, FBS, Cat. # A15-201)
- 500 mL Filter Unit (Nalgene)
- Pipettor
- Aspirator

Procedure (to make 500 mL hMSC media):
1. Add 470 mL DMEM, 25 mL FBS, and 5 mL Pen-Strep to top of filter unit.
2. Connect aspirator to the filter unit and turn on, allowing media to flow into the bottom portion of the filter unit.
3. When all of media is in the bottom, turn off and detach the aspirator.
4. Detach the top portion and dispose in biohazard.
5. Label media and store at 4° C.

Feeding cardiomyocytes

Materials:
- hMSC media
- Sterile phosphate buffered saline (PBS, Cellgro, Cat. # 21-030-CM)
- Serological pipettes
- Pipettor
- Sterile glass Pasteur pipettes and aspirator

Procedure:
1. Place media in water bath to warm.
2. Wearing gloves, place all materials in hood that will be needed for the procedure.
3. Remove cells from incubator and place in biosafety cabinet
4. Aspirate old media using Pasteur pipette
5. Use pipettor to add 2.5 mL fresh media to the well (for 6 well plates). Check the plate with the microscope to be sure cells appear healthy.
6. Return plate to incubator. Repeat every other day.
7. Clean the biosafety cabinet with 70% ethanol.

## Passaging Cardiomyocytes

Materials:

- 5ml 0.25% Trypsin aliquot (Cellgro, Trypsin 1X, Cat. # 25-050-CI)
- 7.5 mL filtered DI water
- Cardiomyocyte Media
- 15 ml conical tube
- 2 T-75 flasks
- Serological pipettes
- Pipettor
- Hemocytometer
- Micropipettor and tips
- Trypan blue

Procedure:

