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Increasing the Presence of Cell Cycle Markers in Adult Cardiac Myocytes for the Treatment of Myocardial Infarction

A Major Qualifying Project Report

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by

_____
Tantra Budiman

_____
Stacie Gutowski

_____
Cara Messier

Date: April 24, 2008

Approved by:

_____
Professor Glenn R. Gaudette, Advisor

1. cardiac myocyte
2. cell cycle marker
3. co-culture

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Professor George D. Pins, Co-Advisor

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Abstract

Myocardial infarction (MI), more commonly known as a heart attack, affects millions of people every year. Infarcted heart tissue quickly undergoes necrosis, leaving the patient with portions of dead heart tissue that cannot function to efficiently pump blood throughout the body. Treatments involving resectioning of the heart muscle only restore form and not function. Other treatments and medications only treat the symptoms of MI and do not address the problem of dead tissue. Cell-based therapies are being pursued in an attempt to grow cardiac myocytes to replace the dead tissue within the heart. The purpose of this project was to develop a system to co-culture mouse cardiac myocytes with human mesenchymal stem cells with the goal of promoting the cardiac myocytes to express cell cycle markers, an indication of proliferative potential in cells.

The team began by developing an agarose barrier that would maintain two separate populations of cells that could remain pure, while still being cultured in the same dish. Results showed that the agarose barrier was effective in preventing cell migration while still allowing media exchange. The team also developed a staining protocol for cyclin D1, a marker expressed during the cell cycle. Additionally, the team was able to stain control tissue for α-actinin, a marker found in cardiac cells. These finding suggest that this agarose barrier co-culture system can be used to co-culture cardiac myocytes with human mesenchymal stem cells in order to promote the myocytes to reenter the cell cycle, and potentially undergo mitosis. These cells could one day be used to regenerate functional myocardium in MI patients.

Executive Summary

Myocardial infarction is a significant problem that affects almost 8 million people in the United States today. During myocardial infarction, also known as a heart attack, the myocytes that compose the heart are deprived of oxygen. This may lead to necrosis (cell death) and scar formation in the infarcted region, and decreased functionality of the heart.

There are several methods that have been used to treat patients after myocardial infarction. Some treatments involve the removal or repositioning of the infracted tissue with ventricular resection or ventricular overlap surgeries. Other treatments involve replacing the damaged tissue with a patch of synthetic material designed to restore structure to the infarcted area. However, one common problem exists with these procedures: although the form of the tissue may be restored, functionality of the infarcted area is not recovered. This highlights the need for a treatment that can restore form and function to the damaged area.

One alternate strategy for treating myocardial infarction is to replace the dead tissue with viable cells that will proliferate and restore complete functionality to the muscle. Some cell therapies involve directly injecting stem cells into the infarcted region with the intention that the stem cells will proliferate and differentiate into myocytes, eventually replacing the infarcted tissue. Another idea in cell therapy is to use stem cells to promote cardiac myocytes to proliferate.

The proliferation of cardiac myocytes has been thought impossible until recently as new research has shown evidence of adult cardiomyocyte proliferation. By using stem cells to promote proliferation of existing cardiomyocytes, a sample of the patient's

own cells could be used to produce a therapy that would both restore functionality to the damaged tissue within the heart, while also eliminating the possibility of rejection from donor cells. Additionally, the cardiomyocytes would be able to replace the damaged tissue without the need to introduce foreign stem cells into the patient, as the stem cells may not always follow a desired path of differentiation to become a cardiac myocyte.

The goal of this project was to develop a design that separates cardiac myocytes from stem cells, while still allowing the two cell types to remain in a co-culture environment. To begin the project, the team completed background research in order to gain a greater understanding of the problem as well as research to date that has been completed by other research groups. Background research consisted of several main areas, namely the effects of myocardial infarction, the structure and function of cardiac myocytes, current approaches for cardiomyocyte regeneration research, and general cell culture techniques. This background aided the team in gaining a greater understanding of the current methods for culturing cells and conducting experiments related to cardiac myocyte regeneration.

Following the background, the team began to approach the project utilizing the process learned in the junior-level biomedical engineering design class. This included interviewing clients, developing an objectives tree, analyzing client-determined pairwise comparison charts, and developing a weighted objectives tree. The team successfully completed design matrices to determine the best methods for animal model choice, cell isolation procedures, media choice, and cell culture system design. The team also completed conceptual designs and moved on to the development of a preliminary design.

The team pursued several designs during the project. After several unsuccessful attempts, the team finally developed a method to produce an agarose-based cell separation system. This system involves the use of a small agarose line placed on a cover slip in a Petri dish. The agarose, which is not harmful to cells, allows media to pass over the barrier in order to be exchanged between the two cell types, while preventing cells from migrating from one side of the dish to the other. Primary isolations were performed to acquire mouse cardiac myocytes from adult CF-1 mice, and human mesenchymal stem cells were obtained from the Gaudette lab. The two cell types were cultured on opposite sides of the agarose barrier and allowed to grow in co-culture for one week.

Results from the control studies showed positive results. The control migration study indicated that the mesenchymal stem cells were unable to migrate past the agarose barrier after 10 days in culture. Additionally, the team successfully isolated cardiac myocytes from adult mouse hearts. The team developed an analysis procedure to assess the effectiveness of the system as well. By developing a staining protocol for cyclin D1, a marker found in cells during the cell cycle, the team identified cells that have re-entered the cell cycle. Additionally, the team was able to modify an existing protocol for α-actinin stain. This staining protocol allows for the identification of cells that express characteristic striations found in cardiac tissue.

The team was able to develop a barrier system that is effective for separating cell populations while still allowing media exchange. Additionally, the staining protocols for cyclin D1 and α-actinin demonstrate the ability to analyze the system's effectiveness in the future. However, the team was unsuccessful in gathering data that confirmed

myocyte cell cycle reentry using the co-culture system for several possible reasons including enzyme batch changes and high passage number stem cells.

In the future, there are several modifications that can be made to this project in order to improve the system. By reducing the size of the system to a 12-well or 24-well plate, more cells can be cultured at one time, possibly producing better results. Media and serum formulations should also be re-evaluated in order to find the most effective combination for this system. Finally, using human heart cells in this system would be the ultimate goal, as human cells would be necessary for treatment of human MI. The team believes that ultimately this system can be used for two purposes. First, the system can be used as a research tool to identify the specific paracrine factors secreted from the stem cells that promote cardiac myocytes to re-enter the cell cycle. Second, this system can be used to develop a population of cardiac myocytes that can undergo mitosis. Once a sufficient number of myocytes has regenerated, they can be delivered to the heart to regenerate the damaged myocardium. Overall, the team believes that this system has great potential for future treatment of damaged myocardium caused by myocardial infarction.

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1. Introduction

Myocardial infarction (MI) affects 7.9 million people in the United States today (Rosamond, 2007). During an MI, more commonly known as a heart attack, a section of the tissue within the heart is deprived of oxygen for an extended period of time due to a blockage in one or more coronary blood vessels. This leads to necrosis, or death of the surrounding tissue, which is made of myocytes. As the myocytes die the functionality of the heart decreases, leaving the patient with a damaged heart that can no longer function as well as before the MI. This causes the heart to do more work in order to keep the patient alive which increases the stress on the heart muscle itself. There are other functional problems such as decreased stroke volume which leads to a decrease in the oxygenated blood being delivered to the rest of the body. The damaged tissue may also lose mechanical integrity, leading to a ventricular aneurism. These factors are often detrimental to patient's health (Rosamond, 2007).

There are several different therapies that are used to treat patients with infarcted heart tissue. These therapies can include several variations of geometrical remodeling via surgery. The endoventricular patch plasty (Dor procedure), septal anterior ventricular exclusion (SAVE), and overlapping procedures are all performed to change the form of the heart in the attempt to strengthen the damaged muscle in order to avoid future problems with aneurism (Ueno et al., 2007). Many surgeons will also perform some sort of revascularization such as a vessel bypass graft in order to restore blood flow to the infarcted area (Bockeria, et al., 2006). Although these procedures try to restore the macroscopic form of the heart, they lack the ability to restore function to the infarcted tissue. This leaves the patient with the problem of reduced general heart

function and most likely a reduced ability to perform normal daily functions as compared to what the patient would have experienced before the myocardial infarction.

There is a specific need for a post-MI treatment that will restore both form and function to the damaged heart tissue. One of the newer treatments being investigated involves the use of cell therapy to repair the damaged heart tissue. One form of cell therapy involves direct injection of stem cells into the necrotic tissue with the intention that the stem cells will proliferate and differentiate into functioning cardiac myocytes (Collins et al., 2007). However, there is no guarantee that the stem cells will all differentiate into cardiac myocytes, and therefore no assurance that complete restoration of the tissue will occur. Research has been conducted to indicate that there is a small population of cardiac stem cells within the human heart, along with a small population of myocytes that can re-enter the cell cycle and proliferate after a traumatic cardiac event (Anversa, 1998). However these myocytes cannot regenerate enough tissue to repair the infracted region, so the affected tissue cannot be replaced and the heart remains injured. The purpose of this project was to investigate the process of co-culturing cardiac myocytes with human mesenchymal stem cells with the goal of increasing cell cycle markers in cardiac myocytes *in vitro*. The increase of cell cycle markers indicates the possibility of stimulating cells to re-enter the cell cycle and to undergo mitosis to proliferate. We anticipated that this *in vitro* culture technique could be used as a protocol for culturing a physiologically significant number of cardiac myocytes to deliver to infracted heart tissue to regenerate the infracted region of the heart.

The team's efforts consisted of a process of engineering design based on the techniques learned in the junior level design class. Matrices were used to analyze various design ideas for their functionality and cost effectiveness. After completing several design analyses, the team pursued several designs in the lab in order to discover the most effective design.

The final design provided a co-culture system suitable for the desired results: compatibility with cell culture (i.e. does not harm cells) while providing an effective co-culture system in which the cells can exchange media while retaining separate populations. Control studies were conducted to prove that the system can effectively retain two separated cell populations for at least one week. The team also developed an analysis procedure that can be used to identify cells that have re-entered the cell cycle. This procedure could also be used to identify cardiac myocytes that have retained striation, a characteristic of cardiac cells. The design was ultimately used to co-culture cardiac myocytes with human mesenchymal stem cells in the attempt to promote cardiomyocytes to produce cell cycle markers, an indicator of the potential for mitosis in living cells.

2. Literature Review

In order to be able to design a system that will allow for the successful increase of cell cycle markers in adult cardiac myocytes, it was necessary to perform a review of relevant literature. The following is a review of the areas of myocardial infarction, cardiac myocytes, current approaches to treating myocardial infarction, and cell culture techniques.

2.1 Myocardial Infarction

Myocardial infarction (MI) is a major health problem in all areas of the world. According to the American Heart Association (AHA), almost 8 million Americans are affected by MI (Rosamond, 2007). The Joint European Society of Cardiology/American College of Cardiology Committee defines MI as a condition involving myocardial necrosis, or the death of heart tissue. Depending on the severity of the MI, up to one third of the cardiac myocytes in the left ventricle can die (MI Redefined, 2000). Without these cardiac myocytes, which are the contractile cells in the heart, the functionality of the heart can drastically decrease. The AHA states that MI will cause death within five years for 33% of men and 43% of women over the age of 40. This decrease in heart function also may lead to heart failure in 5 years for 7% of men and 12% of women between the ages of 40 and 69. For patients age 70 and over, the chances of heart failure within 5 years raises to 22% of men and 25% of women (Rosamond, 2007). It is estimated that 30% of patients who experience heart failure will die within the first year (Engel, 2005).

As these statistics suggest, there is no cure for myocardial necrosis. This is mainly because adult cardiac myocytes in humans do not proliferate (Ahuja, 2007).

Therefore, there is no mechanism in the human heart to regenerate the dead tissue following MI. Considerable research is ongoing in the biomedical field to find a way to promote cardiac myocyte proliferation. The intention of this research is to find a way to either induce the healthy myocytes to proliferate *in vivo* or to promote the cardiac myocytes to proliferate *in vitro* and use those cells to replace the dead cells in the heart.

2.2 Cardiac Myocytes

In order to perform successful experiments with cardiac myocytes, it is important to understand the structure and function of the cells. The following is a review of the structure and function of cardiac myocytes and their life cycle.

2.2.1 Structure and Function

There are roughly 8 billion heart muscle cells, also known as myocytes, in the heart of a healthy adult human (Ford, 2000). Heart muscle cells are short in length as compared to other muscle cells in the body, and they also have projections that branch out from the cell. The diameter of cardiac cells is usually around 5-15μm. The ends of myocytes are connected to each other via intercalated disks. The intercalated disks allow contractile forces and small molecules to flow from one cell through to another. Some cardiac cells have only one nucleus in the center, but some can be bi- or multi-nucleated.

The walls of the heart are formed by cells with connected channels called gap junctions that allow the flow of small molecules and ions between cells. The major function of these passages is to allow electric current carried by the ions in the cells to flow to adjacent cells, thereby propagating the electrical signal to produce a heartbeat.

The action potential of cardiac myocytes increases rapidly from -80mV to 40mV during contraction of the heart muscle. The action potentials then decrease slowly, during which time they cannot generate a full contractile response again until the cells repolarize. Once repolarization has occurred, the cell is ready to contract once again (Ford, 2000).

2.2.2 Life Cycle

The life cycle of a human cardiac myocyte is much different than most other cells in the body. They are similar to other cells in that they proliferate via the cell cycle during fetal life, but that is where the similarities end. Adult cardiac myocytes do not proliferate on their own, where some other cells in the body replicate in order to replace old or damaged cells in the tissue or organ system such as skin.

During the rapid growth period in the fetus, embryonic cardiac myocytes proliferate quickly in order to maintain a steady growth rate with the rest of the body. In general, the cell cycle involves four phases. Cells perform basic functions during G1, S, and G2 phases. Mitosis occurs during M (mitosis) phase, producing two separate daughter cells from one parent cell. (Marieb and Hoehn, 2007).The cell cycle can be seen below in Figure 1.

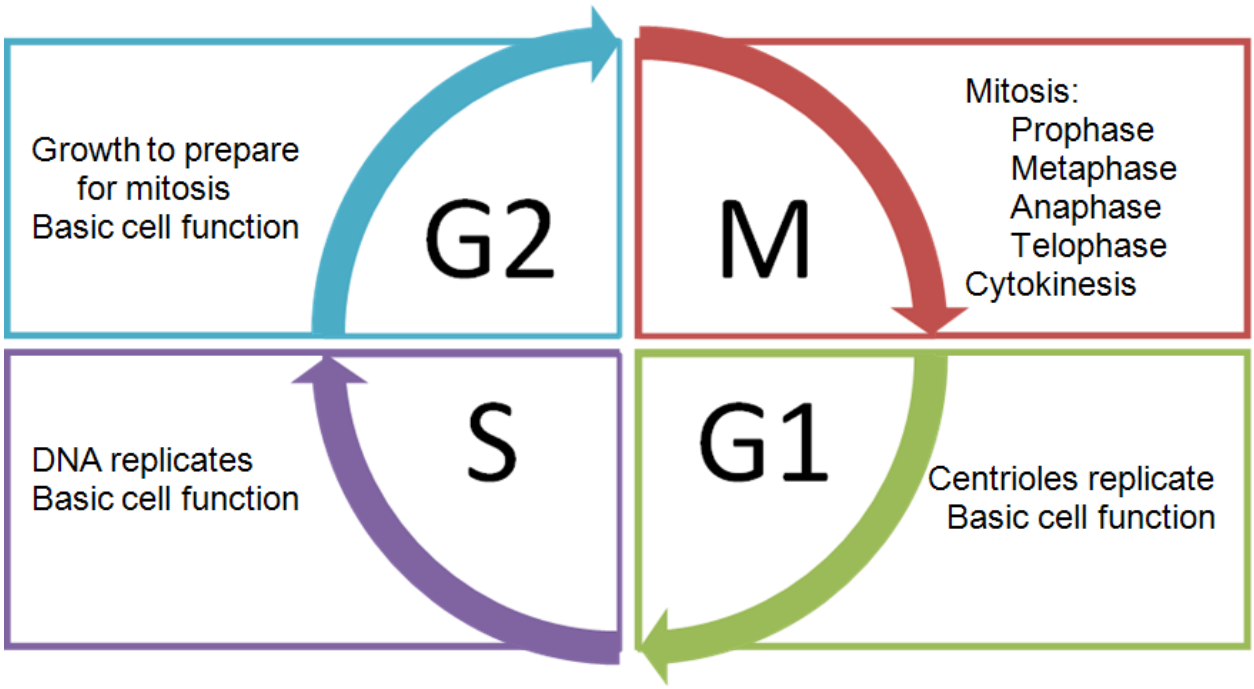


Figure 1: The Typical Cell Cycle.

The cell cycle of a cardiac myocyte is coordinated by various regulating proteins that are expressed in the cell at different stages of the cycle. As can be seen in Appendix A: Cell Cycle Regulator Proteins, these regulatory proteins can be divided into three groups: mediators, inhibitors, and transcription factors. Mediators aid the progress of the cell cycle, where inhibitors attempt to stop the cell cycle by deterring the function of the mediators. The transcription factors help with the transcription process of DNA (Ahuja, 2007).

Immediately after birth, cardiac myocytes still proliferate. After a few days, the cells exit the cell cycle and, until recently, it was believed that they did not begin to proliferate again. Some of the myocytes replicate DNA once more before exiting the cell cycle, but they do not undergo cytokinesis, which causes binucleation. It is estimated that 25 to 57% of adult cardiac myocytes are binucleated (Ahuja, 2007). As the infant

ages, the cells undergo hypertrophy, or growth within the cell, which causes the heart to grow to adult size.

Until recently, it was assumed that there were no adult cardiac myocytes capable of re-entering the cell cycle. In a 2001 study published by doctors at New York Medical College, the University of Udine in Italy, and the University of Trieste in Italy, it was determined that there are myocytes that are actively involved in the cell cycle. In fact, they were able to show that out of the myocytes surrounding an infarcted area, up to 4% were expressing Ki-67, an indicator of cell proliferation. In the regions far from the infarcted region, up to 1% of the myocytes were expressing Ki-67. The evidence showing that more cells around the dead area in the heart were in the cell cycle than in the healthy tissue indicates that the cell death may spur the reentry into the cell cycle. This means that the heart may have some limited ability to regenerate to replace the dead tissue (Beltrami, 2001). However, even if this is the case, the heart would not be able to completely replace the dead tissue without additional help. It is on this hypothesis, that cell cycle reentry is possible, that most of the current research is based.

2.3 Current Approaches

Based on the hypothesis that cardiac myocytes reenter the cell cycle, there are a number of research laboratories around the world trying to promote myocyte regeneration in human ventricular myocytes. The following is a review of relevant literature with respect to this problem, including reasons for why traditional treatments are not completely effective, research into embryonic myocyte development, stem cells and their effect on cardiac myocyte proliferation, and an overview of co-culture systems.

2.3.1 The Problem with Traditional Treatments

Many traditional non-cell related treatments for post-MI patients involve trying to restore form rather than function to the affected myocardium. After a heart attack, the infarcted tissue in the heart is dead, and therefore can no longer function as healthy tissue. In some patients, the infarcted region loses mechanical integrity due to the reduction in healthy tissue. This causes the wall of the heart to expand in an aneurysm, a condition that can further reduce cardiac function if not treated (Ueno et al., 2007). In order to avoid this complication, traditional treatments were developed in the attempt to restore the form of the heart.

There are several different treatments that can be used after MI to restore form to the heart. Ventricular patch plasty, also known as the Dor procedure, involves removing the infarcted tissue and sewing the remaining healthy tissue together (Bockeria et al., 2006). Another procedure known as “septal anterior ventricular exclusion” (SAVE) involves the use of a synthetic patch to restore form to the heart, and is used when the septum is affected by ischemia (Isomura et al., 2006). The overlapping procedure is similar to the Dor procedure with the exception of leaving the infracted tissue in the heart, and overlapping / sewing together the healthy tissue to restore form (Matsui et al., 2002). Some procedures also involve the use of revascularization techniques such as coronary artery bypass grafts (CABG) in order to restore blood flow to the tissue (Ribeiro et al., 2006, Bockeria et al., 2006). Although such a procedure would be beneficial to ischemic tissue, infarcted tissue cannot be restored to a living, functional state through revascularization because the tissue is already dead. Other treatments involve the use of medication and other therapies that treat the symptoms.

The problem with the traditional treatments is that they only treat the symptoms, and some can only restore form to the heart, not function. By observing the structure of the heart muscle itself, it becomes clear that a procedure such as the Dor or SAVE does not yield the same heart function as was present before the infarct. For example, by studying the structure of the "helical ventricular myocardial band", as discovered by Francisco Torrent-Guasp (Kocica et al., 2006), one can observe that cutting and re-sectioning the tissue of the heart will alter the muscle structure, and therefore alter the form and function of the heart itself. Since none of these procedures can restore the functionality of the heart, it is important to research cell-based therapies that may be more effective in repairing myocardial tissue.

2.3.2 Embryonic Cardiac Myocyte Development

Research has been conducted with embryonic cardiac myocytes and the specific roles of the regulatory proteins in myocyte development. One study in particular was published in 2007 by Ahuja, Sdek, and Maclellan. They suppressed or overexpressed certain regulatory proteins (see Appendix A: Cell Cycle Regulator Proteins) in embryonic mice and measured the difference in the heart weight to body weight ratios. As can be expected, the over-expression of mediator proteins and suppression of inhibitor proteins increased the heart weight, sometimes with drastic results. For example, mice suppressing developmental myocardial proteins such as Rb and p130 increased their heart weight/body weight ratio 300% (Ahuja et al, 2007).

Although this is a proven method to proliferating myocytes, it would not be an acceptable means. There is much debate about the ethical issues surrounding the use of embryonic cells for research purposes, and these issues are outside of the scope of

this project. However, this research is useful in indicating that proteins have a significant influence on myocyte proliferation, and it may be worth investigating the effects of proteins on adult cardiac myocytes once a suitable co-culture system is devised.

2.3.3 Stem Cells

Stem cell research is a relatively new and controversial field of scientific research. There has been much hype surrounding stem cell research since it was first developed decades ago, as many people thought that stem cell research and tissue engineering would be the technologies that could cure all disease and ailments within the human body (Ingber and Levin, 2007). To that end, there has been much research conducted using different types of stem cells. One of the main types of research being conducted, and the type most relevant to this project, is using stem cells for organ and tissue regeneration, specifically cardiac regeneration.

It is important to understand that there are two different types of stem cells: embryonic and adult. Embryos are the basis of all mammalian life. All of the tissues found within the mammalian body are products of embryonic growth due to the totipotent nature of embryonic stem cells. Totipotency is the ability of a cell to differentiate into any cell within the body. The goal of scientific stem cell research is to be able to grow any tissue, organ, or body part from the embryonic stem cells in order to cure diseases and replaced damaged tissues. Due to the controversy over the issue (Vats et al., 2005), and a limited cell supply, embryonic stem cells are not a viable option for this project.