1. Place trypsin, media, and PBS in water bath.
2. Dilute 0.25% trypsin using filtered DI water to obtain 0.01% trypsin
3. Remove the desired T-75 flask (with cells) from the incubator and place in cell culture hood.
4. Place trypsin, media, and PBS in cell culture hood.
5. Aspirate off the media using a sterile glass Pasteur pipette.
6. Using a 10 mL pipette, add 5 mL of PBS to the flask to wash the cells.
7. Aspirate the PBS from the flask.
8. Using a pipette, remove 5 mL of trypsin from aliquot and add to the T-75 flask.
9. Place the T-75 back in the incubator for 5-10 minutes. Check the flask under microscope to make sure that the majority of cells have lifted.
10. Add 5 mL of media to the flask.
11. Using a pipette, remove the entire cell suspension and place in a new 15ml conical tube.
12. Centrifuge the cells (be sure to use a counter balance). Centrifuge at 800 rpm for 5 minutes.
13. Aspirate the supernatant (media and trypsin) using a sterile glass Pasteur pipette. Be sure not to disturb the cell pellet.
14. Re-suspend the cell pellet with a known amount of media (1 mL).
15. Remove 10 μL of the cell suspension, combine in an eppendorf tube with 10μl of trypan blue stain (5 μL trypan blue + 5 μL PBS).
16. Mix the cells / trypan blue well using the micropipette.
17. Remove 10 μL of the cells / trypan blue and place in the hemocytometer.
18. Count the cells using the hemocytometer.
19. Cells/ml = (#cells/#squares) x 2 (dilution) x 10^4 (Hematocytometer Volume)
20. Plate the cells with the desired concentration in a new T-75 flask.
21. Add fresh media to the T-75 to achieve the desired final amount of media (10 mL).
22. Write necessary information on the new T-75 (cell type, passage number, date, initials, cell plating concentration), place in incubator.
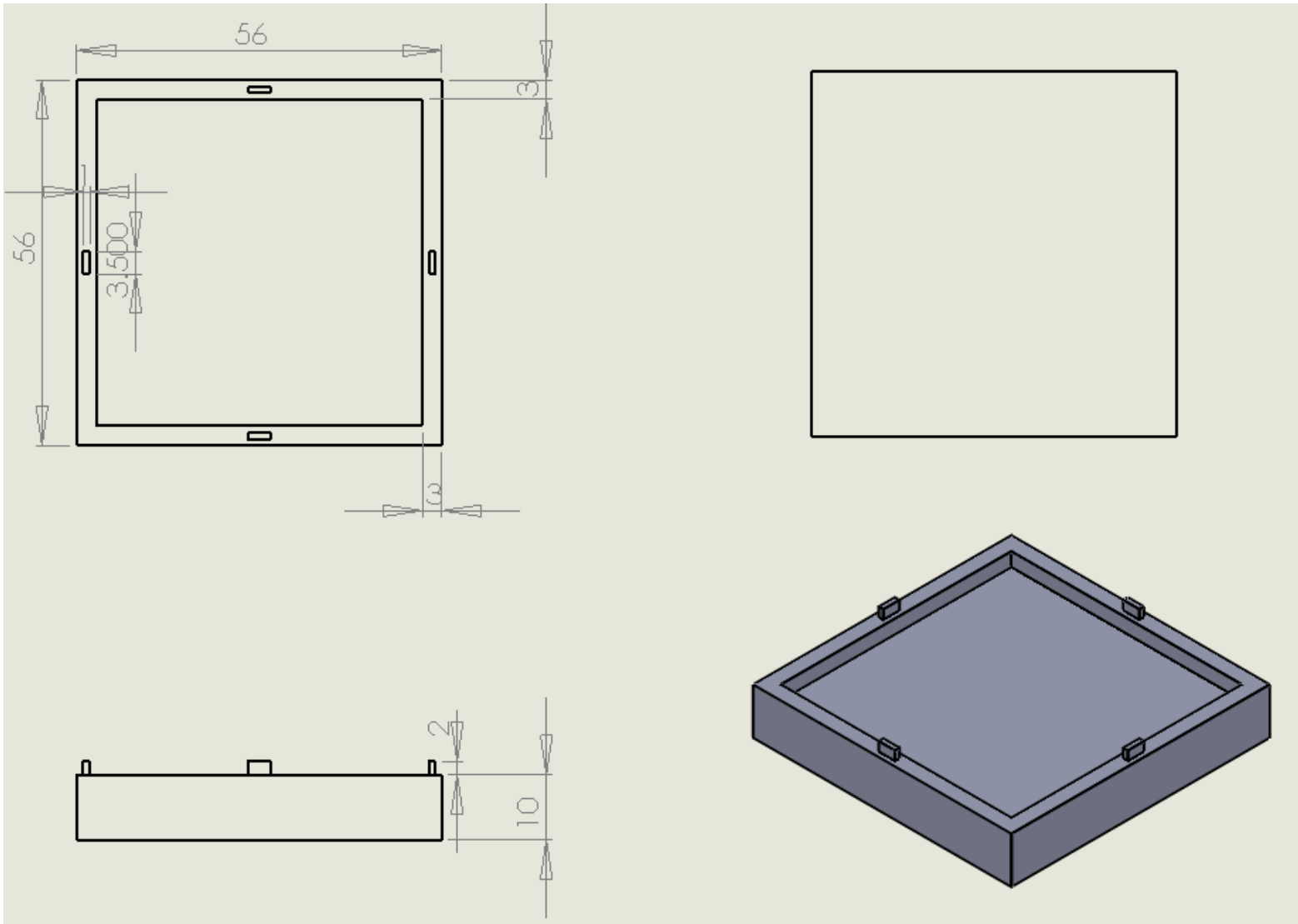23. Dispose of the old T-75 flask in the biohazard bag.

24. Place media and PBS back in refrigerator, clean the cell culture hood with 70% ethanol, turn on UV assuming no other experiments are in hood.
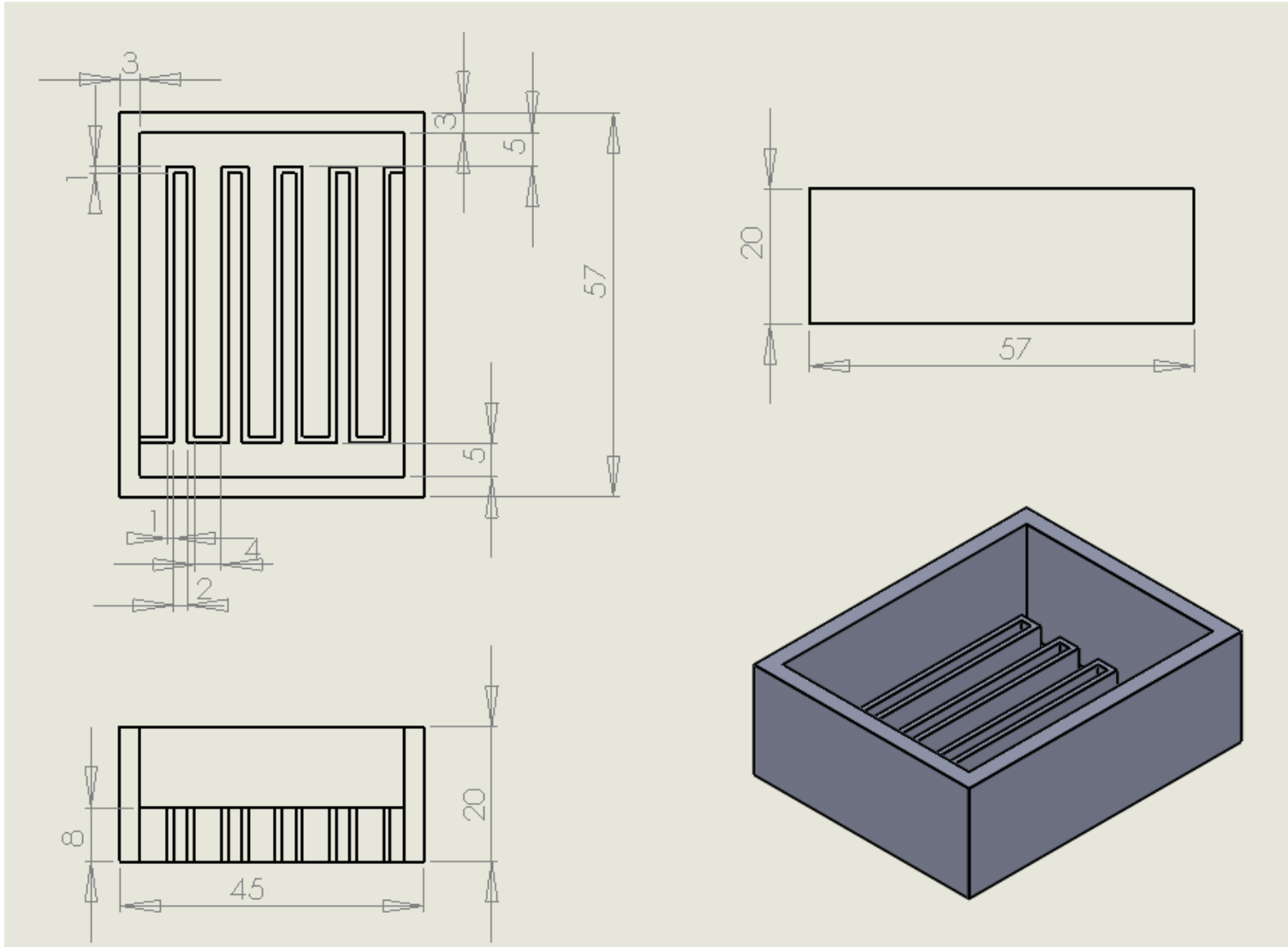
Appendix 5: Velcro Model
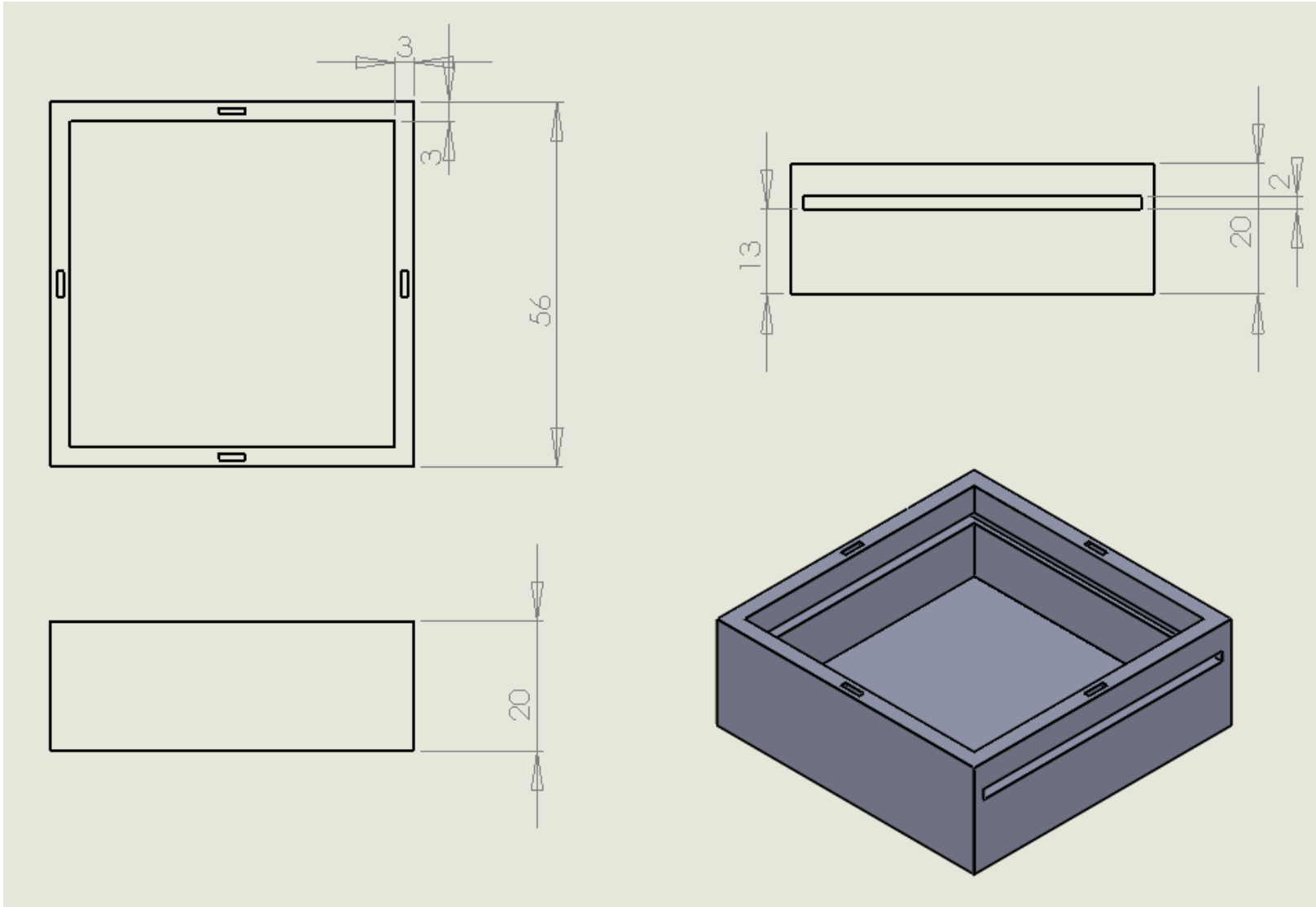