Another major type of stem cell is the adult stem cell. These cells are found within different regions of the body including the hippocampus region of the brain, bone

marrow within long bones, and within the heart, to name a few. Adult stem cells do not have the same totipotent nature as embryonic stem cells, but many do have the ability to differentiate into several different cell types. Many adult stem cells are actually progenitor cells, meaning that they have already begun to differentiate into one tissue type, but have not yet differentiated into a specific type of cell. One example of this is a neural progenitor cell, which can differentiate into neurons, oligodendrocytes, and astrocytes (Czyz et al, 2003).

Until recently, organ and tissue transplants were the only way to treat many significantly damaged or diseased tissues while still allowing the patient to live a full and healthy life. There are two significant problems with organ and tissue transplants; the shortage of available tissue, and immune reactions from the patient's body in response to the foreign tissue that is implanted. For these reasons, the ability to grow an organ or tissue *in vitro* would be extremely useful in the treatment of different diseases and tissue damaging conditions. Cell therapy involves the use of the patient's own cells to grow a tissue or organ *in vitro*, and replace the damaged tissue with the healthy tissue once it has grown to its necessary size and functionality. In theory, this could potentially cure many diseases and life-threatening conditions, but it cannot be done yet (Vats et al, 2005).

The goal of the research presented here is to use a combination of myocytes and stem cells to produce a system that can promote cardiac myocytes to re-enter the cell cycle and proliferate *in vitro*. If this can be accomplished, then the cells may be effectively delivered into damaged heart tissue. It is hypothesized that these cells will direct myocardial regeneration and the heart may regain complete functionality after a

myocardial infarction or other cardiac event, allowing the patient to live a full and healthy life.

In the past, it was believed that a heart responded to MI by cell hypertrophy, or causing the cells to grow in size without actually completing the cell cycle. However, recent research has shown the heart is an organ with a resident population of differentiating myocytes and stem cells within the tissue. In fact, research has shown that in heart transplants from a female donor into a male recipient, the heart contains cell that are positive for the Y-chromosome, proving that there are cells from the body that can promote cardiac myocyte growth (Beltrami et al., 2003). According to some labs, cardiac stem cells can be identified by the presence of three different markers; c-kit (stem cell factor receptor), MDR-1 (multi-drug resistance-1), and Sca-1 (stem cell antigen-1) (Barile et. al, 2007). According to Barile et al., these stem cells also show the presence of telomerase, which is only seen in cells that have the ability to complete cytokinesis, the final step of mitosis in which two cells are formed from one.

There has been much research about the use of stem cells in culture with cardiac myocytes in the attempt to have the stem cells proliferate into cardiac myocytes. Tang and coworkers have shown an effective method of isolating Sca-1+ cells from the heart, promoting cell proliferation, and introducing the cells back into the heart. Promising results were seen as the cells were still present in the heart after one month, and had differentiated into viable heart cells (Tang et al., 2007). Another study by Oh et al. showed similar results with Sca-1+ cells promoting myocardial regeneration in an infarcted heart. Also, the study showed that most of the newly proliferating cells were those that were removed, promoted to differentiate into cardiac myocytes in culture, and

reintroduced into the heart. The introduction of the Sca-1+ cells did not promote the existing myocardium to regenerate (Oh et al., 2003).

Stem cells that are positive for c-kit have been studied extensively by several groups. Beltrami et al. were able to isolate and proliferate c-kit+ cells in culture through P26 (passage 26). The cells also showed preliminary differentiation, although the phenotypic cell type was not the same as what is found in normal tissue. Also, positive results that led to favorable cardiac function were observed when the cells were delivered to the heart and allowed to grow *in vivo* (Beltrami et al., 2003).

Mesenchymal stem cells (MSCs) have also been investigated as possible cardiomyocyte replacements. Xu et al. demonstrated this by establishing a way to isolate the MSCs from surrounding tissue, and promote them to differentiate into cardiomyocyte-like cells. The research showed positive results *in vitro* as chemical and visual analysis showed that the cells were similar to normal cardiomyocytes in that they produced cardiac markers such as α-actinin and desmin which are found in healthy cardiac tissue (Xu et al., 2004). Other labs have proven the effectiveness of co-culturing myocytes with mesenchymal stem cells. Research comparing co-cultured myocytes and MSCs with MSCs grown with conditioned media showed that direct myocyte to MSC contact is necessary in order to promote the MSCs to differentiate into functional myocytes (Rangappa et al, 2003; Wang et al., 2006).

2.4 Cell Culture and Analysis

In order to perform successful cell culture experimentation, it is important to understand some of the basic principles that are applied to cell culture experimentation

and technique. The following section describes cell culture technique and immunocytochemistry which are essential to the success of the project.

2.4.1 Cell Culture

Cell culture is the process of growing cells *in vitro* in an environment that is sterile and provides everything necessary to keep the cells alive. After the cells have been dissociated from the tissue, they are grown in culture dishes with medium. The medium contains all the ingredients necessary to keep the cells alive including carbohydrates and proteins. Most medium also contains at least one antibiotic additive to avoid contamination of the cells. There are also other ingredients that can be added such as fetal bovine serum and growth factors to further promote cell growth and proliferation.

Serums are extracted from the blood of living animals, i.e. fetal bovine serum is extracted from fetal cows. The serum contains a mixture of ingredients normally found within the donor blood serum. Although some of the items in the serum are known, there are things in serum what have not yet been defined. Therefore, any experiment that uses serum introduces another variable, as serum can be slightly different from animal to animal and batch to batch. Furthermore, the use of serum is potentially problematic when experimental protocols are translated to clinical environments.

Other ingredients can be added to medium to develop a substance that is useful for a particular experiment. For example, specific quantities and types of vitamins, growth factors, amino acids, antibiotics, and other ingredients can be added to media preparations. These ingredients are known and have little variability between batches. This type of medium is known as defined medium. Because serum is not fully defined, media with serum cannot be considered defined. Experiments with cardiomyocytes

typically use a medium consisting of Dulbecco's Modified Eagle Medium (DMEM) + 1% penicillin/streptomycin cocktail to prevent contamination, and 5% fetal bovine serum (FBS) (Rangappa et al., 2003, Doronin (submitted)).

Some cells are known as anchorage-dependent cells meaning that they must have a suitable surface to adhere to in order to proliferate. This surface is usually the cell culture dish or flask. Other cells are not anchor-dependent, and can simply survive in a suspension surrounded with essential media. Cells can also be maintained for long amounts of time, such as in a cell line. Although cells from a cell line were originally part of a living organism, they are grown continually in culture in order to maintain a constant viable cell source without having to sacrifice more animals each time an experiment is to be performed (Freshney, 1994). Cells such as human mesenchymal stem cells should be maintained in a simple flask system, with regular medium changes and passaging procedures to maintain the cells and to have cells available when necessary for experiments.

The culture techniques described above are usually used to maintain a particular cell line. Cardiac myocytes that are cultured alone will not proliferate, so a co-culture system must be utilized for promoting cell cycle markers in cardiac myocytes. The co-culture will utilize different media and culture dishes, but more than one cell type will be cultured in one dish. The purpose of the co-culture is to observe the effects of one cell type on the outcome of another cell type.

There are three types of co-culture to be discussed here: contact co-culture, insert co-culture, and conditioned media culture. A cartoon of each of the three types of co-culture can be seen in Figure 2.

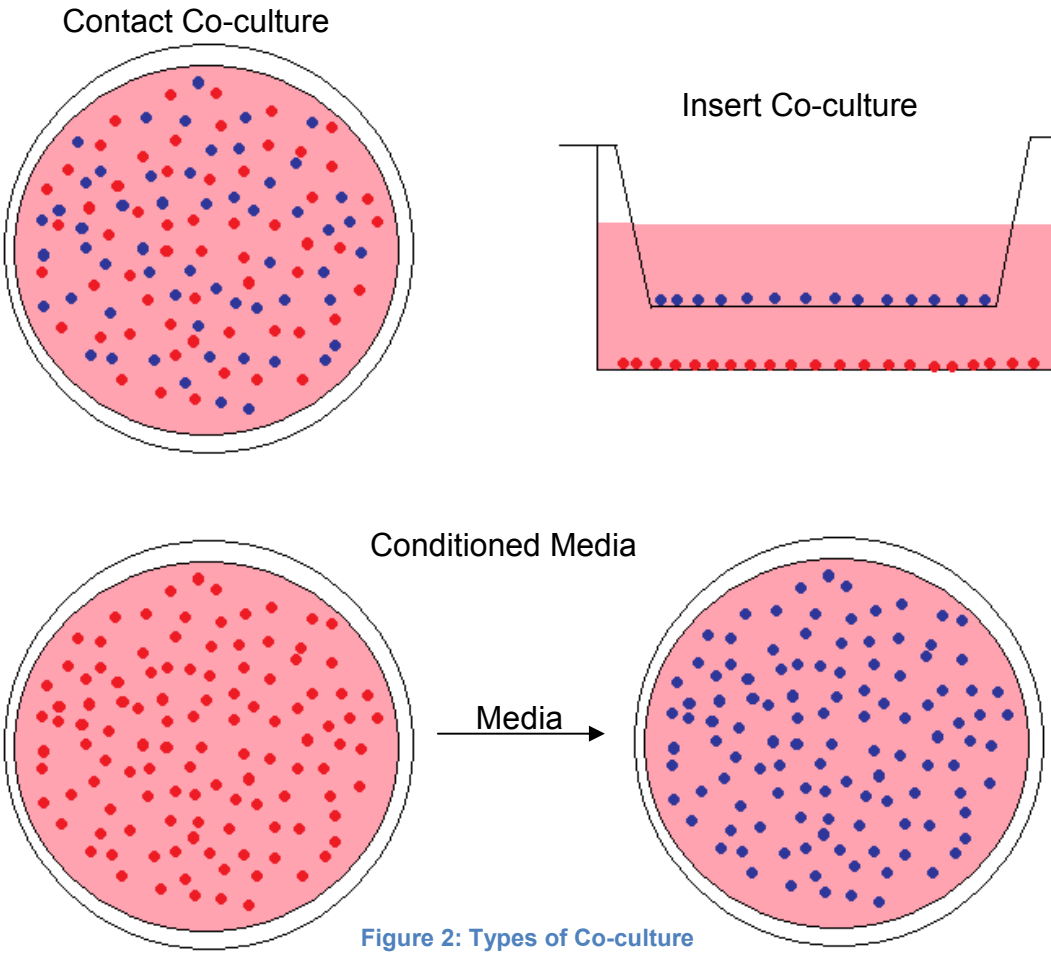


Figure 2: Types of Co-culture

Contact co-culture involves using two different cell types, such as myocytes and stem cells, and plating them together in the same culture dish (Wang, 2006). The cells are both plated on the same surface and in the same area, and there is no attempt to separate the two cell populations as the ”contact” indicates that the two cell types are in contact with each other. The cells are then treated like any other cell culture, being given media to keep them alive and allow proliferation.

Insert co-culture involves the use of a well-shaped insert (i.e. Transwell, Corning Incorporated) which is placed into the culture dish. One type of cell is placed on the insert, while the other is placed on the surface of the cell culture dish (Mensink, 1998).

The insert itself only allows soluble molecules to flow through, while the cells stay in the places where they were initially seeded.

The third culture type, conditioned media, involves separately culturing two different types of cells in different dishes while using the media from one cell type to "feed" the other cell type (Rangappa et al., 2003). This allows any soluble molecules given off from one cell to be received by the other cell with the conditioned media use, while not having the cells in the same culture dish. A study by Rangappa et al. showed the differences between cardiomyocyte and hMSC direct co-culture (two cell types plated together) and cardiomyocyte / hMSC conditioned media experiments. The focus of this experiment was to promote the hMSCs to differentiate into cardiomyocytes, and the results showed that direct cell-cell contact was necessary for this to occur. The conditioned media was less successful than the direct co-culture (Rangappa et al., 2003).

Co-culture systems are not limited to those listed previously. Other forms of co-culture involve the use of a different system than those named above. This involves using some sort of new technique that allows two cells to be cultured in the same dish or well without the cells coming into contact. A system such as this would allow for media to be exchanged freely between cells. The cells are also separated into two distinct areas and allowed to exist separately, which would allow for easy removal of one population if necessary. Depending on the system, cells may be allowed to grow close together (i.e. closer than a Transwell) while still remaining separated.

2.4.2 Immunocytochemistry

Immunocytochemistry is a technique that allows for visualization of specific antigens or proteins in cells. By using immunocytochemistry, the number of cells expressing the protein or presenting the antigen can be determined. Also, the placement of the antigen or protein in the cell can be determined.

The process of immunocytochemistry begins with applying a primary antibody that is specific to the antigen or protein of interest to the cell sample. In direct labeling, the primary antibody is labeled with a fluorescent marker that can be analyzed through microscopy. In indirect labeling, a fluorescent marked secondary antibody binds to the primary antibody and then the sample can be analyzed through microscopy (Principles and Techniques of Practical Biochemistry, 2000). This binding sequence is shown in Figure 3.

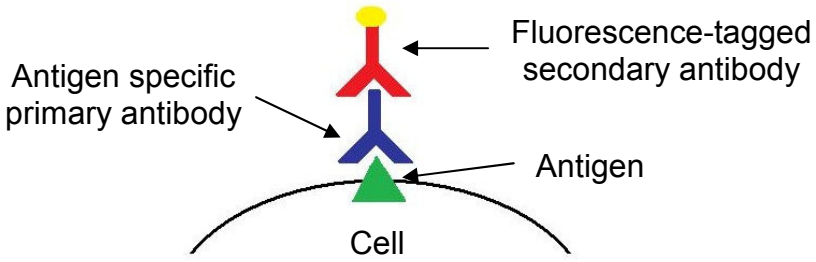


Figure 3: Immunofluorescent Labeling

For the purposes of this project, labels for proteins found during the cell cycle will be useful for visualizing cells that are undergoing mitosis. Ki-67 is a protein that has been used to identify whether or not a cell is or has recently been actively involved in the cell cycle. Ki-67 only appears during cell proliferation and is present during all active phases of cell cycles (G_1, S, G_2, and mitosis). It is absent from resting cells (G_0). During interphase, the protein is exclusively detected in the nucleus. While in mitosis, the

protein relocates to the surface of the chromosomes. The monoclonal antibody used against the Ki-67 protein is MIB-1. MIB-1 overcomes the original Ki-67 antibody because it can be used in paraffin sections of tissues after antigen reassessment by microwave processing (Cattoretti et al., 1992).

Another type of protein that can be found during the cell cycle is the cyclin protein. D-type cyclin proteins are responsible for initiating the response of cyclin-dependent kinases (cdk) which are part of the cascading process for promoting the cell cycle to proceed. The cyclin/cdk combinations are important in every part of the cell cycle (Coqueret, 2002). Cyclin D1 is a cyclin protein that identifies cells that are actively involved in the cell cycle, specifically in G_1 phase (Kim et al., 2008). More specifically, the cyclin D1 protein is present within the cell, but outside of the cell nucleus during G_0, also known as the resting phase. As the cell prepares to enter the cell cycle, cyclin D1 binds with cyclin dependent kinase 4 (CDK4), at which point the assembled product moves into the cell nucleus. While in the nucleus, the cyclin D1/CDK complex promotes a phosphorylation process that prompts the cell to progress through the G_1 phase into the S phase (Gladden and Diehl, 2005). As discussed earlier, G_1 involves centriole replication and prepares the cell for the cell cycle to continue. G_1 occurs just before the S phase in which DNA is replicated, a key step in mitosis. As the cell moves into S phase, the cyclin D1/CDK4 complex relocates outside of the nucleus where the two parts separate and remain until the cell is prepared to enter the G_1 phase again.

Since cyclin D1 is expressed at the beginning of the cell cycle (G_1), it can be used as a marker to detect cells that have recently moved out of the resting phase (G_0) and begun to undergo the steps of the cell cycle. If a cell expresses cyclin D1, then

there is a high likelihood that the cell is progressing through the cell cycle. Because adult cardiomyocytes do not proceed through mitosis naturally, they do not naturally express cyclin D1. Therefore, a co-culture system that promoted cyclin D1 expression in cardiomyocytes would be successful in regards to promoting the cardiomyocytes to reenter the cell cycle.

3. Project Approach

The literature review has provided a comprehensive overview of the past and current techniques and background information related to the problem. This chapter summarizes the processes for developing a design, including hypothesis, assumptions, and specific aims.

3.1 Hypothesis

Currently, no methods exist to completely replace necrotic tissue of the heart after myocardial infarction. Replacing this tissue is the only way to completely restore the functionality that is lost after an MI. Although a small population of proliferating myocytes does exist within the heart, these cells are not enough to repair the heart and replace the dead tissue. We hypothesize that the presence of cell cycle markers can be increased in cardiac myocytes through the use of stem cell/cardiac myocyte co-culture. We believe that the stem cells release a paracrine factor that induces the myocytes to reenter the cell cycle, which is indicated by the cell cycle markers expressed in the co-cultured myocytes. If these myocytes can reenter the cell cycle and be induced to proliferate, the proliferating myocytes could be delivered to the post-MI heart and regenerate the necrotic tissue. This can only happen if the population of myocytes is a pure population, meaning that there cannot be any stem cells mixed into the myocytes that are delivered to the heart. This is due to the fact that we are unsure of the effects of injecting stem cells into the cardiac tissue. However, if a pure population of proliferating myocytes can be produced, a potential treatment for MI can be developed.

3.2 Assumptions

In order for any engineering project to be successful, it is important to define reasonable assumptions that will be used by the team during the design and experimental phase of the project. The assumptions made by the project team are stated below:

- Cells made to proliferate *in vitro* could be effectively delivered back into the heart without harming the cells.
- Cells delivered into the heart would be able to integrate into the heart tissue to improve heart functionality by replacing necrotic tissue.
- Newly proliferated cells have the same structural and biochemical properties of the native cardiac myocytes.

3.3 Specific Aims

In order to develop a solution to the problem presented, there are several specific aims that the team hopes to accomplish through the design and experimentation process:

- Develop a system that can isolate living cardiac myocytes from the animal tissue so that cells can be used in cell culture
- Develop a system that can be used to increase the presence of cell cycle markers in adult cardiac myocytes
- Develop a system that will yield reproducible results

3.4 Project Specifications

To verify the success and effectiveness of the final design, the product must be designed to the following specifications:

- The culturing system must co-culture myocytes with stem cells in an attempt to induce 5% more of the myocytes to express markers than myocytes that are not co-cultured.
- There must be a barrier between the two different co-cultured populations that is at least 200 μm thick to prevent the stem cells from migrating into the myocyte population.
- There must be a barrier between the two different co-cultured populations so the system will yield a myocyte population that is at least 90% pure.
- The culturing system must take less than two weeks to produce results.

4. Design

As with any engineering problem, there must be extensive thought and planning that goes into the design of the solution. By clarifying client needs, specifications, and constraints, it is possible to move forward through the design process to develop a suitable solution to the problem.

4.1 Initial Problem Statement

The problem initially presented to the project team consisted of the following: “Design a system or method that can effectively increase the presence of cell cycle markers in adult cardiac myocytes.”

In order to proceed in the design process, the problem statement needed to be expanded into the revised problem statement. This means that the team needed to identify the clients and users and discuss their needs and wants to complete the first step in the design process.

4.1.1 Clarify Objectives

An important step in the design process is to identify the objectives that the design needs to fulfill. The best way of identifying these objectives is by discussing the design with the clients and the users of the design. In order to do this, the clients and users must be first identified. Using the ideas presented in the design book written by Dym and Little in 2004, the team formed a designer-client-user triangle, as seen in Figure 4, to help visualize the relationship.

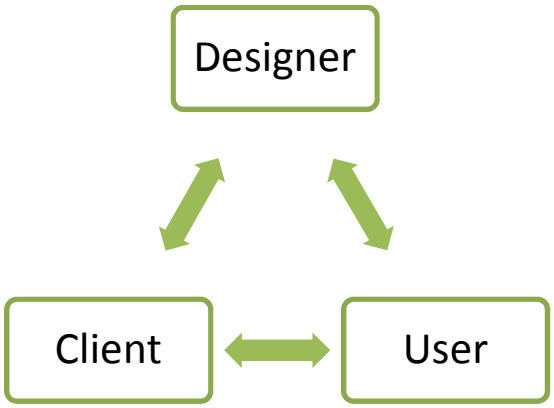


Figure 4: The designer-client-user triangle. (Dym, 2004)

To help with the identification of these important parties, the team decided to define each role involved. The designer is the person or entity that will be developing the end product or methods. The client is the person or entity that presented the problem and is looking for a solution. The user is the person or entity that will be utilizing the end design in real world applications. Using these definitions, the team was able to identify the participants as shown in Figure 5.

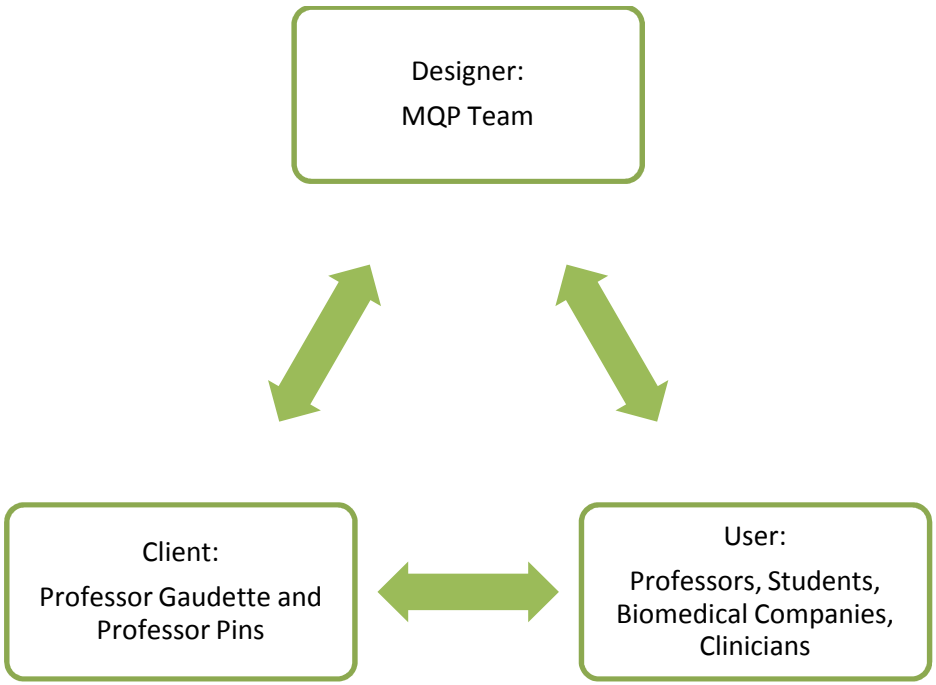


Figure 5: Identified designer-client-user triangle

After the designer-client-user relationship was established, the team developed a list of questions to understand the client and user wants and needs. These questions, with their answers, are provided in Appendix B: Interview with the Client and the User. Both clients in Figure 5 were interviewed and a Biomedical Engineering graduate student, Jacques Guyette, was interviewed as a user. From their answers to the questions the team was able to establish a list of objectives, constraints, and user requirements. The list of objectives can be seen in Table 1 below and the constraints and user requirements can be found in the next two sections.