*All dimensions are in millimeters
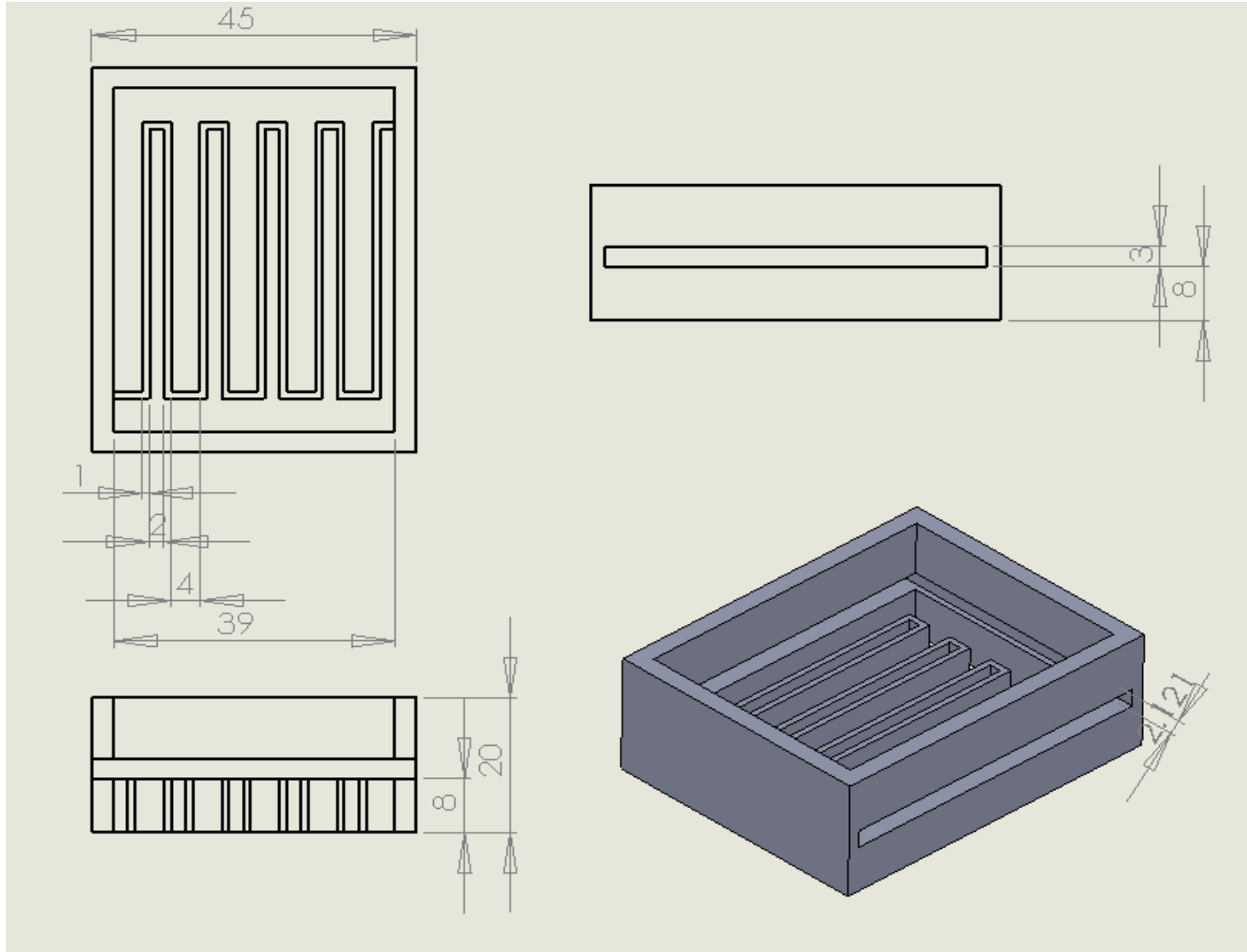
Appendix 6: Velcro Model, Top
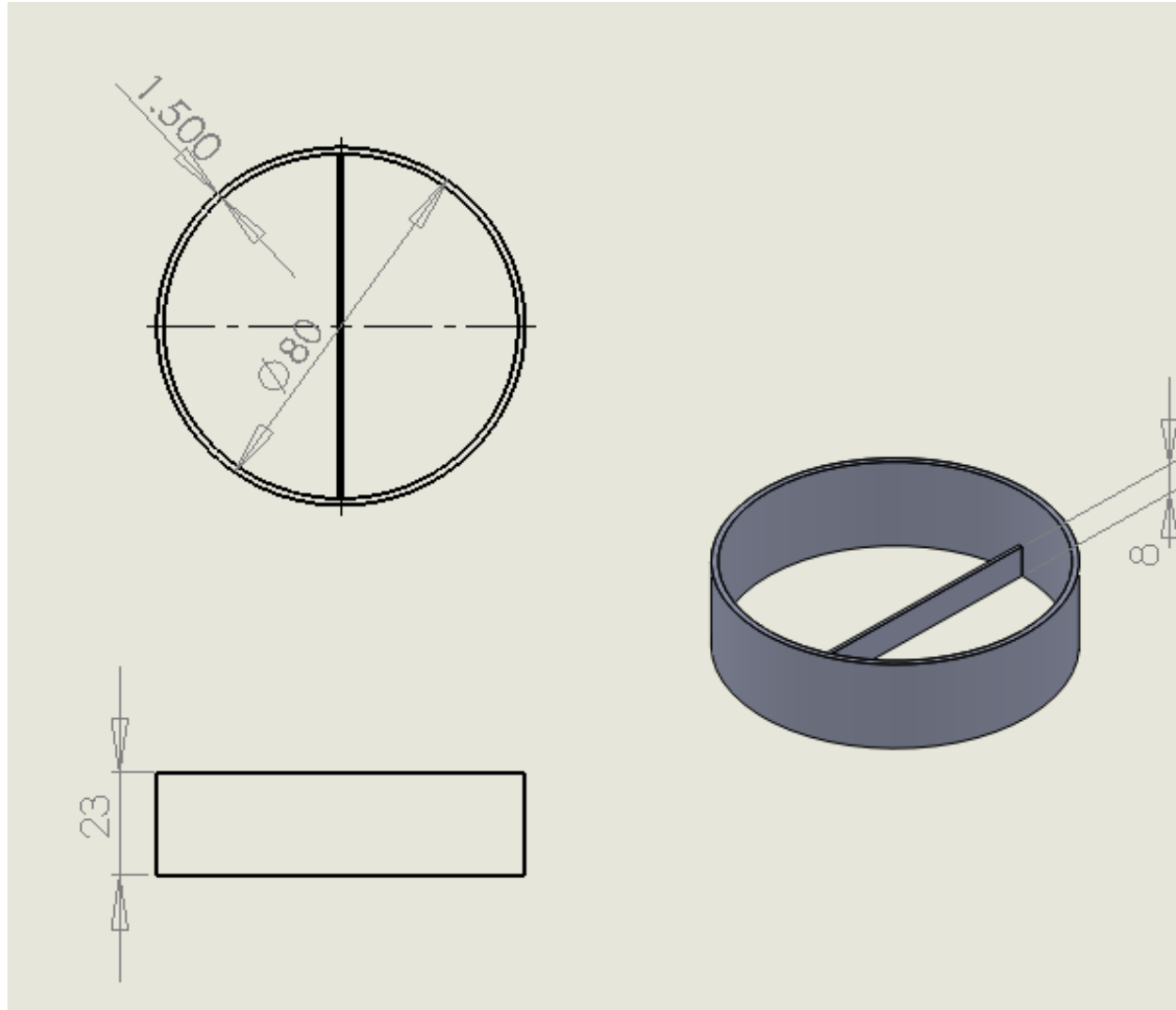