Table 1: Design objectives

Design Objectives		
Primary Objective	**Secondary Objective**	**Tertiary Objective**
Express cell cycle markers	Repeatable	
	Effective	
Safe	For cells	Non-toxic
	For users	
Efficient	Minimize materials	Media
		Cells
		Equipment
Cost		
Ease of Use	Interface	
	Simplicity	
	Time	
	Size	

From the list of objectives, the team created the objectives tree seen in Figure 6. This objective tree was approved by the client and allowed the team to make pairwise comparison charts, which are included in Appendix C: Pairwise Comparison Charts. These charts were sent to the client and user to be completed and sent back. The analyses of these charts, as seen in the Appendix, lead to the weighted objectives tree as seen in Figure 7. The weights were determined by averaging the client and user weights for each objective. These weights are used in the design process to determine which objectives are the most important to meet. In the weighted objectives tree, the secondary and tertiary objectives have two weights. The first is the percent of the above objective and the second is the weight overall.

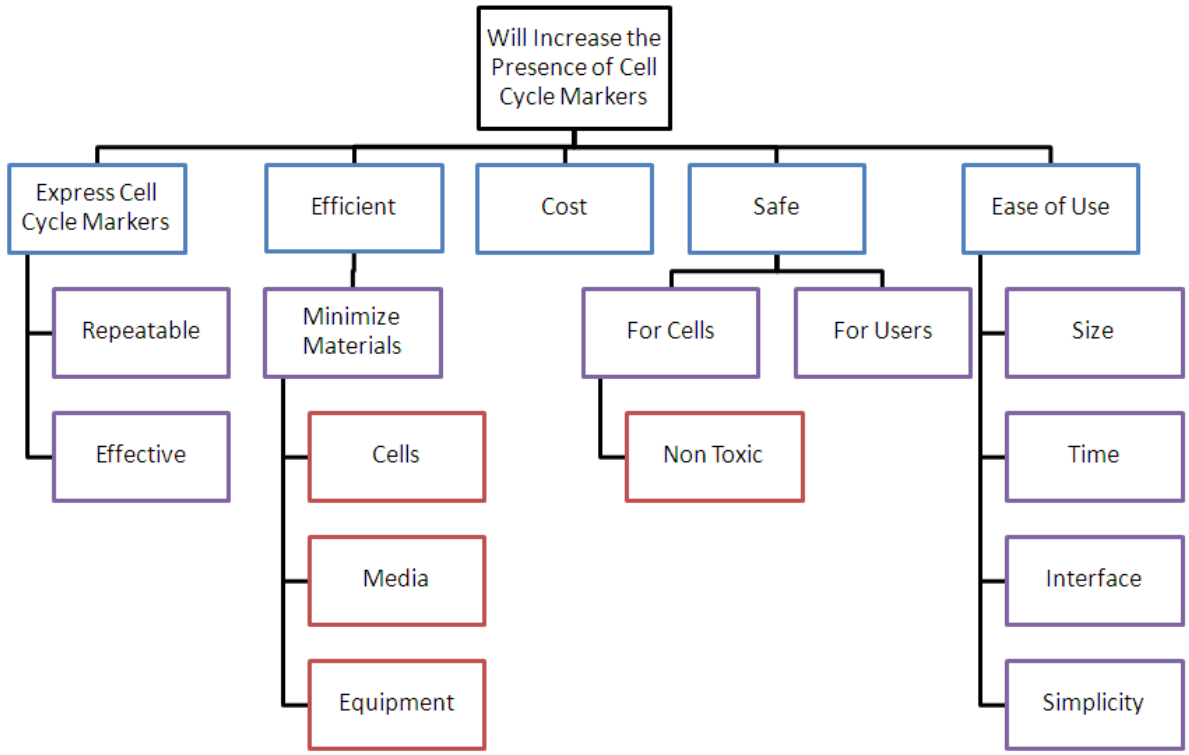


Figure 6: Objectives Tree

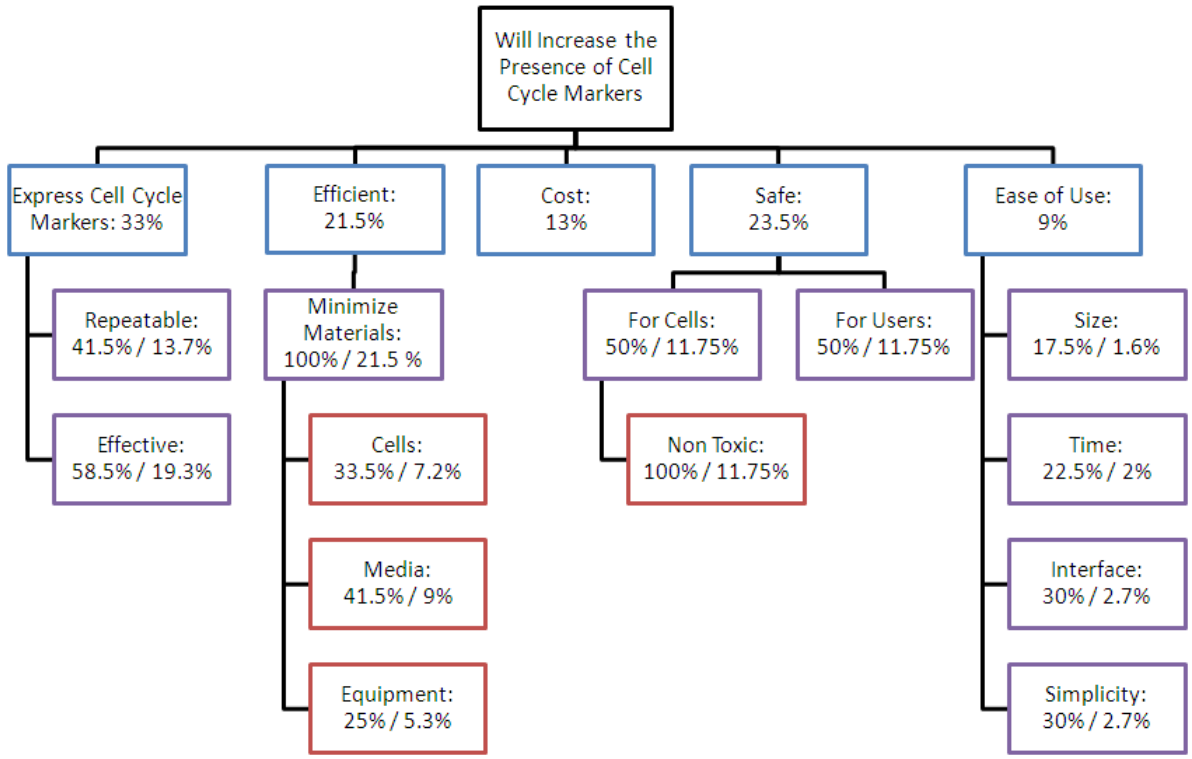


Figure 7: Weighted Objectives Tree

The weighted objectives tree allows the team to prioritize the objectives. The prioritized list of objectives can be seen below in Table 2.

Table 2: Prioritized list of design objectives

Design Objectives					
Primary Objective		**Secondary Objective**		**Tertiary Objective**	
33%	Express cell cycle markers	19.3%	Effective		
		13.7%	Repeatable		
23.5%	Safe	11.75%	For cells	11.75%	Non-toxic
		11.75%	For users		
21.5%	Efficient	21.5%	Minimize materials	9%	Media
				7.2%	Cells
				5.3%	Equipment
13%	Cost				
9%	Ease of Use	2.7%	Interface		
		2.7%	Simplicity		
		2%	Time		
		1.6%	Size		

4.1.2 Establish User Requirements

Based on the client and user interviews as described in the previous section, the team developed a list of user requirements as follows:

- Must be able to fit into a cell culture hood and incubator
- Must cost less than $100 per experiment
- Must not use an excessive amount of resources
- Must take 8 hours or less to prepare and produce results within 2 weeks
- Must produce 5%-10% of cells expressing cell cycle markers

4.1.3 Identify Constraints

Based on the client and user interviews, the team developed a list of design constraints:

- Size: Must fit into a cell culture hood
- Time: Must be able to complete culturing within two weeks.
- Design Time: Prototype must be completed by April 1, 2008.
- Cost: Prototype must cost less than the amount allotted by the Biomedical Engineering department, $468.
- Sterile: Must provide a sterile environment for the cells to grow.
- Migration: Must stop stem cell population from migrating into the myocyte population in order to produce a pure population of cultured myocytes.

4.1.4 Establish Functions

Based on the client and user interviews, the team developed a list of functions that the design must accomplish:

- Must isolate at least 5000 living cardiac myocytes from a heart tissue sample
- Must be able to maintain viable myocytes in culture for two weeks
- Must be able to co-culture stem cells with cardiac myocytes to increase the presence of cell cycle markers in the cardiac myocytes over myocytes that are not cultured using this system
- Must be able to prevent the stem cells from migrating into the myocyte population
- Must include an analysis process that can prove that the cell cycle markers are being increased

4.2 Revised Problem Statement

Based on the completed weighted objectives tree, as well as the lists of user requirements, constraints, and functions, the following revised problem statement was developed:

Design a system or method that can be used to increase in the presence of cell cycle markers in adult mouse cardiac myocytes through a co-culture system with human mesenchymal stem cells. The design must provide a distance of at least 200 μm between the cell types and keep the population of myocytes at least 90% pure. The isolation and culturing process should be able to take place inside a biosafety cabinet and the culture system must be able to fit inside an incubator. The process must contain an analysis procedure that can give proof of the increase in cell cycle markers.

4.3 Conceptual Design

The revised problem statement allows the team to brainstorm and develop conceptual designs. The purpose of creating a set of conceptual designs is to develop a set of ideas that can be analyzed for their strengths and weaknesses. Once the ideas are analyzed, a design that is suitable for our particular application can be determined. There were two areas of the project that needed design alternatives, the cell isolation and the cell culture design. First, alternatives were generated and assessed for the cell isolation procedure using design matrices. Then, a functions-means tree was generated to aid the brainstorming for the cell culture design, after which the design alternatives were generated and assessed using design matrices.

4.3.1 Design Matrices for Cell Isolation

The first step in creating a conceptual design is to brainstorm for ideas about how to accomplish our goals and aims while fulfilling our objectives. The team generated three areas that needed to be addressed for the cardiac myocyte isolation. We needed to choose an animal model, the medium that will be used for cell culture, and the technique that will be used for the myocyte isolation from the animal heart.

After brainstorming, the team identified 6 possible animal models: rat, mouse, human, goat, cow, or pig. The rat and mouse would be bought alive from Charles River Laboratories, Inc. in Wilmington, Massachusetts. They would be euthanized by the team and the hearts extracted. The advantage to lab animals is that they are raised in a controlled environment, but the hearts are much smaller, and therefore, harder to handle. The large animal models, goat, cow, and pig, would not be purchased whole. The team would go to a slaughterhouse and purchase the heart. This means that the heart would not be as fresh as the laboratory animals. They also would have been pasture grown, which means that there will be more variations from animal to animal. However, their hearts would be easier to handle than the small animal models and the hearts would yield more experiments per heart. The final animal model considered was the human, which has all of the advantages and disadvantages of the large animal models. Human hearts, however, would not be taken whole from cadavers. The tissue that the team would be able to obtain would be taken from the patients during heart procedures where the tissue is normally removed. This means that the tissue would most likely be from old, diseased hearts. On the other hand, humans are the end users

of this design, so using human tissue would mean that there would need to be less interspecies adaptation if the design is successful.

The ranking system that was developed to quantitatively assess these advantages and disadvantages can be seen in Table 3. These criteria were used to complete the design matrix, which can be seen in Table 4.

Table 3: Ranking Criteria for choosing the animal model

Ranking Criteria										
Design Constraints	Yes	No								
C. Size	Heart must produce at least 240,000 cells	Heart produces less than 240,000 cells								
C. Cost	Costs less than $25 per animal	Costs more than $25 per animal								
Design Objectives	**0.1**	**1**								
O. Repeatable	Pasture Grown (non-controlled environment)	Lab Raised (controlled environment)								
O. Safe for Users	Possible diseases because pasture grown (non-controlled environment)	No diseases because lab raised (controlled environment)								
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1**
O. Ease of Use: Size	Animals weighs less than 25g	Animals weighs 25g-100g	Animal weighs 101g-500g	Animal weighs 501g-1kg	Animal weighs 1<g-50<g	Animal weighs 51kg-100<g	Animal weighs 101kg-150kg	Animal weighs 151<g-200<g	Animal weighs 201kg-250kg	Animal weighs over 251 kg
O. Ease of Use: Time	Must retrieve ourselves									Delivered
O. Ease of Use: Interface	Animals weighs less than 25g	Animals weighs 25g-100g	Animal weighs 101g-500g	Animal weighs 501g-1kg	Animal weighs 1<g-50<g	Animal weighs 51kg-100<g	Animal weighs 101kg-150kg	Animal weighs 151<g-200<g	Animal weighs 201kg-250kg	Animal weighs over 251 kg

Table 4: Design Matrix including rankings for choosing the animal model

Animal Model													
Design Constraints		Rat		Mouse		Human		Goat		Cow		Pig	
C. Size		Y		Y		Y		Y		Y		Y	
C. Cost		Y		Y		Y		N		N		N	
Total	2	2		2		2		1		1		1	
Design Objectives	Weight (%)												
O. Repeatable	13.7	1	13.7	1	13.7	0.1	1.37						
O. Safe for Users	11.75	1	11.75	1	11.75	0.1	1.175						
O. Ease of Use: Size	1.6	0.2	0.32	0.1	0.16	0.6	0.96						
O. Ease of Use: Time	2	1	2	1	2	0.1	0.2						
O. Ease of Use: Interface	2.7	0.2	0.54	0.1	0.27	0.6	1.62						
Total	31.75		28.31		27.88		5.325						

Although the rat was the highest ranking animal model, the team chose to use the mouse instead. It was discovered that a graduate student, Christine Lima, was euthanizing mice on campus on a regular basis. She was willing to allow the team to have the hearts from her mice. This means that the team was able to acquire hearts for free leaving more money in the budget for other purposes. Since the ranking of the mouse was only slightly below the ranking of the rat, the team felt that this was an acceptable choice.

The brainstorming led the team to three possible choices for media to be used in the cell culture experiments: medium (Dulbecco's Modified Eagle Medium, DMEM) + fetal bovine serum (FBS), conditioned medium from mesenchymal stem cells, and defined medium with proteins. The DMEM + FBS has been used in the past to culture hMSCs with cardiac myocytes (Doronin et al., submitted). The conditioned media experiment would be done by culturing the stem cells in a medium for a period of time, then aspirating that medium out of the culture dish and using it to culture the cardiac myocytes. This means that any products the stem cells secrete will be in the medium that is used for the myocytes. The defined medium with proteins would be the basic medium with specialized proteins added that are expressed during the myocyte cell

cycle. The hypothesis is that the cell cycle proteins would induce the myocytes to enter that phase of the cell cycle. An overview of these proteins can be seen in Appendix A: Cell Cycle Regulator Proteins.

These attributes of the media types were quantified in the ranking chart seen as Table 5 and were used to assess the media types in the design matrix, included as Table 6.

Table 5: Ranking Criteria for choosing the medium

Ranking Criteria										
Design Constraints	**Yes**	**No**								
C. Size	Must fit within a culture hood	Does not fit within a culture hood								
C. Sterile	Must be sterile	Is not sterile								
Design Objectives	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
O. Repeatable	Can not control precise amount of additives				Limited control on amounts of additives					Precise control on amounts of additives
O. Effective	No									Yes
O. Minimize Materials: Media	More than 10 additives				Some additives					no additives necessary
O. Cost	More than 10 additives				Some additives					no additives necessary
O. Safe for Cells	No				Moderate					Yes
O. Safe for Users	No				Moderate					Yes
O. Ease of Use: Simplicity	Need to change media multiple times a day				Need to change media once a day					Only need to change media every 2-3 days

Table 6: Design Matrix for choosing medium

Media							
Design Constraints		**DMEM with FBS**		**Conditioned Medium**		**Defined Medium with Proteins**	
C. Size		Y		Y		Y	
C. Sterile		Y		Y		Y	
Total	2	2		2		2	
Design Objectives	**Weight (%)**						
O. Repeatable	13.7	0.5	6.85	0.5	6.85	1	13.7
O. Effective	19.3	1	19.3	1	19.3	1	19.3
O. Minimize Materials: Media	9	1	9	1	9	0.5	4.5
O. Cost	13	1	13	1	13	0.5	6.5
O. Safe for Cells	11.75	1	11.75	1	11.75	1	11.75
O. Safe for Users	11.75	1	11.75	1	11.75	1	11.75
O. Ease of Use: Simplicity	2.7	1	2.7	0.5	1.35	0.5	1.35
Total	**81.2**		**74.35**		**73**		**68.85**

The medium selected was the DMEM + FBS. This is mostly due to the fact that this media has been shown to be effective in previous experiments (Doronin et al, submitted). Conditioned media may limit the amount of paracrine factors that can reach the cardiac myocytes if the paracrine factors have a short life span. The protein-enhanced medium would have been carefully controlled by the team to ensure uniformity between experiments, but it would have been expensive to produce the amount needed for each experiment.

The team established four methods that could be used to isolate the cardiac myocytes from the host tissue: trituration, scalpel, enzymes, and cell migration. During trituration, small clumps of cells are pulled in and out of a pipette. The shear forces in the pipette causes the cells to break apart. This allows the tissue to be broken down

into individual cells, but would damage a percentage of the cells in the process. The scalpel would be used to manually mince the tissue into small pieces. This would not yield individual cells and would damage cells as well. Enzymatic methods are commonly used in cell culture. The tissue is placed into a bath of enzymes, typically collagenase, and the enzyme breaks the bonds between the cells. This would yield individual cells without damage. The cell migration technique involves putting the tissue into culture and allowing the cells to spread. The spreading causes some of the cells to detach from the tissue. Although this method would yield some individual cells with no damage, it would take a long time to obtain enough individual cells for our purposes.

The advantages and disadvantages of these isolation techniques were quantified in the ranking criteria chart, included as Table 7. These numerical ratings were used to complete the design matrix as seen in Table 8. The highest-ranking technique was the enzymatic process.

Table 7: Ranking Criteria for choosing method of cardiac myocyte isolation

Cell Isolation Ranking Criteria										
Design Constraints	**Yes**	**No**								
C. Size	Fits within cell culture hood	Does not fit within cell culture hood								
C. Experiment Time	Less than 6 hours	More than 6 hours								
C. Cost	Costs less than $5 per experiment	Costs more than $5 per experiment								
C. Sterile	Sterile	Not sterile								
Design Objectives	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
O. Repeatable	No									Yes
O. Effective	No cells are isolated		1-49% are isolated				50 to 99% are isolated			All cells are isolated
O. Minimize Materials: Equipment	10+ tools	9	8	7	6	5	4	Uses 3 tools	Uses 2 tools	Uses only one tool
O. Cost	10+ tools	9	8	7	6	5	4	Uses 3 tools	Uses 2 tools	Uses only one tool
O. Safe for Cells	All cell death				Some cell death					No cell death
O. Safe for Users	Not safe				Moderately safe					Very safe
O. Ease of Use: Size	Outside of hood									Within hood
O. Ease of Use: Time	45+ min	40-45 min	35-40 min	30-35 min	25-30 min	20-25 min	15-20 min	10-15 min	5-10 min	0-5 min
O. Ease of Use: Interface	10+ tools	9	8	7	6	5	4	Uses 3 tools	Uses 2 tools	Uses only one tool
O. Ease of Use: Simplicity	10+ tools	9	8	7	6	5	4	Uses 3 tools	Uses 2 tools	Uses only one tool

Table 8: Design Matrix for choosing the method for myocyte isolation

Cell Isolation									
Design Constraints		**Trituration**		**Scalpel**		**Enzyme**		**Cell Migration**	
C. Size		Y		Y		Y		Y	
C. Experiment Time		Y		Y		Y		N	
C. Cost		Y		Y		Y		Y	
C. Sterile		Y		Y		Y		Y	
Total	4	4		4		4		3	
Design Objectives	**Weight (%)**								
O. Repeatable	13.7	1	13.7	1	13.7	1	13.7		
O. Effective	19.3	0.7	13.51	0.3	5.79	1	19.3		
O. Minimize Materials: Equipment	5.3	0.9	4.77	1	5.3	1	5.3		
O. Cost	13	0.9	11.7	1	13	1	13		
O. Safe for Cells	11.75	0.5	5.875	0.5	5.875	0.5	5.875		
O. Safe for Users	11.75	1	11.75	0.5	5.875	1	11.75		
O. Ease of Use: Size	1.6	1	1.6	1	1.6	1	1.6		
O. Ease of Use: Time	2	0.9	1.8	0.9	1.8	0.5	1		
O. Ease of Use: Interface	2.7	0.9	2.43	1	2.7	1	2.7		
O. Ease of Use: Simplicity	2.7	0.9	2.43	1	2.7	1	2.7		
Total	**83.8**		**69.565**		**58.34**		**76.925**		

4.3.2 Design Matrix for Choosing Cell Culture System

The second part of the conceptual design process was brainstorming for possible means of cell culture. From our initial research and our revised problem statement, we knew that we wanted to co-culture the myocytes with stem cells. We needed to determine the type of co-culture we would want to do. There were four possibilities for co-culturing the two cell types: Transwell®, conditioned medium, random co-incubation with the mesenchymal stem cells (MSCs), and non-random co-incubation with the MSCs. The Transwell® and conditioned medium methods were described in Section 2.4.1 Cell Culture. The Transwell® system would keep the cell types separated, but the distance between the cell types is fairly large meaning that the paracrine factors

secreted by the stem cells may never reach the myocytes. The conditioned medium method would also keep the cell types apart, but the paracrine factors may denature before the medium is given to the myocyte population. The random co-incubation method would entail plating both cell populations in the same dish, making no attempt to keep the populations separated. This would decrease the distance between the cells and ensure that the myocytes would be exposed to the paracrine factors. The non-random co-incubation would require the team to develop a new method of co-culturing cells that would keep the cell types apart, but reduce the distance between the cell types. The four methods were ranked according to the criteria listed in Table 9 and the results can be seen in Table 10.

Table 9: Ranking criteria for choosing the cell culture system to be used.