*All dimensions are in millimeters

*All dimensions are in millimeters

Appendix 8: Interconnected Rows with slider

*All dimensions are in millimeters

Appendix 9: Circular Media Exchange

*All dimensions are in millimeters

Appendix 10: Project Materials and Budget

Account Number: 17020-BE

Total Funds for project: $624

The following is a list of project materials that will be used and the estimated cost of each:

- Quantum Dots

- Cardiomyocyte Isolation:

    - Gentamicin:1, 10 mL package: $51.80

    - Liberase Blendzyme:1, 90 mg package: $220.00

    - Adenosine 5'-triphosphate disodium salt solution (ATP): 1 package: $73.00

- Prototype culture dishes: $45.64

- Gelatin for cell attachment

- Materials for cell culture & overflow media

    - Penicillin: streptomycin
    - Fetal Bovine Serum (FBS) stock solution
    - Dulbecco's Modified Eagle Medium (DMEM)

- Materials for plating cells:

    - Poly-Styrene: ($28 to $45)/(0.5-1.0kg) MW: 190,000-260,000 g/mol

    - 90mm with a cell growth area of 55cm2
    - Dow Corning 976V silicone high vacuum grease - $16-23 per 150 g
    - Vaseline petroleum jelly (low-cost alternative to silicone grease) - $4.50 per 150g