Ranking Criteria										
Design Constraints	**Yes**	**No**								
C. Size	Will fit into the cell culture hood	Will not fit into the cell culture hood								
C. Experiment Time	Will take two weeks or less to culture	Will take over two weeks to culture								
C. Cost	Will cost less than $78 per experiment set	Will cost more than $78 per experiment set								
C. Purity	Will produce a 90% pure population	Will not produce a 90% pure population								
C. Sterile	Will not inhibit standard sterile procedures	Will inhibit standard sterile procedures								
Design Objectives	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
O. Repeatable	No									Yes
O. Effective	No myocyte expression		Few myocytes express markers			Portion of myocytes express markers		Many myocytes express markers		All myocytes express markers
O. Miminize Materials: Cells	uses more than 1 million cells	uses 750,000-1million cells	uses 500,000 - 750,000 cells	uses 250,000-500,000 cells	uses 100,000-250,000 cells	uses 75,000-100,000 cells	uses 50,000-75,000 cells	uses 25,000 - 50,000 cells	uses 10,000-25,000 cells	uses 0-10,000 cells
O. Minimize Materials: Media	50+ ml	45-50ml	40-45ml	35-40ml	30-35ml	25-30ml	20-25ml	15-20ml	10-15ml	less than 10 ml
O. Minimize Materials: Equipment	10+ tools	9	8	7	6	5	4	Uses 3 tools	Uses 2 tools	Uses only one tool
O. Safe for Cells	All Cells die				Half of Cells die					No Cells die
O. Ease of Use: Time	Takes 10 hours or more to set	Takes 9 hours to set up	Takes 8 hours to set up	Takes 7 hours to set up	Takes 6 hours to set up	Takes 5 hours to set up	Takes 4 hours to set up	Takes 3 hours to set up	Takes 2 hours to set up	Takes 1 hour or less to set up
O. Ease of Use: Interface	Hard to understand process				Moderately hard to understand process					Easy to understand process
O. Ease of Use: Simplicity	10+ steps	9 steps	8 steps	7 steps	6 steps	5 steps	4 steps	3 steps	2 steps	1 step

Table 10: Design matrix for evaluating the four options for the cell culture system.

Cell Culture System									
Design Constraints		Transwell		Conditioned Medium		Random Co-incubation with MSCs		Non-random Co-incubation with MSCs	
C. Size		Y		Y		Y		Y	
C. Experiment Time		Y		Y		Y		Y	
C. Cost		Y		Y		Y		Y	
C. Migration		Y		Y		N		Y	
C. Sterile		Y		Y		Y		Y	
Total	5	5		5		4		5	
Design Objectives	Weight (%)								
O. Repeatable	13.7	1	13.7	1	13.7			1	13.7
O. Effective	19.3	0.3	5.79	0.3	5.79			0.6	11.58
O. Miminize Materials: Cells	7.2	0.7	5.04	0.7	5.04			0.8	5.76
O. Minimize Materials:	9	1	9	0.8	7.2			1	9
O. Minimize Materials: Equipment	5.3	0.9	4.77	0.9	4.77			0.9	4.77
O. Safe for Cells	11.75	1	11.75	1	11.75			1	11.75
O. Safe for Users	11.75	1	11.75	1	11.75			1	11.75
O. Ease of Use: Time	2	1	2	1	2			0.8	1.6
O. Ease of Use: Interface	2.7	0.5	1.35	0.5	1.35			0.5	1.35
O. Ease of Use: Simplicity	2.7	0.9	2.43	0.9	2.43			0.8	2.16
Total	85.4		67.58		65.78				73.42

The non-random co-incubation with MSCs method was ranked the highest among the four possibilities. This means that the team would need to continue the design process in order to design a new method for co-culturing cells.

4.3.3 Functions-Means

Since the cell culture system chosen required additional designing by the team, we needed to brainstorm for possible means of generating a non-random co-incubation culturing system. To aid with brainstorming, the team produced a functions-means tree as can be seen as Figure 8. In the figure, the square boxes represent functions and the ovals represent possible means.

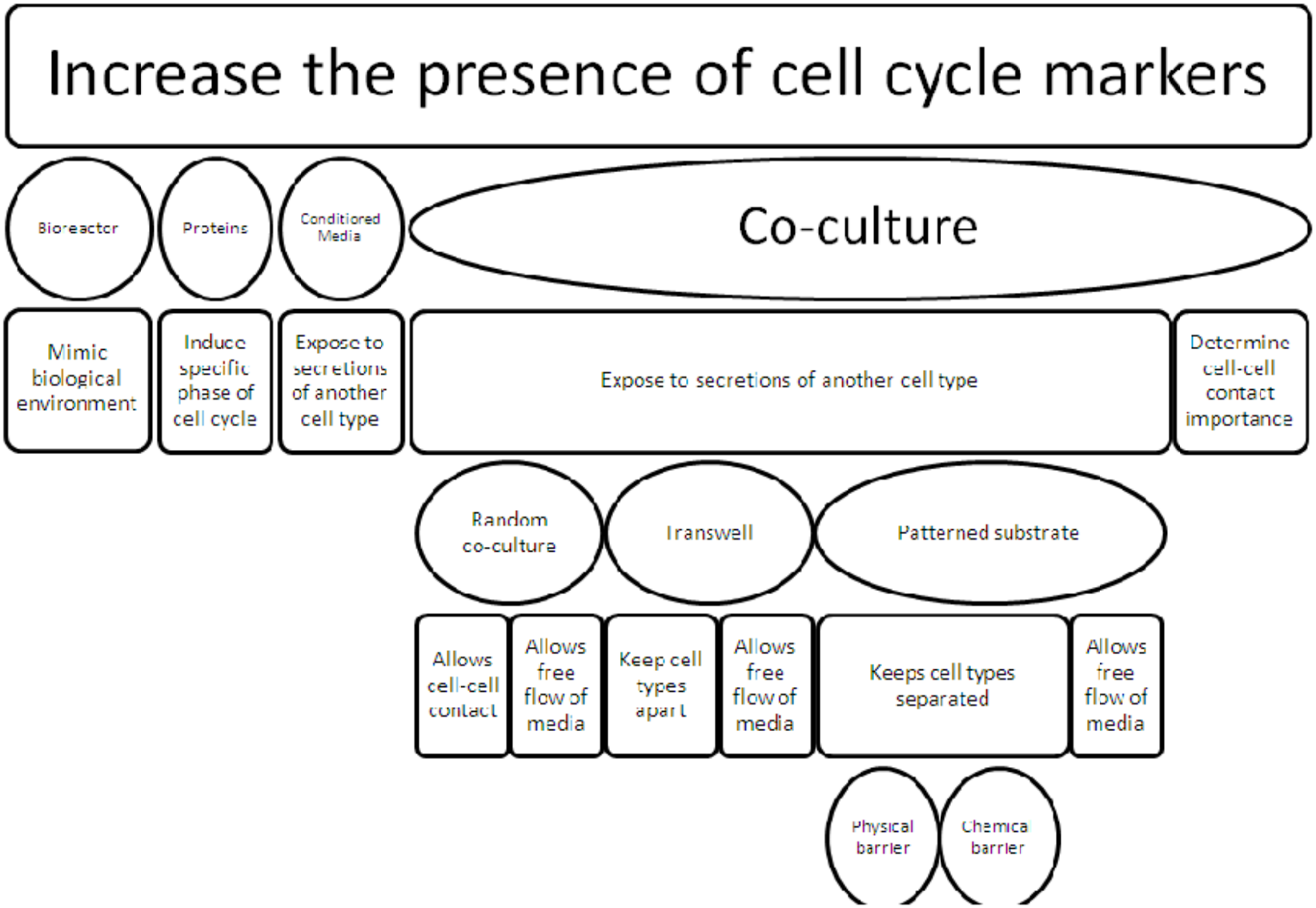


Figure 8: Functions-Means Tree

From the Functions-Means tree, two possible means of patterning the culture system were determined: a physical barrier or a chemical barrier. A physical barrier system would entail generating some sort of material that could be placed between the cell types. In a chemical barrier system, some sort of chemical would need to be placed between the cell types to prevent the cells from migrating. With this information in mind, the team was able to brainstorm for barrier options and developed Table 11, as can be seen below. These ideas are expanded in Section 4.3.4 Design Alternatives.

Table 11: Brainstorming ideas for cell culture design

Possible Methods for Patterning the Cell Culture	
Physical Barrier	**Chemical Barrier**
vertical cover slips	PDMS stencil → protein patterning on surface
agarose gel	PDMS stamping on gold slides
PDMS cloning rings	

4.3.4 Design Alternatives

The team generated five design alternatives during the brainstorming session as can be seen in Table 11. These five conceptual designs were further expanded by the team in an effort to understand the logistical issues behind these ideas.

The first conceptual design that was discussed was the vertical cover slip design. In this setup, a rectangular glass slide would be used as the cell culture area. Glass cover slips would be oriented vertically on the glass slide and held in place in order to form a barrier between the different sides of the slide, as can be seen in Figure 9. The slide would be divided into multiple sections, like wells, allowing a different cell type to be cultured in each section. The cells would then be put in their respective sections.

This would allow for alternating stem cell / myocyte culture sections. The cover slips would be removed which would allow the cell types to freely share media.

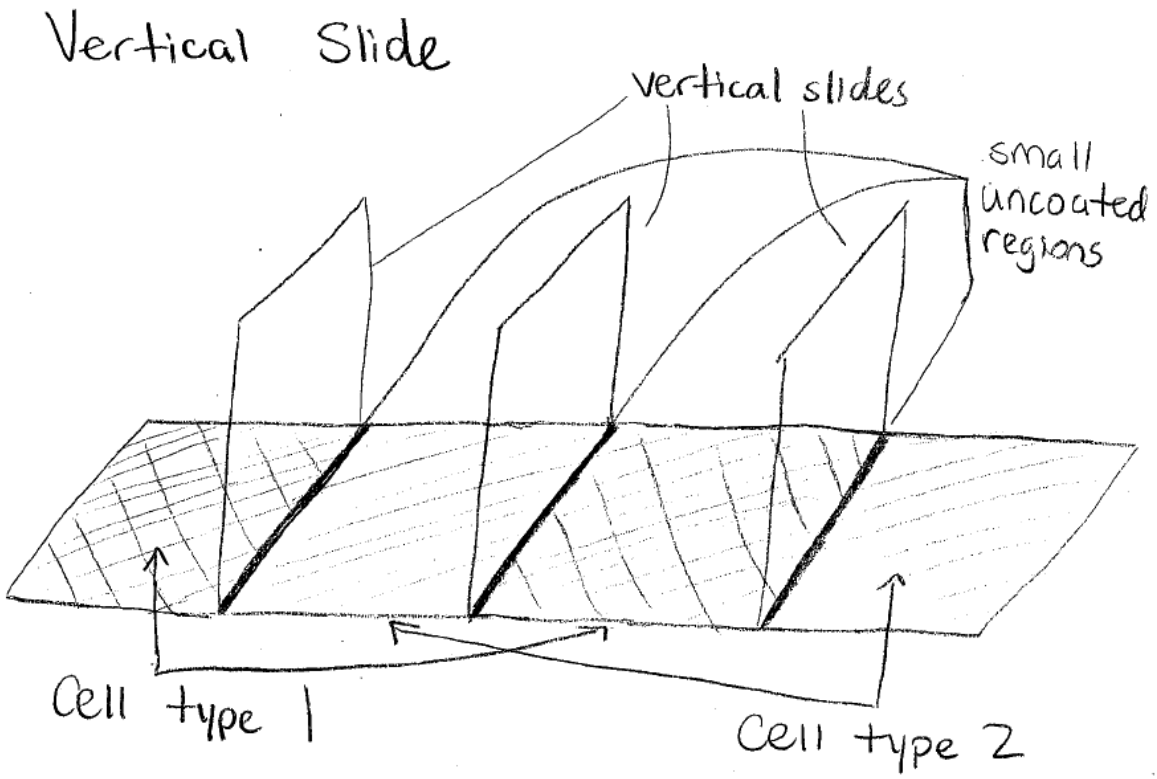


Figure 9: Conceptual design idea using vertical slides

Another alternative involves using agarose gel to create physical barriers between the cell types, as seen in Figure 10. The gel would be applied to the clean culture dish to create individual "wells" and allowed to set. The cardiac myocytes and the stem cells would be pipetted into separate wells. The agarose gel would act as a wall in between the two cell types. The benefit of agarose gel is that it allows proteins to pass through the gel (Gutenwik, 2003). This would have the benefits of a Transwell® experiment, with the media and proteins being shared between the cells, but the cells would be much closer together.

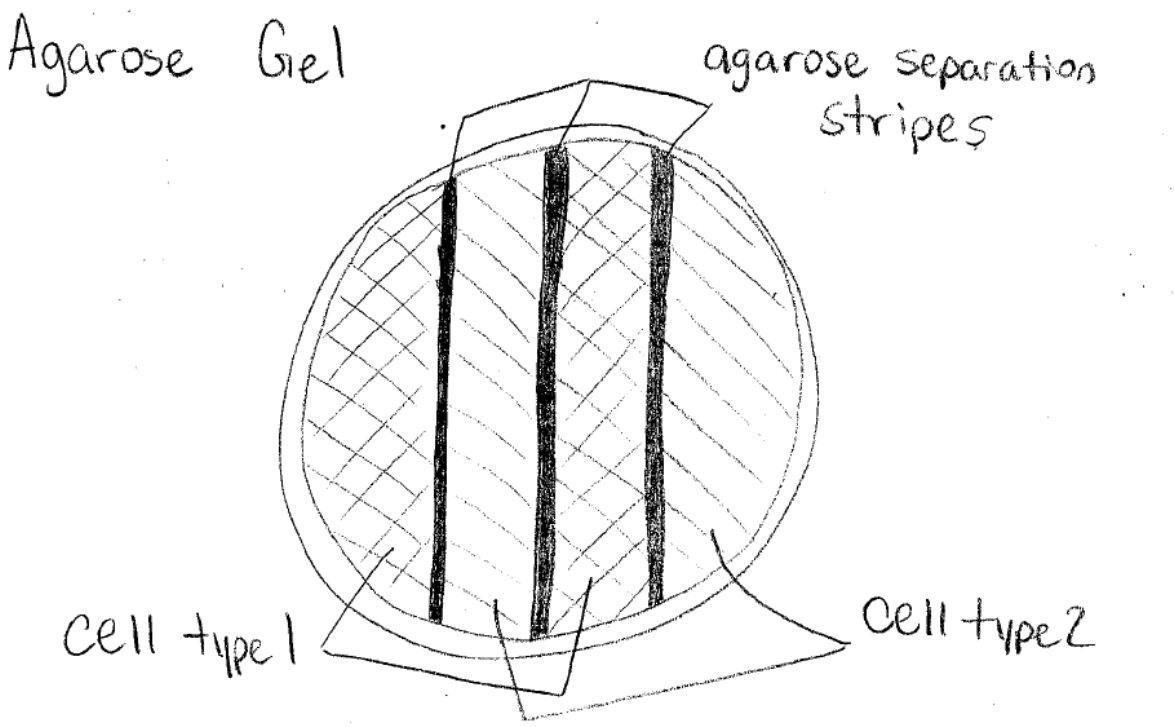


Figure 10: Conceptual design idea using agarose gel

Another conceptual design utilizes the cloning cylinder, also known as the cloning ring, to produce a co-culture system. The purpose of the cell cloning cylinder is to allow researchers to remove small colonies of cells that have grown in Petri dishes (McFarland, 2000). Although they are effective for separating cells, the cloning rings must be used with sterile vacuum grease in order to contain the media and cells to one specific area. The vacuum grease is not easily removed from the culture dish once it has been applied, so another method must be utilized for the design of this experiment. We propose using a ring made from polydimethyl siloxane (PDMS) to create a pseudo—cloning ring. PDMS is a polymer that has been shown to create a seal with cell culture dishes (Folch, 2000). The PDMS ring would allow for easy adherence to the culture dish without the addition of vacuum grease, while also allowing the cells to

remain separated by the barrier that the ring provides. A sketch of this design idea can be seen in Figure 11 below.

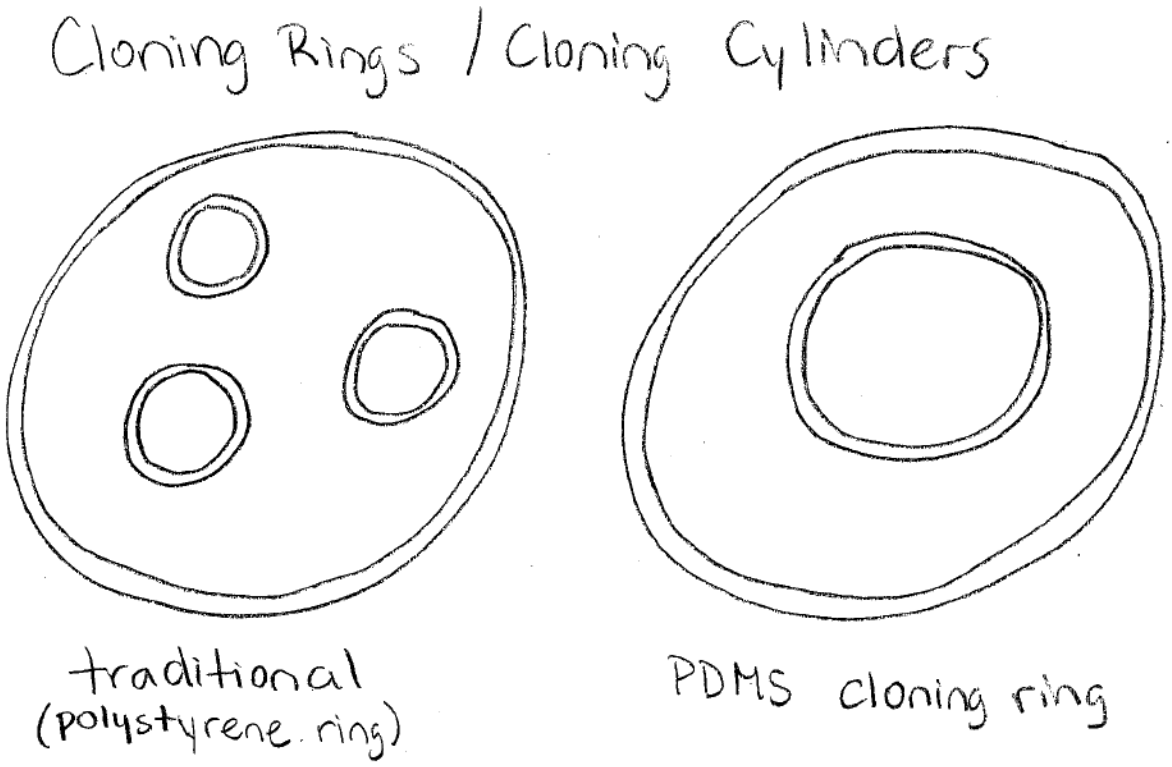


Figure 11: Conceptual design idea using cloning rings (traditional and PDMS)

The fourth design alternative generated focuses on creating a stencil that can be adhered to the surface of the cell plate and then removed once the cells have adhered. The stencil would be made of PDMS, which will create a seal with the culture dish. The stencil would be a wafer that is fitted to the bottom of the culture dish and a pattern could be cut out of it. This pattern would create individual wells for the cell types, as seen in Figure 12. The stem cells and cardiac myocytes would be plated in their respective wells. The stencil would be made thick enough to prevent the media from entering neighboring wells. Then, after the cells have adhered to the surface, the PDMS

stencil would be removed. It has been demonstrated that PDMS stencils can be removed without damaging the surrounding cells (Folch, 2000).

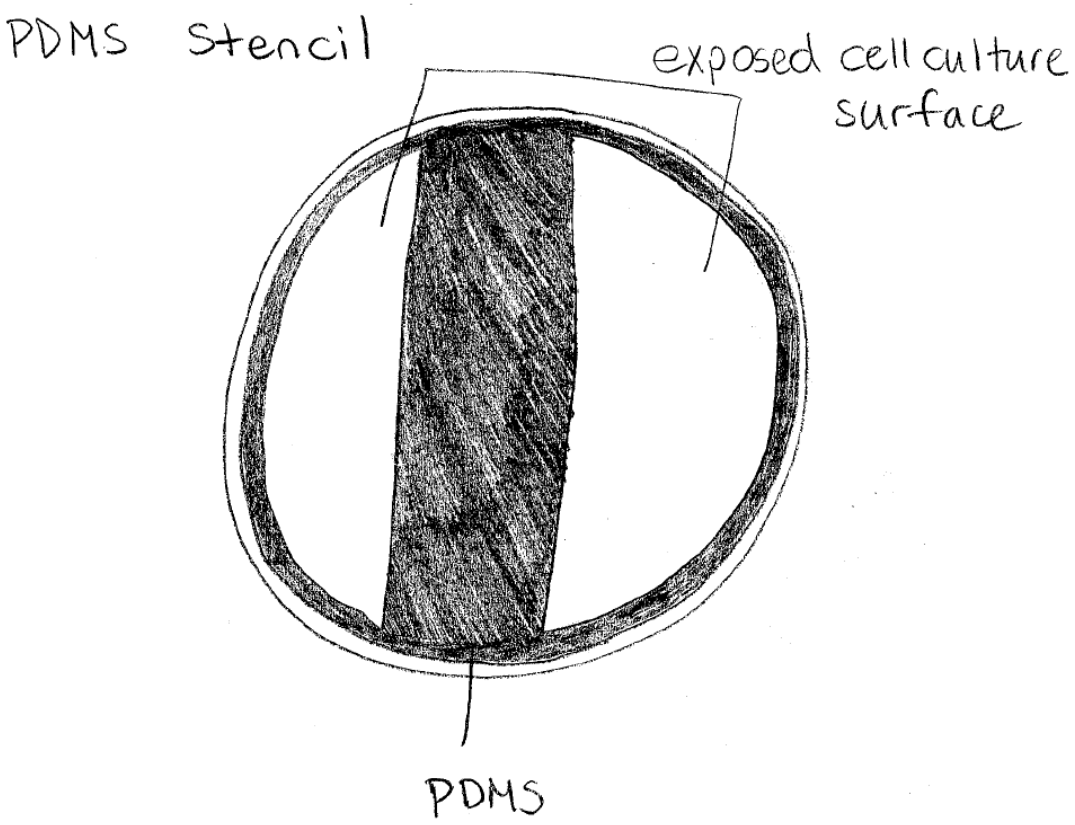


Figure 12: Conceptual design idea using PDMS stencil

The final design alternative involves PDMS stamping on gold slides. In a study by Heller et al., neural cells were patterned using PDMS microcontact printing on gold slides (Heller et al., 2004). We reasoned that a similar process could be used to pattern a gold slide for our co-culture system. The team spoke with Jonathan Charest, a student from Worcester Polytechnic Institute who spent the summer of 2007 developing a protocol for PDMS stamping on gold slides. We would need to design our own PDMS stamp, as can be seen in Figure 13. The square parts would extrude from the surface, which is indicated by the darker region. According to Jonathan's protocol, the stamp is

coated with dodecanethiol and is pressed onto the slide and removed after 30 seconds. Then the slide is coated with (1-mercaptoundec-11-yl)tri(ethylene glycol) (EG3), which binds to the surface of the slide that is not coated in the dodecanethiol. The slide can then be coated with fibronectin, an extracellular matrix protein that is commonly used for cell adhesion (Ogawa, 2000). The fibronectin only adheres to the dodecanethiol, so the result is a pattern similar to Figure 13 where the squares are coated with fibronectin and the darker regions are coated with EG3. The EG3 is extremely hydrophobic, so the cells will not migrate onto the EG3 and should stay in their individual regions. The cells could then be plated with myocytes on the top row and stem cells in the bottom region.

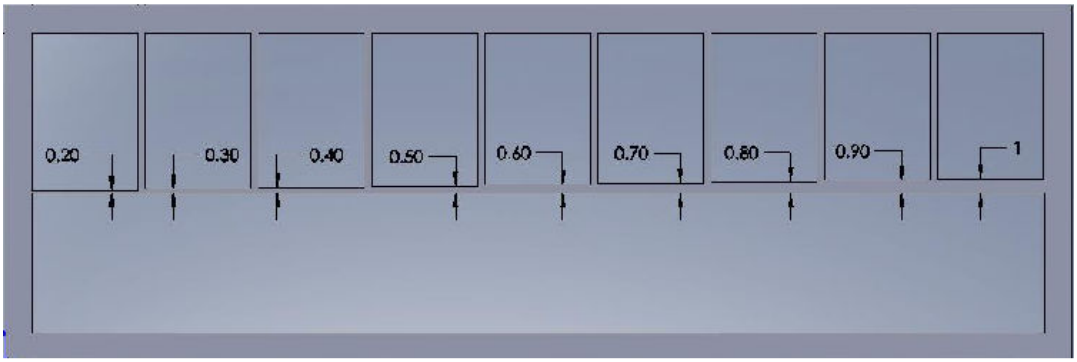


Figure 13: Conceptual design idea for a PDMS stamp to be used on a gold slide.

4.3.5 Design Matrix for Design Alternatives

To decide which of the design alternatives for the cell culture system the team would use, a design matrix was generated to evaluate the alternatives. The alternatives were evaluated using the ranking criteria that can be found in Table 12. The agarose design was ranked the highest, as can be seen in Table 13.

Table 12: Ranking criteria for choosing the design alternative

Ranking Criteria											
Design Constraints	**Yes**	**No**									
C. Size	Will fit into the incubator	Will not fit into the incubator									
C. Sterile	All materials can be sterilized	Some materials can not be sterilized									
C. Migration	Prevents cells from migrating into different	Cells can migrate into different regions									
Design Objectives	**0.1**	**1**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1**	
O. Repeatable	No	Yes	Media flow very hindered				Media flow somewhat hindered			Media flows freely between regions	
O. Safe for Cells	No	Yes	uses 500,000 - 750,000 cells	uses 250,000-500,000 cells	uses 100,000-250,000 cells	uses 75,000-100,000 cells	uses 50,000-75,000 cells	uses 25,000 - 50,000 cells	uses 10,000-25,000 cells	uses 0-10,000 cells	
O. Safe for Users	No	Yes	40-45ml	35-40ml	30-35ml	25-30ml	20-25ml	15-20ml	10-15ml	less than 10 ml	
O. Effective	**0.1**	**0.2**	8	7	6	5	4	Uses 3 tools	Uses 2 tools	Uses only one tool	
O. Minimize Materials: Cells	Media can not flow between regions	uses 750,000-1million cells								Within hood	
O. Minimize Materials: Media	uses more than 1 million cells	45-50ml	6-7 hrs	5-6 hrs	4-5 hrs	3-4 hrs	2-3 hrs	1-2 hrs	30 min - 1 hr	0-30 min	
O. Minimize Materials: Equipment	50+ ml	9			Moderately hard to understand process					Easy to understand process	
O. Ease of Use: Size	10+ tools	9 steps	8 steps	7 steps	6 steps	5 steps	4 steps	3 steps	2 steps	1 step	
O. Ease of Use: Time	Outside of hood										
O. Ease of Use: Interface	8+ hrs										
O. Ease of Use: Simplicity	Hard to understand process										

Table 13: Design Matrix including rankings for choosing from the design alternatives

Design Alternatives											
Design Constraints		**Verticle Cover Slip**		**Agarose Gel**		**PDMS Cloning Ring**		**PDMS Stencil**		**PDMS Stamping on Gold Slides**	
C. Size		Y		Y		Y		Y		Y	
C. Sterile		Y		Y		Y		Y		Y	
C. Migration		N		Y		Y		N		Y	
Total		2		3		3		2		3	
Design Objectives	**Weight (%)**										
O. Repeatable	13.7			1	13.7	1	13.7			1	13.7
O. Safe for Cells	11.75			1	11.75	1	11.75			1	11.75
O. Safe for Users	11.75			1	11.75	1	11.75			1	11.75
O. Effective	19.3			0.7	13.51	0.1	1.93			1	19.3
O. Minimize Materials: Cells	7.2			0.7	5.04	0.7	5.04			0.7	5.04
O. Minimize Materials: Media	9			0.9	8.1	0.9	8.1			0.9	8.1
O. Minimize Materials: Equipment	5.3			0.9	4.77	0.9	4.77			0.5	2.65
O. Ease of Use: Size	1.6			1	1.6	1	1.6			1	1.6
O. Ease of Use: Time	2			0.9	1.8	1	2			0.5	1
O. Ease of Use: Interface	2.7			1	2.7	1	2.7			0.1	0.27
O. Ease of Use: Simplicity	2.7			0.7	1.89	0.8	2.16			0.1	0.27
Total	**87**				**76.61**		**65.5**				**75.43**

4.4 Preliminary Design

The design for co-culturing cardiac myocytes with hMSC's was a three-step process, as can be seen in Figure 14. First, the team needed to isolate cardiac myocytes from an animal source. Then the co-culture took place in a system that was designed by the team. The final step involved analyzing the co-culture to determine if cell cycle markers were expressed by the myocytes.

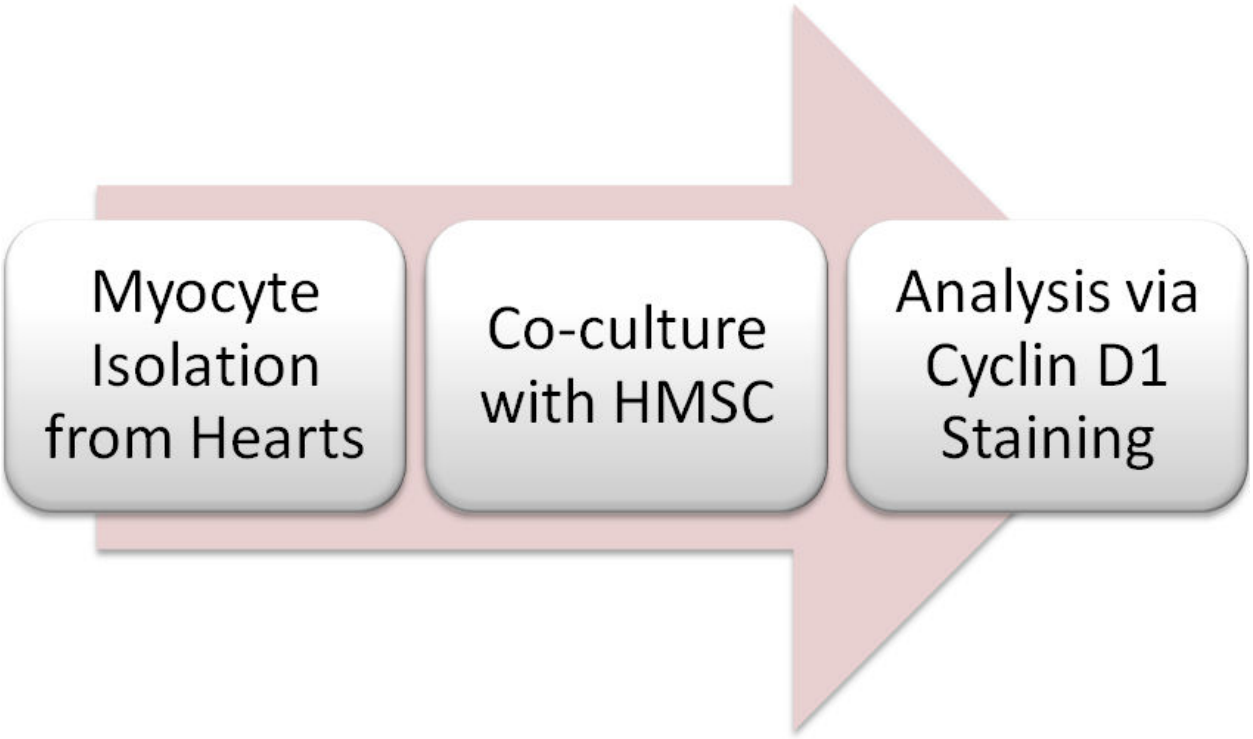


Figure 14: Three-step process for the design

4.4.1 Preliminary Cardiac Myocyte Isolation Procedure

The first step in our design was isolating the cardiac myocytes from the animal source. The design matrix for choosing the species (Table 4) resulted in using rats, however a graduate student at Worcester Polytechnic Institute, Christine Lima, was willing to donate mice for our use. Since mouse was only slightly lower ranked than the rat in the design matrix and the donation would save money, the team determined that this would be acceptable. The mice that were used were 2 to 3 month old female CF-1 mice and were euthanized via CO_2 asphyxiation. Victoria Huntress, a biology staff member at Worcester Polytechnic Institute, showed the team how to extract the heart from the mouse. The hearts were extracted from the mice within 10 minutes of their death. The hearts were rinsed three times in a Tyrode's solution. The Tyrode's solution

was made following the protocol provided in an article from the First Hospital of Xi 'an Jiaotong University (Wang et al., 2006).

It was determined through the isolation design matrix (Table 8) that the cardiac myocyte isolations would be done using enzymes. A protocol written by Joan Zuckerman from the State University of New York at Stony Brook that isolates myocytes from mouse hearts (see Appendix D: Mouse Myocyte Isolation Protocol) uses an enzyme called Blendzyme 4. This was the enzyme that was chosen by the team. We determined that in order to use the enzyme, the heart tissue would need to be broken down into small pieces to increase the surface area and there would need to be some physical agitation to help the enzyme to work the most efficiently. We decided to use a scalpel to mince the tissue into small pieces and to triturate the tissue while it is in the Blendzyme solution. Following the trituration, the solution was spun twice at 1000 rotations per minute (rpms) and the pellet was resuspended in a KB solution after both spins. The KB solution was generated by combining the protocols provided by the Wang et al. article and the protocol provided by Joan Zuckerman.

Following the isolations, the myocytes were plated in KB for four hours. The KB was then replaced with DMEM containing 1% penicillin-streptomycin. The media was replaced with DMEM containing 1% penicillin-streptomycin and 5% fetal bovine serum the following day.

4.4.2 Preliminary Cell Culture Design

The second step of the design was the co-culture system developed to allow the myocytes to culture with HMSC's. The design matrix for choosing the design alternative indicated that our design should use agarose as a physical barrier between the cell

types. The agarose was laid in a strip down the center of a cover slip using the process described below:

First, a PDMS mold was prepared by making a 10:1 mixture of silicone elastomer base: silicone elastomer curing agent. This mixture was poured into a 35mm diameter Petri dish to a thickness of 3-5 mm. The bubbles were removed by placing the dish into a vacuum for 20 minutes. A 22x22 mm cover slip was placed vertically into the PDMS as can be seen in Figure 15. The Petri dish was placed in a 60°C oven for 1 to 2 hours to cure the PDMS.

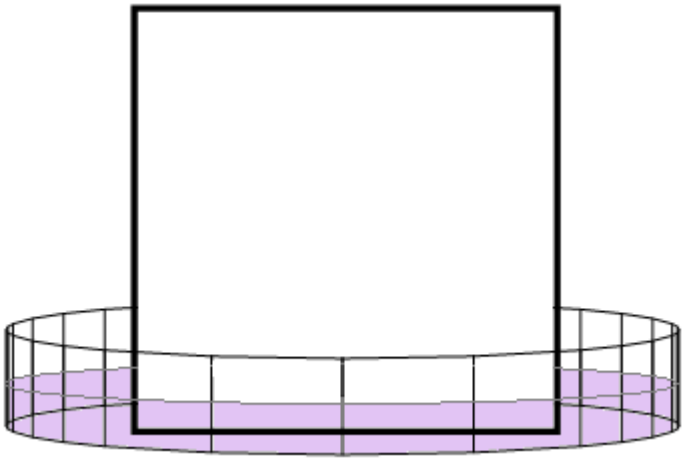


Figure 15: The PDMS mold with vertical cover slip

After the PDMS cured, the cover slip was removed revealing a slit that went completely through the mold. The mold was then placed in a fresh Petri dish on top of a 22x22mm cover slip. The slit in the PDMS was lined up to exactly match the width of the cover slip as can be seen in Figure 16. A 1% agarose in PBS solution was heated to 90°C and pipetted into the slit. After the agarose set and gelled, the PDMS mold was removed. This revealed a line of agarose down the center of the cover slip as can be seen in Figure 17.

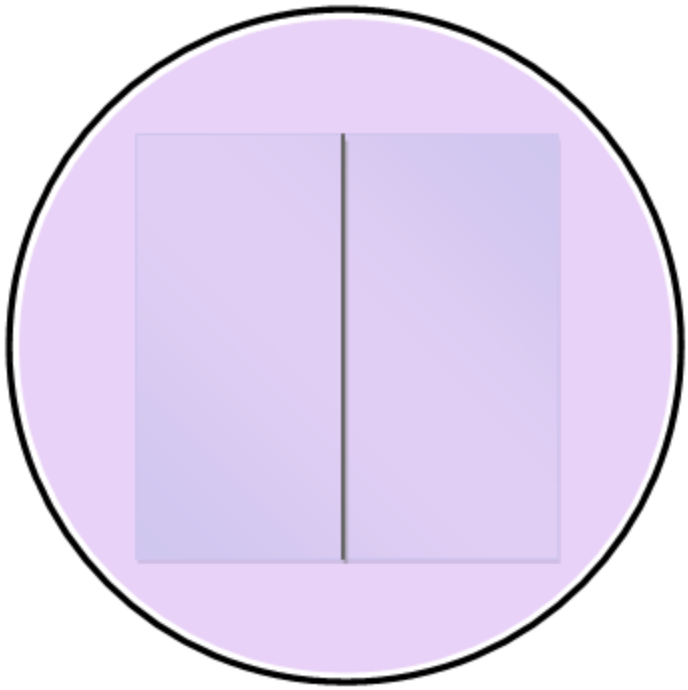


Figure 16: The PDMS mold placed on top of a cover slip

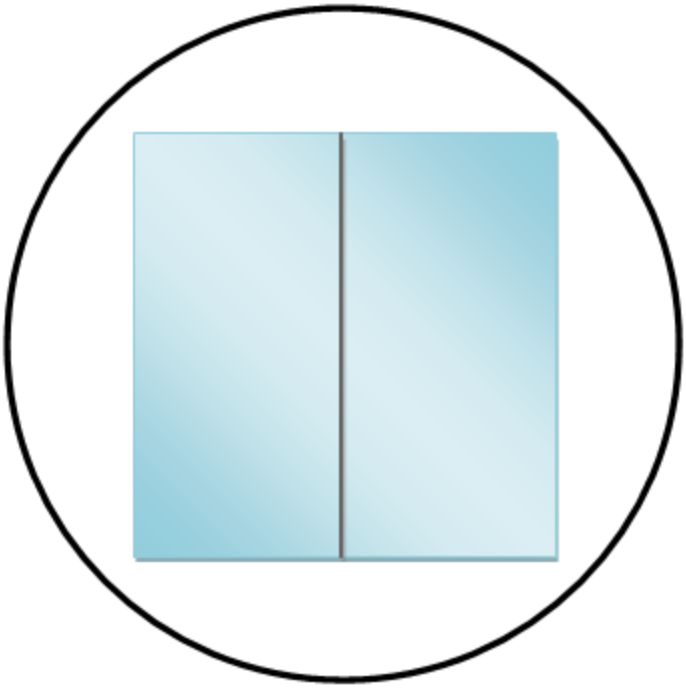


Figure 17: PDMS mold removed, leaving a line of agarose down center of cover slip

This system allowed for myocytes to be plated on one side of the agarose line and HMSCs to be plated on the other. We were able to vary the width of the line by adding multiple cover slips into the PDMS while it was setting. In addition, after the culturing, the cover slip could be removed and analyzed on a slide. In practicality, however, the line was not homogeneous. The variation in widths along the line was noticeable with the naked eye. Furthermore, the line never went all the way to the edges of the cover slip. This means that the HMSC's would have been able to migrate into the myocyte population. This led us to our final design, which is described in Section 4.5.2 Final Cell Culture Design.

4.4.3 Preliminary Analysis Procedure

The third portion of the design is the analysis procedure. In order to show that the myocytes began expressing cell cycle markers, the team decided to stain the cells for a specific marker, Cyclin D1. The myocytes also needed to be stained for α-actinin, and a Hoescht nuclear dye needed to be used to identify cell nuclei. All of the myocytes were stained with the Hoescht nuclear dye, while half of those had the additional Cyclin D1 stain and the other half had the additional α-actinin stain. After the staining procedure was complete, the myocytes were examined with a microscope and images were taken.

4.5 Final Design

The trials with the preliminary design revealed the weaknesses of the design. The team reevaluated the processes used and developed the final design. The final

design uses the same concepts as the preliminary, but it was able to fix the gaps that were discovered during the initial testing.

4.5.1 Final Cardiac Myocyte Isolation Procedure

The preliminary isolation procedure was only modified slightly. The procedure was adjusted so that the trituration would be completed using a water jacket at 37°C. This ensured that the Blendzyme would be more efficient than at room temperature. We added a second trituration to the procedure as well. The first trituration would be done in the Tyrode's solution containing Blendzyme. After the cell layer was removed to be spun, KB was added to the remaining tissue and the tissue was triturated again. The results from the Tyrode's trituration were labeled H1 (H for heart) and the results from the KB trituration were labeled H2. Also, many miscellaneous substances were in our end product, so the second spin cycle was decreased to half the speed (500 rpms) in an attempt to remove the unwanted material. The first spin cycle was maintained at the original speed (1000 rpms) to ensure that we did not lose any of the yield from the solution. For details on the final procedure and the materials used, see the protocol in Section 5.4.

4.5.2 Final Cell Culture Design

The preliminary cell culture design yielded an agarose barrier that was not uniform in thickness and did not reach the edges of the cover slip. This meant that the hMSCs would be able to migrate around the barrier. A new method for laying down an agarose barrier was developed. Through experimentation, the team discovered that by using a micro-capillary pipette and depressing the plunger at a steady rate, an agarose

line could be laid down that was far more homogenous than using the PDMS mold approach. The agarose was changed to a 2% solution. The cover slip on the bottom of the Petri dish was still used to aid with the staining process. The final design involved using the micro-capillary pipette to lay down a line of agarose that completely bisected a 22x22 mm cover slip on the bottom of a 35mm Petri dish. In order to be sure that the thickness of the agarose line was sufficient, we use bright field microscopy to analyze three different dishes. Each of the lines had a similar thickness which measured to be approximately 370 μm, which is above the 200 μm needed. A representative image can be seen in Figure 18 below. A cartoon and image of the entire design can be seen in Figure 19 and Figure 20, respectively.

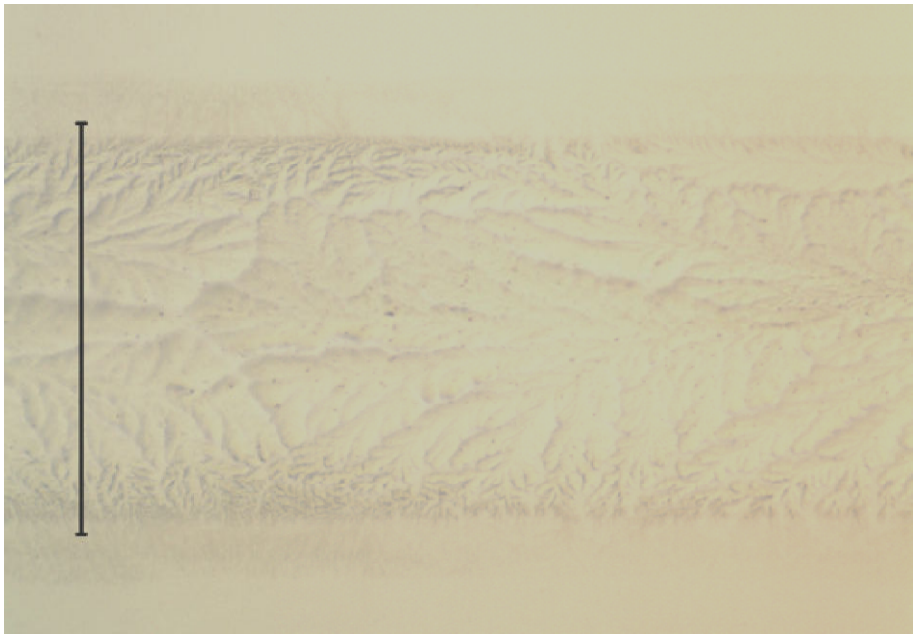


Figure 18: Agarose Barrier (scale bar = 400 microns)

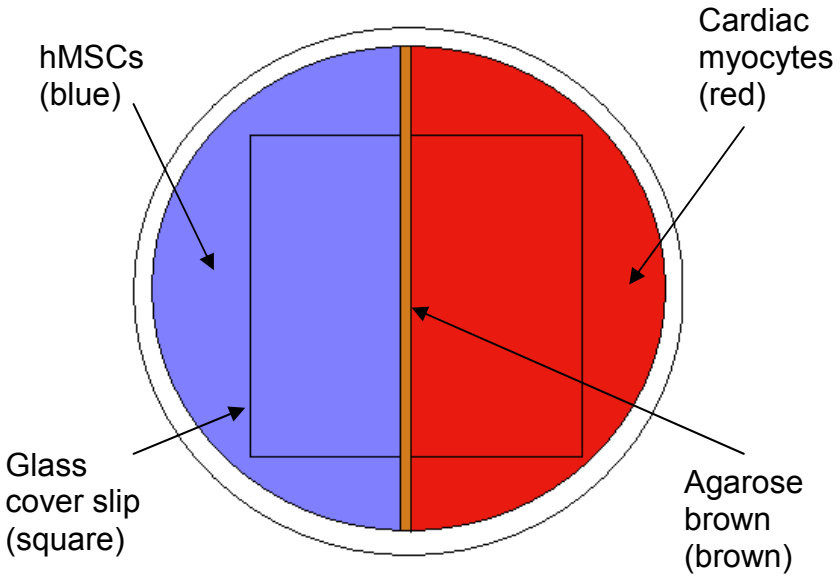


Figure 19: Agarose Design Cartoon

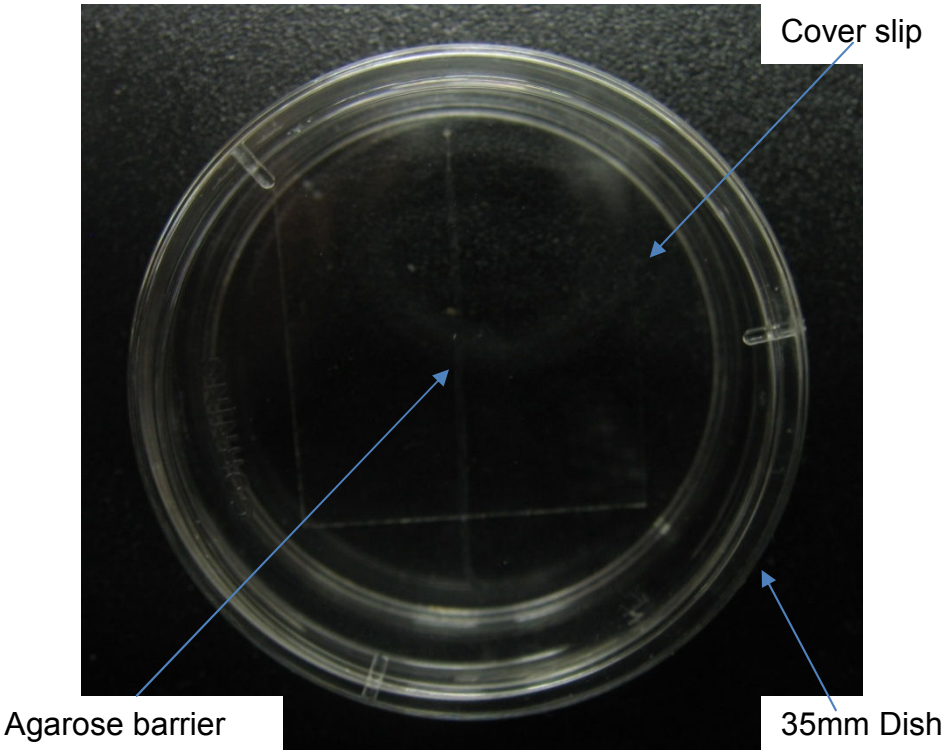


Figure 20: Agarose Design Image

In preparation for the myocyte isolations and the co-cultures, we prepared fourteen Petri dishes with agarose barriers. Ten dishes were plated with quantum-dot loaded hMSCs on one side of the barrier the day before the myocyte isolations, and two of those were set aside as the hMSC controls. The hMSCs were quantum-dot loaded so that we would be able to distinguish between hMSCs and myocytes during the analysis procedure. After the myocyte isolation, the remaining eight hMSC plated dishes had myocytes plated on the opposite side of the barrier, four from H1 and four from H2. The remaining four dishes that were not plated with hMSCs were plated with only myocytes, two from H1 and two from H2. These were used as the myocyte controls. For details on the final procedure and the materials used, see the protocol in Sections 5.4 and 5.5.

4.5.3 Final Analysis Procedure

For the final analysis procedure, the team decided to stain for two different proteins. The first is cyclin D1, which is found in cells that have entered the cell cycle, indicating the potential for mitosis. The other protein, α-actinin, is found only in cells that are cardiac-derived. The purpose of using this stain is to show that the myocytes are still expressing a cardiac marker during the cell culture process. For details on the final procedure and the materials used, see the protocol in Section 5.6.2.

5. Methodology

This section contains detailed descriptions of the methods used during the project. These methods include: cell culture, cardiac myocyte isolation, and making agarose barrier lines in plates/wells. Methods for analyzing data will also be discussed.

5.1 Overview

There were several important goals that were essential for the project to demonstrate the effectiveness of the design. First, it was essential that the team learn how to culture, passage, and feed cells using sterile techniques. Also, it was necessary to learn the process of isolating cardiac myocytes from fresh mouse hearts. The third goal was to be able to reproducibly create uniform agarose barriers on cover slips within culture dishes. Finally, it was important to develop an analysis method to demonstrate the effect of the co-culture on the different cell populations. The strategy for achieving each goal is described in the sections that follow.

5.2 Maintenance of Cells in Culture

The research performed as part of this project involved the use of human mesenchymal stem cells (Lonza Group Ltd.) as the cells to be co-cultured with mouse cardiac myocytes. In order to have a set of hMSCs ready to be used for the co-culture experiments, it was important to maintain a line of hMSCs in culture for the duration of the project. The following are protocols for feeding and passaging cells. The feeding protocol also contains a step regarding quantum dot loading in hMSCs, with the quantum dots being used to identify hMSCs in culture.

5.2.1 Feeding Cells

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)
- 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 5ml 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 make sure cells look healthy.
7. Place flask in incubator. Clean the biosafety cabinet with ethanol.
8. In order to load quantum dots into hMSCs, it is necessary to feed the cells with medium containing quantum dots. For this, 10µl of QuantumDot 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.

5.2.2 Passaging Cells

Materials:

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

Procedure:

1. Place trypsin and media in water bath.

2. Remove the desired T-75 flask (with cells) from the incubator and place in cell culture hood.
3. Place trypsin and media in cell culture hood.
4. Aspirate off the media using a sterile glass Pasteur pipette.
5. Using a pipette, remove 5ml of trypsin from aliquot and add to the T-75 flask.
6. Place the T-75 back in the incubator for 5-10 minutes. Check the flask to make sure that the majority of cells have lifted.
7. Add 5ml media to the flask.
8. Using a pipette, remove the entire cell suspension and place in a new 15ml conical tube.
9. Centrifuge the cells (be sure to use a counter balance). Centrifuge at 800 rpm for 5 minutes.
10. Aspirate the supernatant (media and trypsin) using a sterile glass Pasteur pipette. Be sure not to disturb the cell pellet.
11. Re-suspend the cell pellet with a known amount of media (2ml).
12. Remove 10µl of the cell suspension, combine in an eppendorf tube with 90µl of trypan blue stain (50 µl trypan blue + 40 µl PBS).
13. Mix the cells / trypan blue well using the micropipette.
14. Remove 10µl of the cells / trypan blue and place in the hemocytometer.
15. Count the cells using the hemocytometer.
    Cells/ml = (#cells/#squares) x 10 (dilution) x 10^4
16. Plate the cells with the desired concentration in a new T-75 flask.
17. Add fresh media to the T-75 to achieve the desired final amount of media (usually about 10-15ml).
18. Write necessary information on the new T-75 (cell type, passage number, date, initials, cell plating concentration), place in incubator.
19. Dispose of the old T-75 flask in the biohazard bag.
20. Place media back in refrigerator, clean the cell culture hood, turn on UV assuming no other experiments are in hood.

5.3 Making the Agarose Barrier Line

In order to effectively utilize the agarose, it was essential to first try making different concentrations of agarose in order to observe the concentration that would yield the best degree of firmness for the experiments. Figure 21 and Figure 22 show the process of making the agarose solution, and Figure 23 shows the process of making the agarose barrier using the microcapillary pipet.

Figure 21: Making Agarose Solution

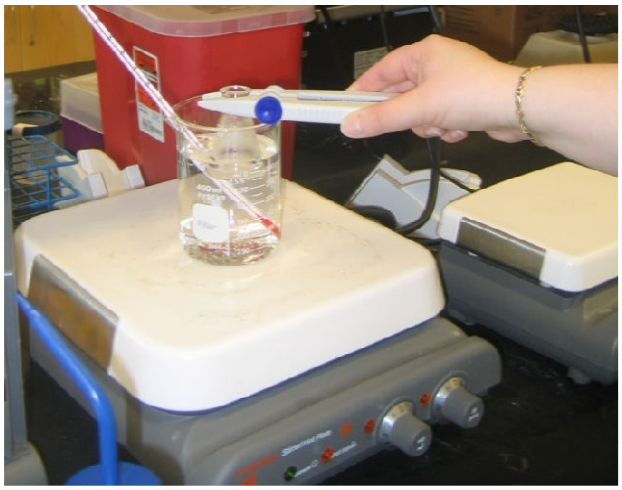


Figure 22: Warming Agarose

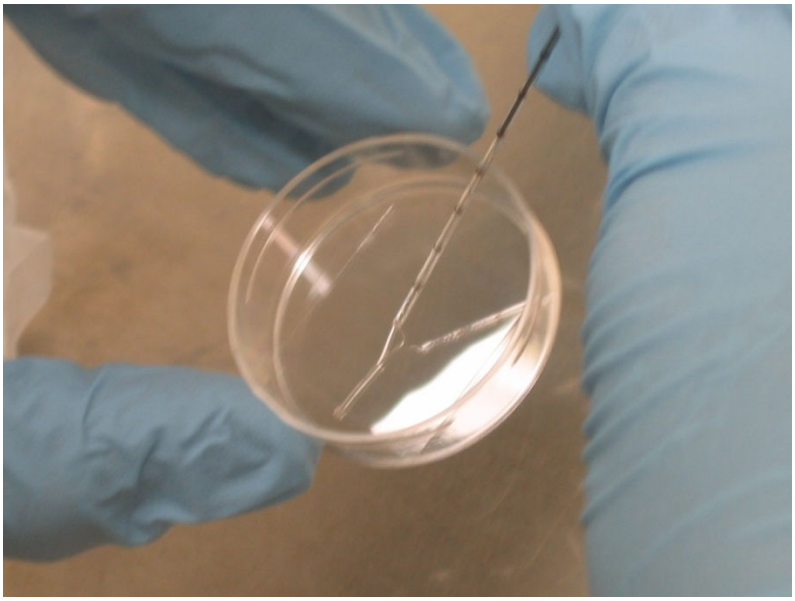


Figure 23: Making Agarose Barrier

Agarose was poured into a round mold, allowed to set, and removed for observation. Agarose concentrations of 1%, 2%, 4%, 6%, 8%, and 10% were used. Images from these preliminary tests can be found in Appendix F: Making Agarose. The final concentration of 2% was chosen because it stays liquid long enough to work with easily, yet stays firm when formed into a mold or line. The protocol for making the agarose barriers on cover slips can be found below.

Materials

- Agarose-HGT (USB, cat. 32805)
- PBS (Fluka Biochemika, Cat. # 79382)
- Serological pipette
- Microcapillary pipette (Stratagene, 10µl volume)
- Pipetter
- Hot plate
- Thermometer
- Tongs
- 250mL beaker of water
- Precision metric scale
- Weighing paper
- Spatula
- 25mL beaker
- Mold
- Autoclavable bottle
- Cell culture materials (well plate and/or petri dishes)
- Biosafety cabinet

1. Put beaker of water (at least 80% full) on the hot plate. Insert the thermometer and let the water heat to 90°C.
2. Make a 2% agarose solution by combining 0.2g of agarose per 10ml PBS. Use the pipettor with the serological pipette to measure out the desired amount of PBS into the beaker. Weigh the amount of agarose needed and combine the agarose and PBS in the ependorf flask. Gently swirl the flask to mix the solution
3. Use the tongs to hold the small beaker in the beaker of water. Swirl the flask occasionally and do not allow any water to get into the flask. You will know that the gel is ready when you can not see any particles remaining in the solution

(look directly down into the flask, looking from the side is inadequate). The gel should easily swirl and should be slightly clearer than the original mixture.

4. Pour the agarose solution into an autoclavable bottle that is significantly larger than the volume of agarose. Autoclave the agarose using a liquid cycle.

The following steps occur in the biosafety cabinet

5. Reheat the agarose in the cell culture hood using a hot plate. Transfer a small amount of the agarose from the large bottle to a smaller container, such as a small beaker.
6. Use a microcapillary pipet to draw up 10µl of agarose. Hold for 10 seconds to allow the agarose to gel.
7. Expel the agarose gel slowly and continuously onto the glass cover slip in the Petri dish. Be sure to keep the line as uniform as possible until you reach the other side. Dispose of excess agaose left in microcapillary pipette.
8. Using the microcapillary pipet, quickly draw up more agarose and quickly expel onto the ends of the agarose line you just created. This is done to ensure that the agarose meets the side of the dish, and provides a full barrier between the different cell types that will be plated.
9. Make more wells/dishes as necessary for the experiments.
10. Seed hMSCs on one side of the cover slip the day before cardiomyocyte isolation. Allow the cells to sit for 4-6 hours, then remove the media and replace with fresh (cell-free) media. Repeat again before cardiomyocytes are plated. This is to keep the hMSCs on one side of the dish/well.

5.4 Cardiac Myocyte Isolation from CF-1 Mice

The isolation of cardiac myocytes from adult mouse hearts is a detailed process involving a series of steps that can take several hours to complete from the time of obtaining the hearts until the cells are plated. Although the procedure is long, the cardiac myocytes can be isolated and plated on cell culture surfaces. The procedure involves the use of an enzyme called Liberase Blendzyme (Roche Diagnostics, Cat # 11988468001). This enzyme contains collagenase and other enzymes in a specific blend used for tissue dissociation applications. The exact formulation is not published.

5.4.1 Making Tyrode's and KB Solutions

There are two solutions used during the cardiac myocyte isolation procedure. These solutions and others like them have been used in similar research with primary isolations (Wang et al., 2006). Their purpose is to keep the myocytes alive during the isolation and plating process and to allow for the cells to be gradually introduced into the cell culture environment.

Tyrode's solution is made by combining the ingredients listed below in the appropriate amounts. All ingredients are weighed and mixed with water. The solution is filtered into an autoclaved (sterile) bottle with a bottle top filter in a biosafety cabinet.

Table 14: Tyrode's Solution Formula

Tyrode's Solution	molecular weight	mM	g/L	g/500 mL	g/250 ml
NaCl	58.3	140	8.162	4.081	2.0405
KCl	74.5	5.4	0.4023	0.20115	0.100575
$MgCl_2$	95.1	1.8	0.17118	0.08559	0.042795
HEPES	238	10	2.38	1.19	0.595
Glucose	180	10	1.8	0.9	0.45

The KB solution involves a few more steps than the Tyrode's solution. All of the necessary ingredients are listed in the table below. All ingredients in Table 15: KB Solution Formula(EXCEPT ATP) are added into a flask with the appropriate amount of water. The entire solution is mixed on a stir plate/hotplate, with the heat turned on. The solution must be watched so that the solution temperature does not rise above 37ºC. The ingredients will not dissolve without the combined stirring/heat. Once the solution is clear, it is filtered using a bottle top filter into an autoclaved (sterile) bottle while in the biosafety cabinet. On the day of the isolation, the appropriate amount of ATP should be added to a small amount of KB (only what will be used that day). The KB+ATP solution should then be filtered using a syringe filter. The ATP is not stable in the KB solution for

more than a few days, so the ATP should be added as close to the isolation as possible (morning of isolation).

Table 15: KB Solution Formula

KB Solution	molecular weight	mM	g/L	g/500 mL	g/250 ml
KCl	74.5	25	1.8625	0.93125	0.465625
KH_2PO_4	136.1	10	1.361	0.6805	0.34025
$MgCl_2$	95.1	3	0.2853	0.14265	0.071325
Glucose	180	10	1.8	0.9	0.45
EGTA	380	0.5	0.19	0.095	0.0475
Taurine	125	20	2.5	1.25	0.625
L-glutamic acid	147	70	10.29	5.145	2.5725
HEPES	238	10	2.38	1.19	0.595
ATP	551.14	0.5	0.276	0.138	0.069

5.4.2 Setting Up

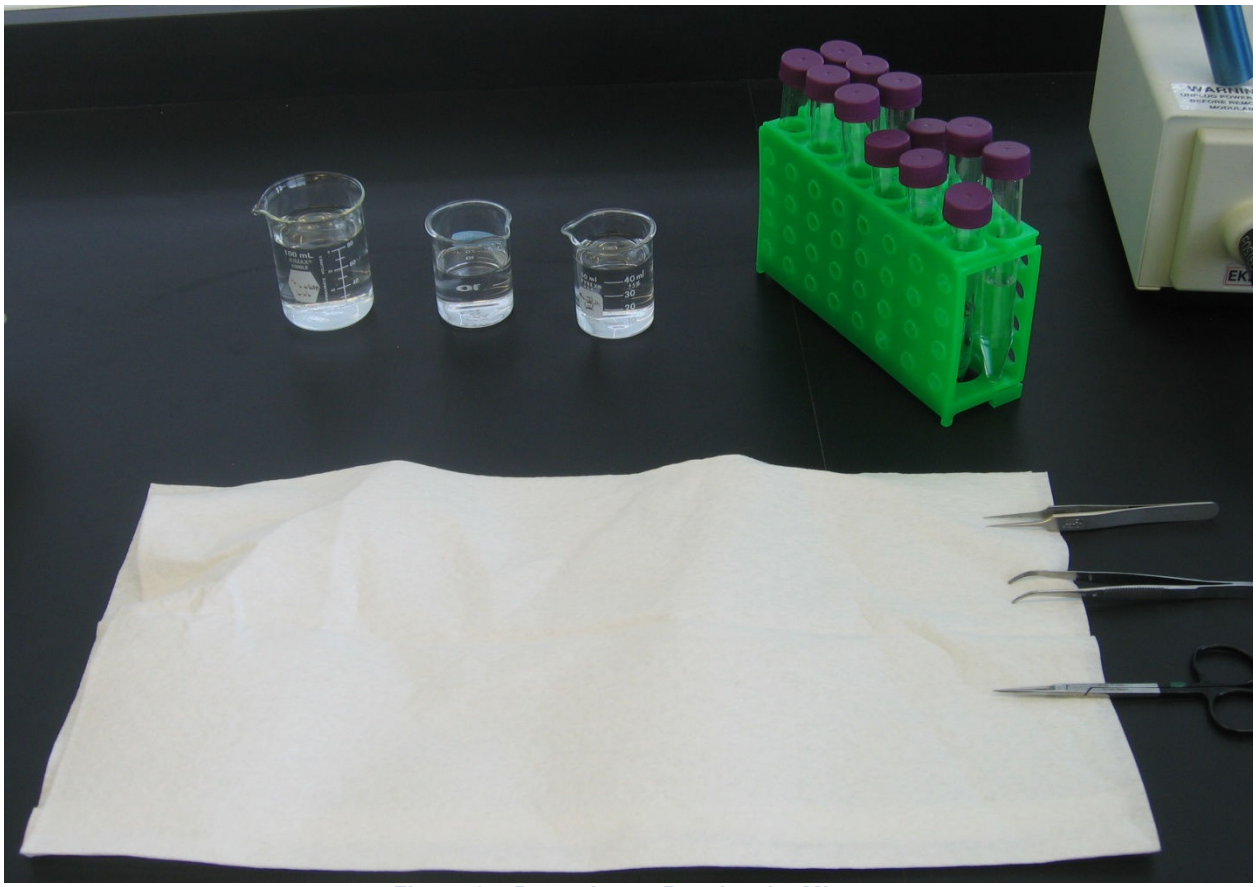


Figure 24: Preparing to Receive the Mice

Materials:

- Biosafety cabinet
- Water bath (consists of water pump and glass water jacket)
- Pipettor and serological pipettes (10ml and 25ml)
- Conical tubes (15ml and 50ml)
- Petri dishes
- Scapel (#10 blade)
- Forceps
- Micropipettors and tips
- Tyrode's solution, KB Solution
- Oxygen tank, regulator, and tubing
- Sterile syringe with syringe filters

Setting Up

1. Oxygenate Tyrode's solution in the biosafety cabinet using medical grade oxygen. Place sterile Tyrode's solution bottle into the hood. Spray tubing with ethanol and wipe down, immediately place one end in the Tyrode's solution. Turn on the oxygen flow, and allow the Tyrode's solution to oxygenate for 7 minutes. Repeat with sterile KB solution.
2. After oxygenation of Tyrode's solution, fill 15 ml conical tubes with 7-8ml of Tyrode's solution. Have 3 conical tubes per heart. Set aside another 35 ml of Tyrode's for later use.
3. After oxygenation of KB solution, remove 50-75 ml of KB and place in non-sterile bottle. Add ATP as necessary per volume of KB, mix. Sterilize the solution into new 50 ml conical tubes using a syringe and syringe filters.
4. Spray the glass water jacket with ethanol, wipe down, and place in biosafety cabinet. Be sure that the pump (placed outside of the hood) is filled to an adequate level (the water should be no more than a few inches from the top). Tube 1 should be connected from the outflow nozzle on the pump to the upper nozzle on the water jacket and tube 2 should be connected from the bottom nozzle on the water jacket to the inflow nozzle on the pump. Turn on the pump and set to 37°C.
5. Sterilize water using a syringe and syringe filter, placing water into inner bath of the water jacket. Fill the inner bath to roughly ¾ of the total volume.
6. Prepare the dissection area: fill three small beakers with DI water, ethanol, and PBS to clean tools between heart extractions. Obtain scissors and forceps. Place paper wiper on table for dissection surface. Have one 15mL conical tube with 7mL of sterile Tyrode's solution for each heart that will be extracted.
7. Prepare the biosafety cabinet (everything sterile): Have each of the following per heart – 2 small Petri dishes with lids, 2 conical tubes with 7mL of Tyrode's solution. Also, have scalpel, forceps, 20ml Tyrode's solution per 4 hearts (in 50 mL conical tube), pipettor, 25mL serological pipettes, 10ml serological pipettes, Pasteur pipettes for aspirating, waste bucket.

5.4.3 Extracting the Heart

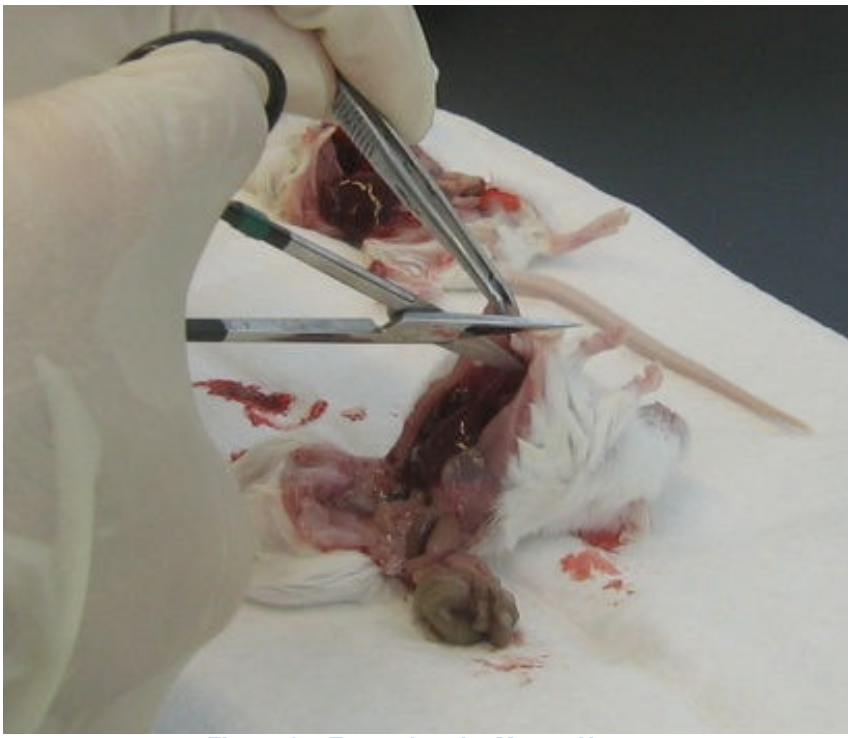


Figure 25: Extracting the Mouse Heart

Materials

- Mouse
- Spray bottle of ethanol
- Small beaker (50ml) of DI water
- Small beaker (50ml) of ethanol
- Small beaker (50ml) of PBS
- Gloves
- Mask
- Scissors
- Forceps
- 15mL conical tube with 7mL of Tyrode's solution (one per heart)

1. Be sure to wear a mask and gloves for this procedure. A lab partner will also be useful to ensure that the conical tube does not stay open for longer than necessary (see step 7). This entire process should be done as quickly as possible without damaging the heart.
2. Obtain a euthanized mouse and use the spray bottle of ethanol to spray the skin.
3. Use the scissors to cut through the skin, moving from the abdominal cavity upwards to the neck. Peel the skin back to move it out of the way (remove pieces if necessary).

4. Use the scissors to cut through the sternum, removing pieces if necessary, to expose the heart.
5. Gently grasp the apex with the forceps and lift it out of the chest cavity, being careful not to pull too hard.
6. Cut the connective tissue surrounding the heart until it comes free.
7. Have a lab partner open the conical tube of Tyrode's solution, drop the heart into the tube, and quickly cap. After recapping, keep the tube inverted so that the heart will not sit in / become caught in the small end of the conical tube.
8. If obtaining more hearts, clean the surgical tools by rinsing in the beakers of DI water, ethanol, and PBS in between mice.

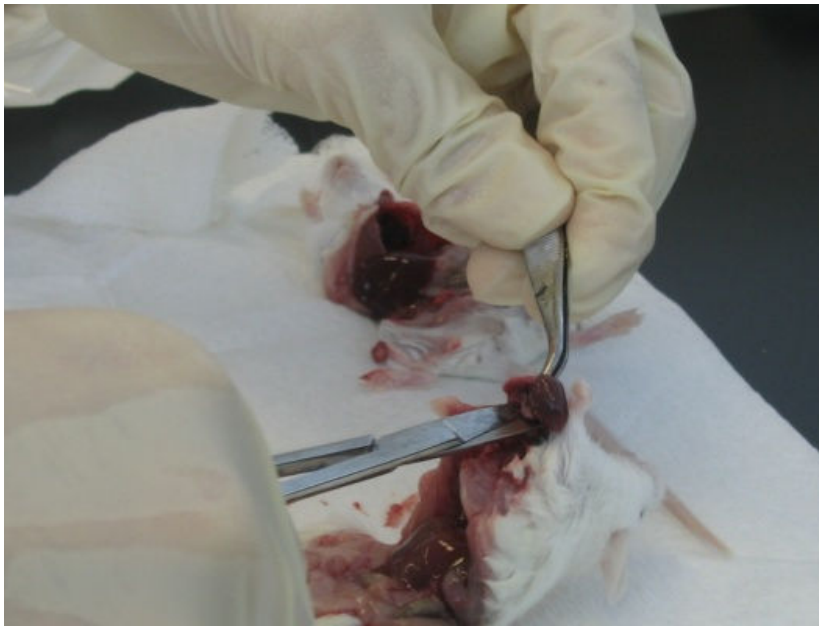


Figure 26: Retrieving the Heart

5.4.4 Dissociating the Tissue

Materials

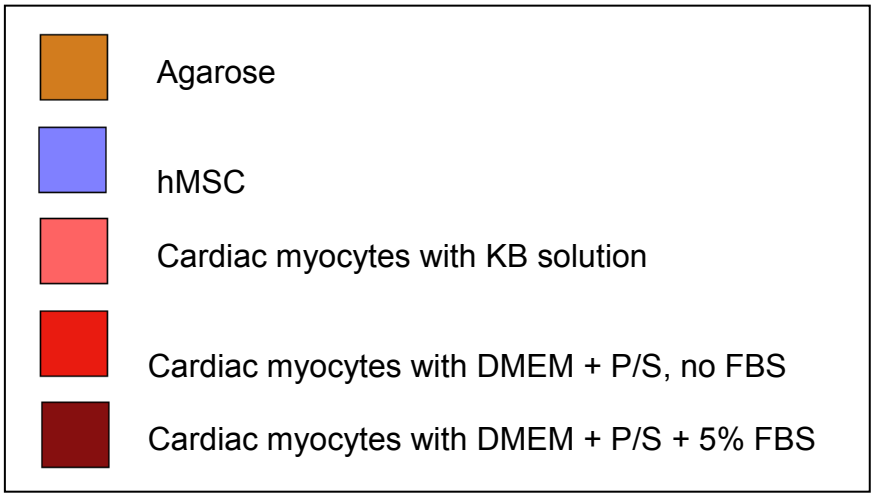
- Scalpel
- Forceps
- Petri dishes
- 10ml and 25 ml serological pipettes and pipettor
- Tyrode's solution
- KB solution
- Blendzyme (Liberase Blendzyme 4, Roche Diagnostics, Cat. # 11988468001)

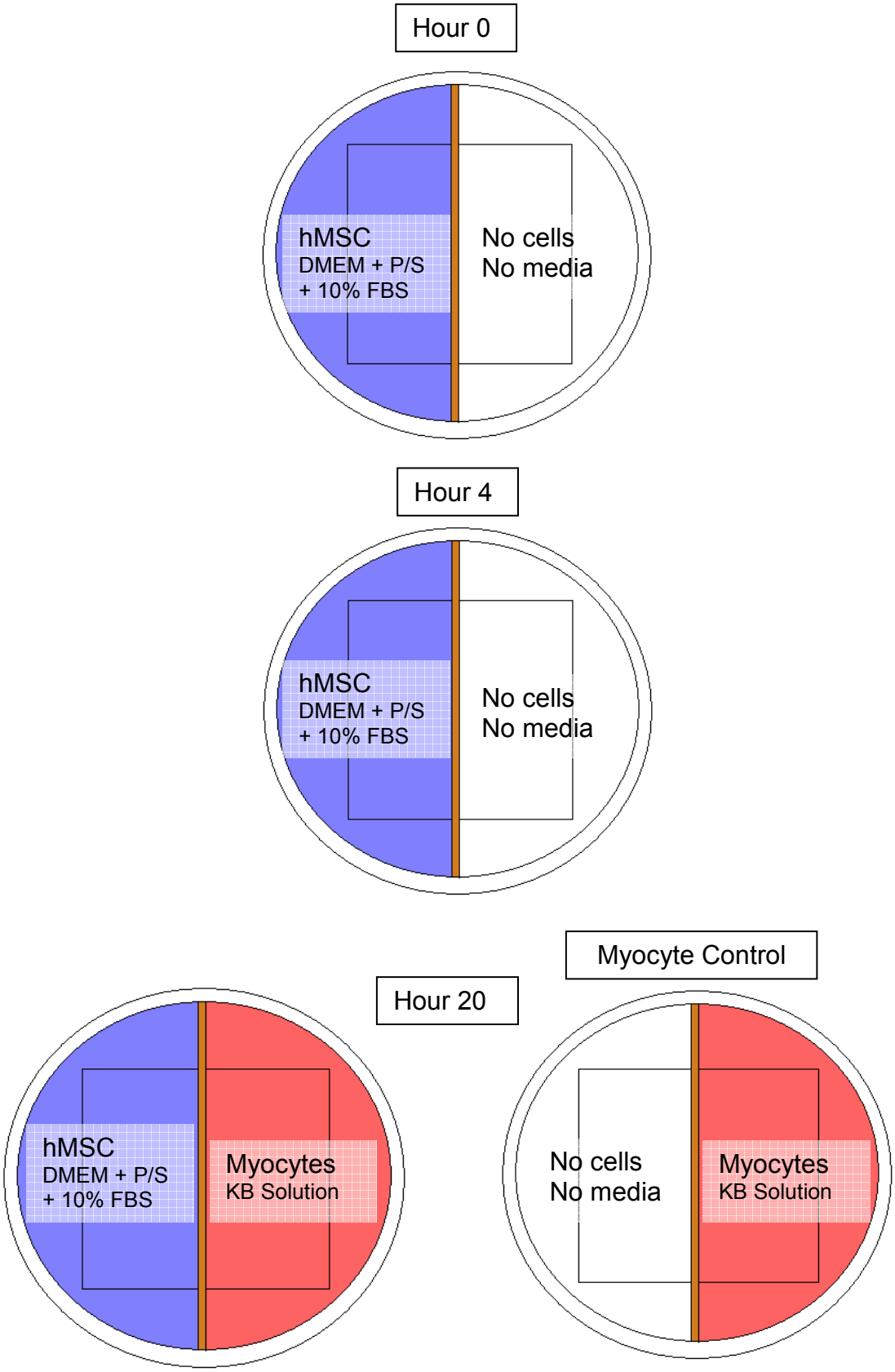
- 50 ml conical tube (empty)
- 15 ml conical tube with Tyrode's (2 per heart)
- 15 ml conical tube (empty, 3)

1. This procedure should be completed in a biosafety cabinet while wearing gloves. This should also be done as quickly as possible in order to obtain the best yield of viable cardiac myocytes.
2. Set up two Petri dishes with the lids acting as dishes (should have 4 dishes total). Gently invert the conical tube containing the heart several times, then pour the contents into the first dish. Use the forceps to lightly squeeze the heart to expel blood.
3. Transfer the heart into a 15mL conical tube containing 7mL of Tyrode's solution and cap. Gently invert the tube several times and pour the contents into the second dish.
4. Transfer the heart into a 15mL conical tube containing 7mL of Tyrode's solution and cap. Gently invert the tube several times and pour the contents into the third dish. Use the scalpel to cut away the atria and other tissue above. Gently squeeze the ventricles to expel any remaining blood.
5. Pour ~5mL of fresh Tyrodes into the last Petri dish. Transfer the ventricles into this fluid and mince the tissue. Repeat steps 2-5 for all hearts, mincing all ventricular tissue in one final dish.
6. Mix the aliquot of Blendzyme (200 µl) with 20mL of Tyrode's solution in a 50mL conical tube. Use the pipetter with a 25mL serological pipette to transfer the minced tissue and surrounding fluid into the Blendzyme solution. Triturate for 7 minutes with the conical tube in the water jacket at 37°C.
7. Allow the tissue to settle and transfer the liquid layer to a new 15mL conical tube (use more than one 15ml conical tube if necessary). Cap and label this tube H-1 (H for heart, 1 for first trituration). Spin the conical tube(s) in the centrifuge at 1000 rpm for 5 minutes.
8. You should still have a 50mL conical tube containing the minced tissue in a small amount of fluid. Add 10mL of the KB solution and triturate for 7 minutes. Allow the tissue to settle and transfer the liquid layer to a new 15mL conical tube. Cap and label this tube H-2 (H for heart, 2 for second trituration). Spin the conical tube in the centrifuge at 1000 rpm for 5 minutes.
9. From this point on H-1 and H-2 treated the same way. After spinning, there should be a pellet formed at the bottom of the conical tube. In the biosafety cabinet, aspirate the fluid layer without disturbing the pellet. Resuspend in 5mL of KB solution and spin again at 500 rpm for 5 minutes.

5.5 Plating hMSCs and Mouse Cardiac Myocytes in Co-culture

The following (Figure 27) is a series of images following the step-by-step process for co-culturing cardiac myocytes and hMSCs together. This process involves advanced plating of the hMSCs the day before cardiomyocyte isolation. Each image is marked with an approximate hour, starting from the time of initial hMSC plating. Each image also shows the type of media that is used for each cell type at any given time. Starting at hour 20, there are two dishes, one for the co-culture, and one for the myocyte control experiment. The last image shows the control dish in which hMSCs are plated at hour 0 and allowed to grow for the duration of the experiment with no cardiac myocytes added.




Hour 0
hMSC
DMEM + P/S
+ 10% FBS
No cells
No media
Hour 4
hMSC
DMEM + P/S
+ 10% FBS
No cells
No media
Hour 20
hMSC
DMEM + P/S
+ 10% FBS
Myocytes
KB Solution
Myocyte Control
No cells
No media
Myocytes
KB Solution

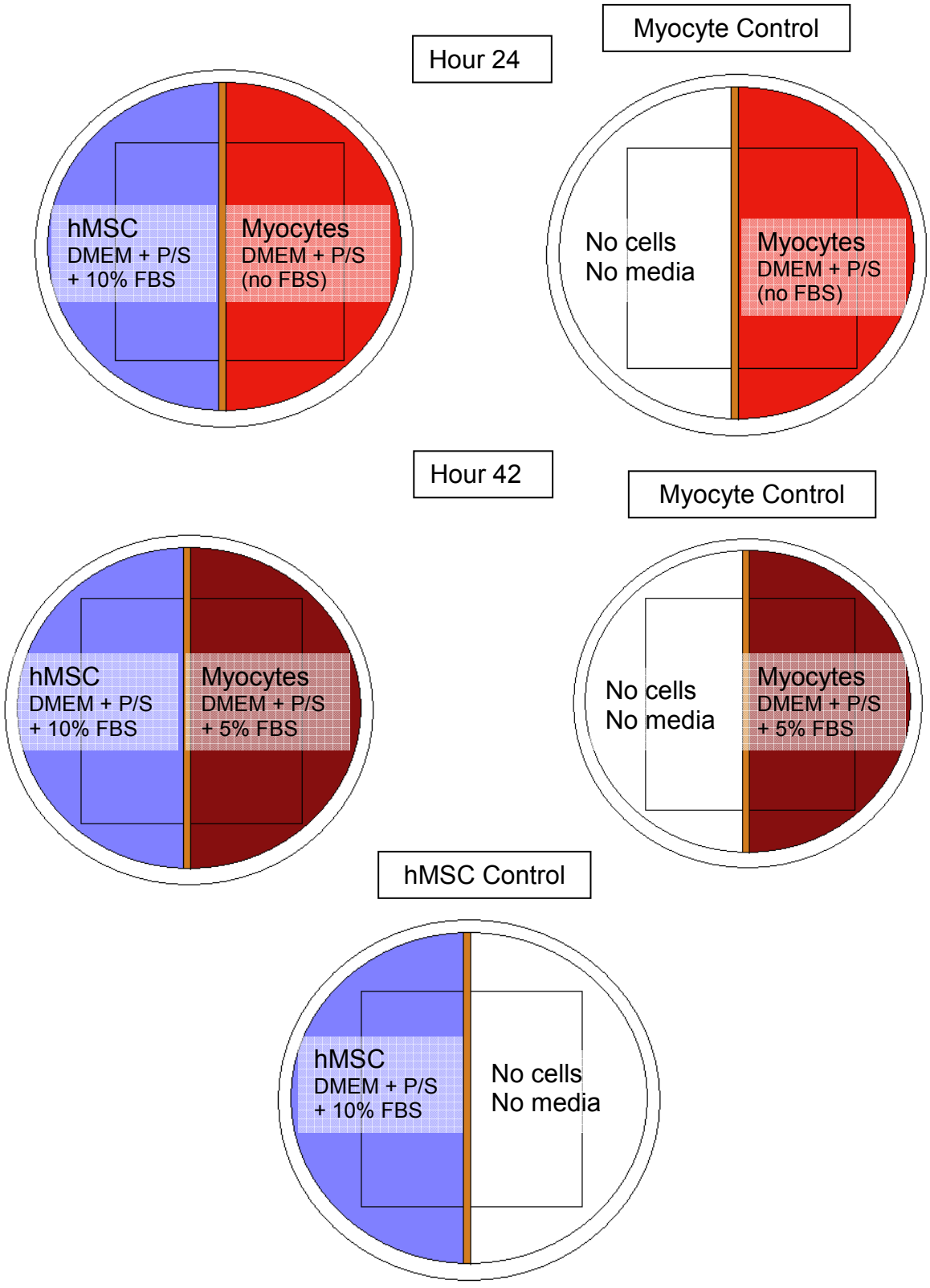


Figure 27: Co-culture Plating Over Time

In order to effectively plate the cells as describe above, it is essential to follow a specific procedure during the first two steps of the hMSC plating. When the hMSCs are plated initially (hour 0), the dish is placed flat on the culture surface. The cell suspension is then plated in a small dot on one half of the cover slip. It is essential that the media not spill over the barrier because doing so will allow the hMSCs to plate on both sides of the barrier. Optimal plating would involve the cell suspension covering the entire half of the cover slip, however this is nearly impossible as the media can very easily spill over the barrier if it is placed too close to the agarose. Once the dot of media (~200µl total) is plated, the dish is placed in the incubator. It is helpful to place the dishes on a small tray or other flat surface (such as the top of a well plate) for easy transport.

After four hours, the media must be changed for two reasons. First, the amount of media placed on the dish at hour 0 is not sufficient to keep the cells healthy for longer than a few hours. Second, removing the media also removes floating cells that have not plated successfully. By replacing the old media with fresh (cell-free) media, the chance of having un-attached cells is reduced. At this point, the dishes must be tilted slightly, and more media must be placed in the dish. The media is filled in the tilted dish until almost reaching the agarose barrier. Again, this media must not cross the barrier in order to reduce the chances of hMSCs being plated on the wrong side. The top and bottom of 35mm cell culture dishes work well as a prop to hold up the tilted culture dish with the cells.

During the cardiac myocyte plating the next day, the dish is returned to a flat surface. The hMSCs receive fresh media once again, although in a dot similar to that which was plated at hour 0. The cardiac myocytes are also plated in a similar fashion

using a dot of KB/cell suspension. Although mixing of the KB and DMEM + 1% P/S +10% FBS is discouraged, there is a chance that these solutions will mix during plating. This is not a significant problem as the only population that needs to remain pure is the myocytes. Once the cardiac myocytes have been stepped up to a DMEM + 1% P/S + 5% FBS medium, the entire dish is filled with 2 ml of DMEM + 1% P/S + 5% FBS to cover the entire cell culture surface.

5.6 Analysis of Co-culture

In order to prove the effectiveness of the system, it was important to develop protocols for staining the cells. The first protocol below was used for staining cyclin D1 to identify cells that were actively involved in the cell cycle. The second protocol was used to stain for α-actinin, a protein found in cardiac cells (Doronin et al., as submitted), to identify that the myocytes can retain their cardiac characteristics through the cell culture process.

5.6.1 Fixing the Cells

All cells were fixed in methanol for 10 minutes, rinsed with PBS for 10 minutes, and stored in PBS in the cold room / refrigerator until microscopy analysis took place.

5.6.2 Immunocytochemistry

In order to analyze the effectiveness of the co-culture, the cells were stained for cyclin D1 and α-actinin. Due to the difficult nature of using two different stains in the same dish, half of the dishes were stained for cyclin D1 to indicate cells in the cell cycle and the other half were stained for α-actinin to indicate that the primary isolation cells

are indeed cardiac myocytes. All cells were stained with Hoescht Nuclear Dye to indicate that the cells contain nuclei.

5.6.2.1 Cyclin D1 Protocol

The Gaudette lab had an existing protocol for cyclin D1 staining that was used as the basis for the following protocol. The first attempt at staining for cyclin D1 was not successful, as all of the cells fluoresced under the microscope. After repeated trial and error, and with the help of the advisers, we determined that a new secondary needed to be purchased that would match the primary better than the existing secondary antibodies available in the Gaudette lab. The control cells used for this stain were Swiss Mouse NIH 3T3-J2 fibroblasts (Lindberg and Badylak, 2001). These cells were readily available in the lab and undergo mitosis under normal cell culture conditions.

Cyclin D1 Staining Protocol for Immunocytochemistry

Note: This procedure is done using cells that have been fixed with methanol. If fixed with paraformaldehyde, the cells will need to be permeabilized using a Triton-X solution.

Normal Goat Serum (Invitrogen, 01-6201)
Dilute serum in PBS to obtain 10% solution for blocking

Primary Antibody - Cyclin D1, IgG_{2b} (Santa Cruz, SC-8396)
Dilute in PBS to obtain 1:100 dilution

Secondary Antibody – goat anti-mouse IgG_{2b} AlexaFluor 488 (Invitrogen A21141)
Dilute in PBS to obtain 1:100 dilution

Hoescht 33342 Nuclear Dye
Dilute in PBS to obtain 1:6000 dilution

Procedure:

1. Fix cells using 100% methanol (10 min)
2. Rinse cover slips with PBS (10 min x 2)
3. Block cells using 10% goat serum (30 min)

4. Rinse with PBS (5 min)
5. Prepare parafilm for primary antibody application. Remove necessary length of parafilm from holder, do not remove paper backing. Mark parafilm with each cell/culture type for identification. Remove the paper backing, being careful NOT to touch the clean side (that which was covered by the backing). Place 50µl of primary antibody solution on the clean side of the parafilm.
6. Gently remove cover slips from the Petri dish and place them face down on the drop of primary antibody on the parafilm. Repeat for all cover slips, placing them in the appropriately marked areas. Place a wet paper towel next to (but not touching) the parafilm strips to avoid evaporation. Cover the setup with a tray or box. Allow cover slips to incubate with 1º for 2 hours.
7. Remove cover slips from parafilm, placing face up in their respective Petri dishes. Rinse with PBS (5 min).
8. Repeat steps 5. and 6. for the secondary antibody. Be sure to keep the antibody and cover slips in the dark as much as possible from this point forward as the secondary antibody is light sensitive. Allow the cover slips to incubate with the secondary antibody for 1 hour.
9. Remove cover slips from parafilm, placing face up in respective Petri dishes. Rinse with PBS (5 min).
10. Aspirate PBS and apply Hoescht dye to the cover slip (2 min)
11. Rinse with PBS (5 min x 2)
12. Setup slides for mounting. Obtain as many slides as you have cover slips, mark each slide with pertinent information (cell type, stain, date) and apply 2 drops of aqueous mount onto each slide.
13. Remove one slide from the Petri dish and carefully place face down on the aqueous mount drop on the slide. Repeat for all cover slips.
14. Once the aqueous mount has had a chance to set, seal the slides using clear nail polish to outline the edge of the cover slip. Store the slides in a cool dark place (preferably a slide folder or slide box).

5.6.2.2 α-actinin Protocol

The staining protocol was very similar to the one used the in Gaudette lab for tissue sections, with only minor modifications made for use with cells on cover slips rather than tissue sections on slides. The control tissue used for this stain was canine heart tissue sections.

α-actinin Staining Protocol for Immunocytochemistry (adapted from Gaudette lab)

Blocking Buffer – 2% IgG-free bovine serum albumin (BSA) in PBS
Wash Buffer – 0.5% IgG-free bovine serum albumin (BSA) in PBS

Permeabilization Buffer – 0.25% Triton X-100 in PBS

Primary Antibody – Mouse monoclonal α-actinin, sarcomeric (Sigma A-7811)
Dilute in PBS to obtain 1:100 dilution

Secondary Antibody – goat anti-mouse IgG(H+L) AlexaFluor 546 (Invitrogen A11003)
Dilute in PBS to obtain 1:100 dilution

Hoescht 33342 Nuclear Dye
Dilute in PBS to obtain 1:6000 dilution

Procedure:

1. Fix cells using 100% methanol (10 min)
2. Wash cover slips with wash buffer (5 min x 3)
3. Permeabiliz with Triton X-100 (10 min)
4. Wash with wash buffer (5 min x 3)
5. Block cells using blocking serum (45 min)
6. Wash with wash buffer (5 min x 3)
7. Prepare parafilm for primary antibody application. Remove necessary length of parafilm from holder, do not remove paper backing. Mark parafilm with each cell/culture type for identification. Remove the paper backing, being careful NOT to touch the clean side (that which was covered by the backing). Place 50µl of primary antibody solution on the clean side of the parafilm.
8. Gently remove cover slips from the Petri dish and place them face down on the drop of primary antibody on the parafilm. Repeat for all cover slips, placing them in the appropriately marked areas. Place a wet paper towel next to (but not touching) the parafilm strips to avoid evaporation. Cover the setup with a tray or box. Allow cover slips to incubate with 1º for 1 hour.
9. Remove cover slips from parafilm, placing face up in their respective Petri dishes. Rinse with wash buffer (5 min x 3).
10. Repeat steps 5. and 6. for the secondary antibody. Be sure to keep the antibody and cover slips in the dark as much as possible from this point forward as the secondary antibody is light sensitive. Allow the cover slips to incubate with the secondary antibody for 1 hour.
11. Remove cover slips from parafilm, placing face up in respective Petri dishes. Rinse with wash buffer (5 min x 3).
12. Wash with PBS (5 min x 3)
13. Aspirate PBS and apply Hoescht dye to the cover slip (2 min)
14. Rinse with PBS (5 min x 2)
15. Setup slides for mounting. Obtain as many slides as you have cover slips, mark each slide with pertinent information (cell type, stain, date) and apply 2 drops of aqueous mount onto each slide.
16. Remove one slide from the Petri dish and carefully place face down on the aqueous mount drop on the slide. Repeat for all cover slips.

17. Once the aqueous mount has had a chance to set, seal the slides using clear nail polish to outline the edge of the cover slip. Store the slides in a cool dark place (preferably a slide folder or slide box).

5.6.3 Microscopy

Microscope images were taken of the control dishes to analyze the effectiveness of the agarose barrier. These images were taken using a light microscope to observe the placement of the hMSCs with relation to the agarose barrier.

Fluorescence microscopy was used to analyze the immunostaining for cyclinD1 and α-actinin. Images were taken of each cover slip, as well as the control cells/tissue to observe the success of the staining and of the co-culture system.

6. Results

The following section provides the results for the experiments that were tested. This includes results from the myocyte isolation, agarose as an effective barrier studies, and staining of both control and experimental cells.

6.1 Myocyte Isolation

The isolation of cardiac myocytes was completed successfully on several occasions. Using the first batch of Blendzyme that the team had purchased, we were able to obtain single myocytes from the mouse heart as seen in Figure 28. These myocytes still had some indication of striations. Unfortunately, the attempt of myocyte isolation proved ineffective once the group switched to a new batch of Blendzyme after the first batch was used.

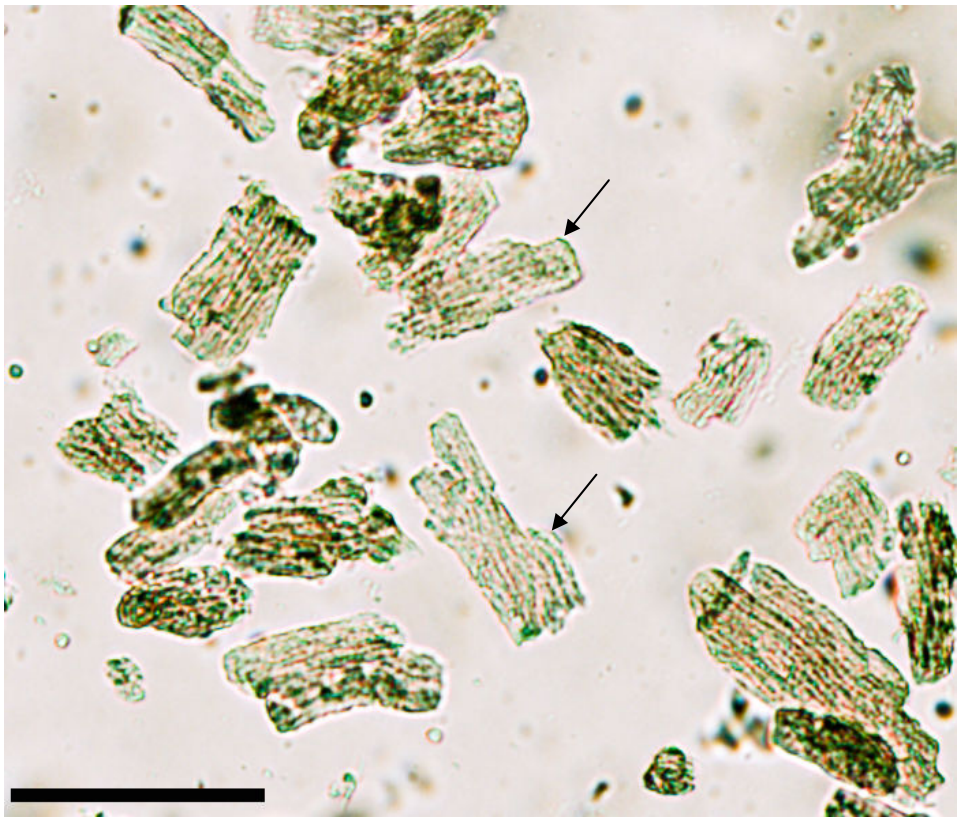


Figure 28: Cardiac myocytes–arrows indicate cells with striations (Scale bar = 100 microns)

6.2 Control Data for Cell Migration

The figures below show the results at day 10 for the migration control study. In Figure 29, the hMSC side of the cover slip is shown with the cells approaching the edge of the barrier, indicated by the black line in the figure. The agarose was mostly removed during the fixing process, as the methanol degraded the agarose. However, the placement of the line was determined through scanning the rest of the cover slip and placing it in the same position as on areas on the cover slip where the barrier still existed. The pictures were not taken at those locations because the mounting fluid had coated the top of the cover slip, lowering the quality of the images in those locations. The opposite side of the cover slip was scanned completely and no cells were found past the barrier. Figure 30 is a representative picture of the control side of the cover slip.

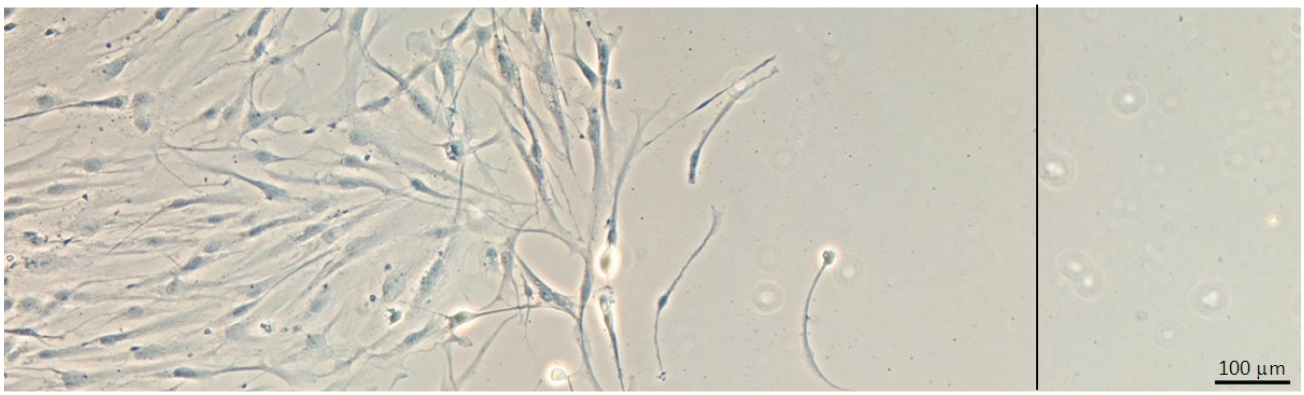


Figure 29: hMSCs approaching the agarose barrier (Day 10)

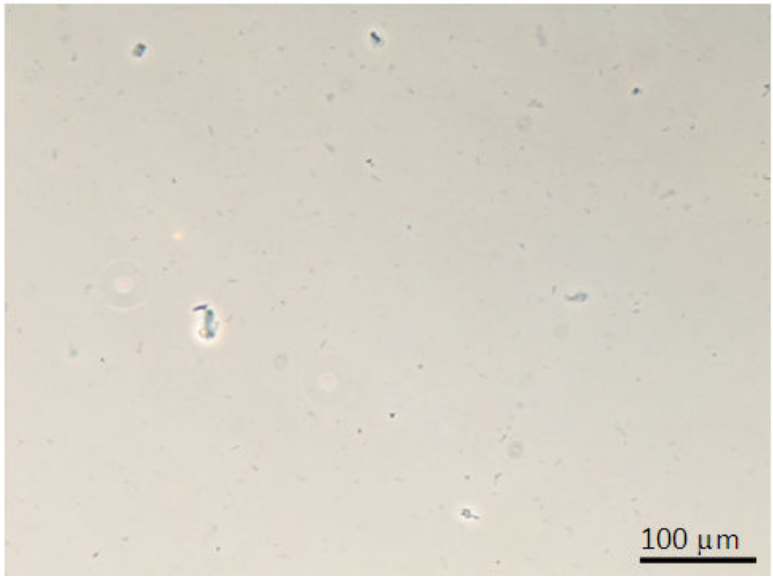


Figure 30: Representative image of the control (no cells plated) side of the (Day 10)

6.3 Staining Data

This section discusses the results of the staining procedure developed. Results include images from cyclin D1 and the α-actinin control as well as experimental data.

6.3.1 Cyclin D1 Control

The following is a set of pictures taken of NIH 3T3-J2 fibroblasts stained for the cell cycle marker cyclin D1. Figure 31 indicates the cyclin D1 staining as noted by the concentrated fluorescent green (surrounded by red boxes). Figure 32 indicates the blue Hoescht nuclear stain. Figure 33 is the combination of the two images to show the correspondence between the nuclei and cell body. Comparisons between staining for +/- cyclin D1 primary antibody can be found in Appendix G: Cyclin D1 Stain: Positive vs. Negative Stain. Note that all three images are taken at the same magnification.

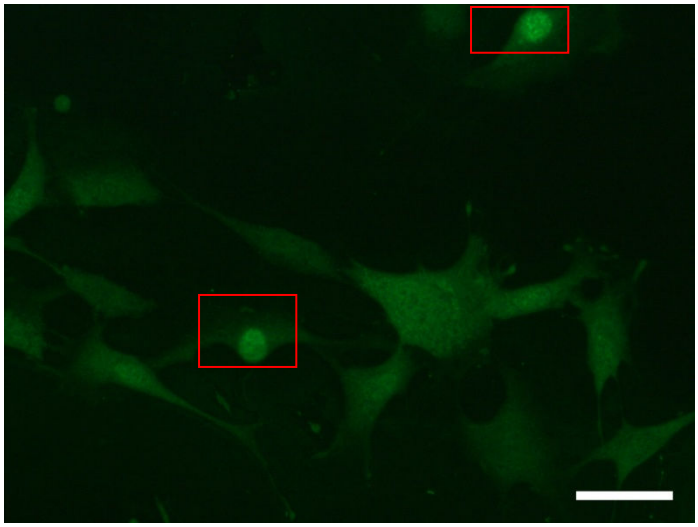


Figure 31: Cyclin D1 Stain (scale bar = 50 microns)

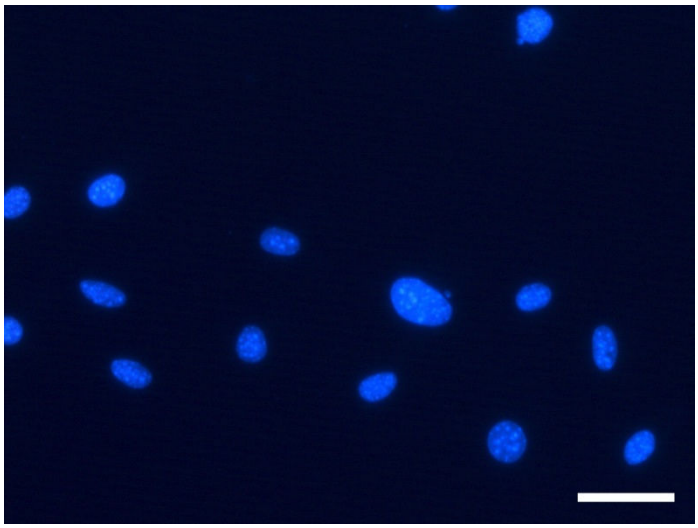


Figure 32: Hoescht Nuclear Dye (scale bar = 50 microns)

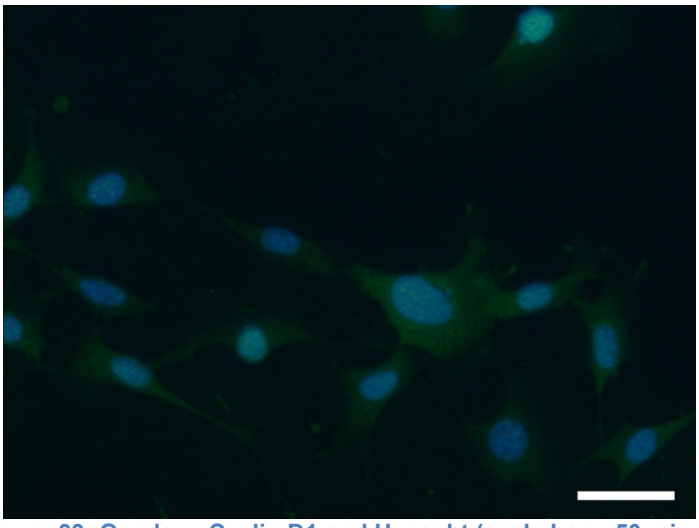


Figure 33: Overlay - Cyclin D1 and Hoescht (scale bar = 50 microns)

6.3.2 α-actinin Control

The following is a set of pictures taken of canine heart tissue sections that were stained for α-actinin. Figure 34 shows the α-actinin stain while Figure 35 shows the Hoescht Nuclear dye. Figure 36 shows the combination of the α-actinin and Hoescht stains. Figure 37 is a blow-up of the α-actinin stain to show the striations in the tissue. These striations indicate the stain of the α-actinin protein in the cardiac tissue. Comparisons of staining for +/- α-actinin primary antibody can be found in Appendix H: α-actinin Stain: Positive vs. Negative Stain. Figs. 34-36 are all the same magnification.

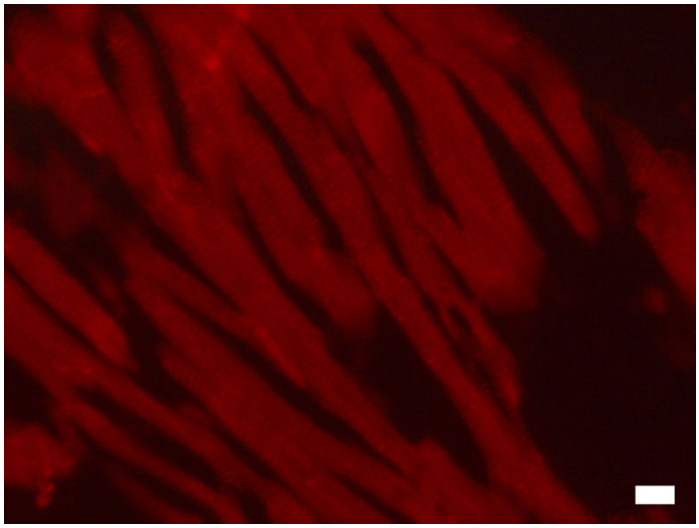


Figure 34: α-actinin Stain (scale = 10 microns)

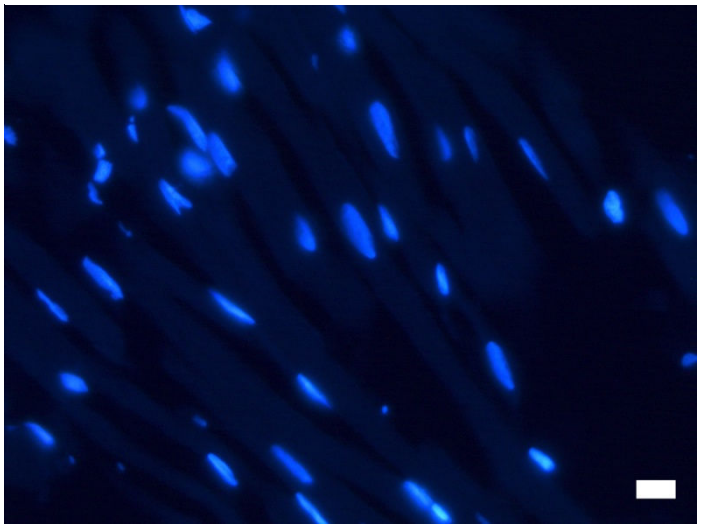


Figure 35: Hoescht Nuclear Stain (scale = 50 microns)

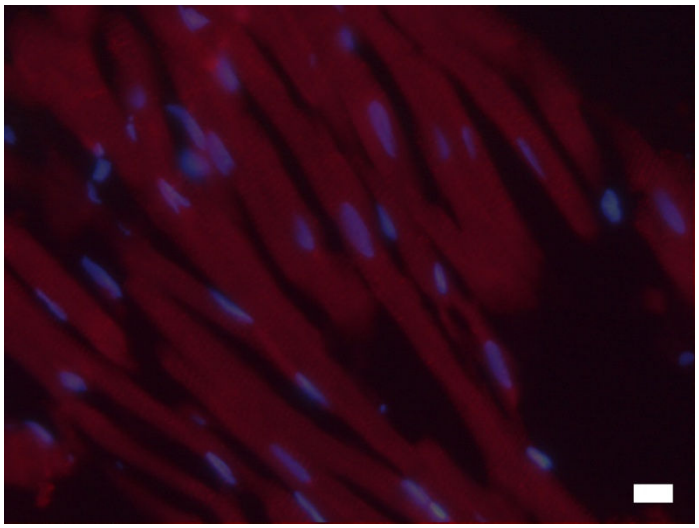


Figure 36: Overlay - α-actinin and Hoescht (scale bar = 10 microns)

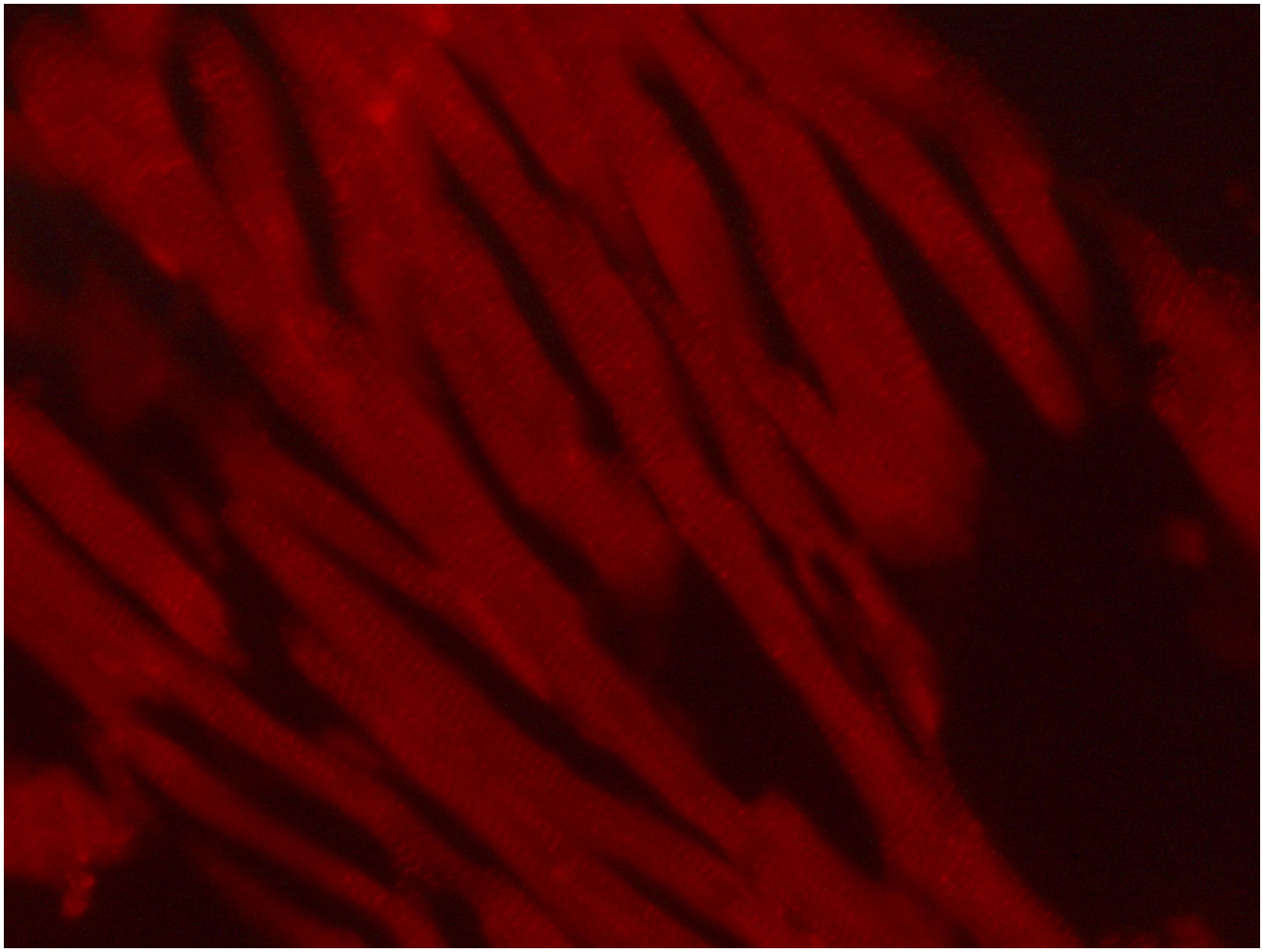


Figure 37: Striations in Cardiac Tissue

6.3.3 Experimental Data

The following pictures are from the experimental system. Both pictures are from the myocyte side of the co-culture system. The DAPI filter revealed no fluorescing nuclei. The line at the bottom of the picture represents the edge of the agarose. Figure 38 shows the myocyte control culture dish (myocytes alone) while Figure 39 shows the myocyte/hMSC co-culture dish. Notice that there is no cell matter in the agarose barrier region.

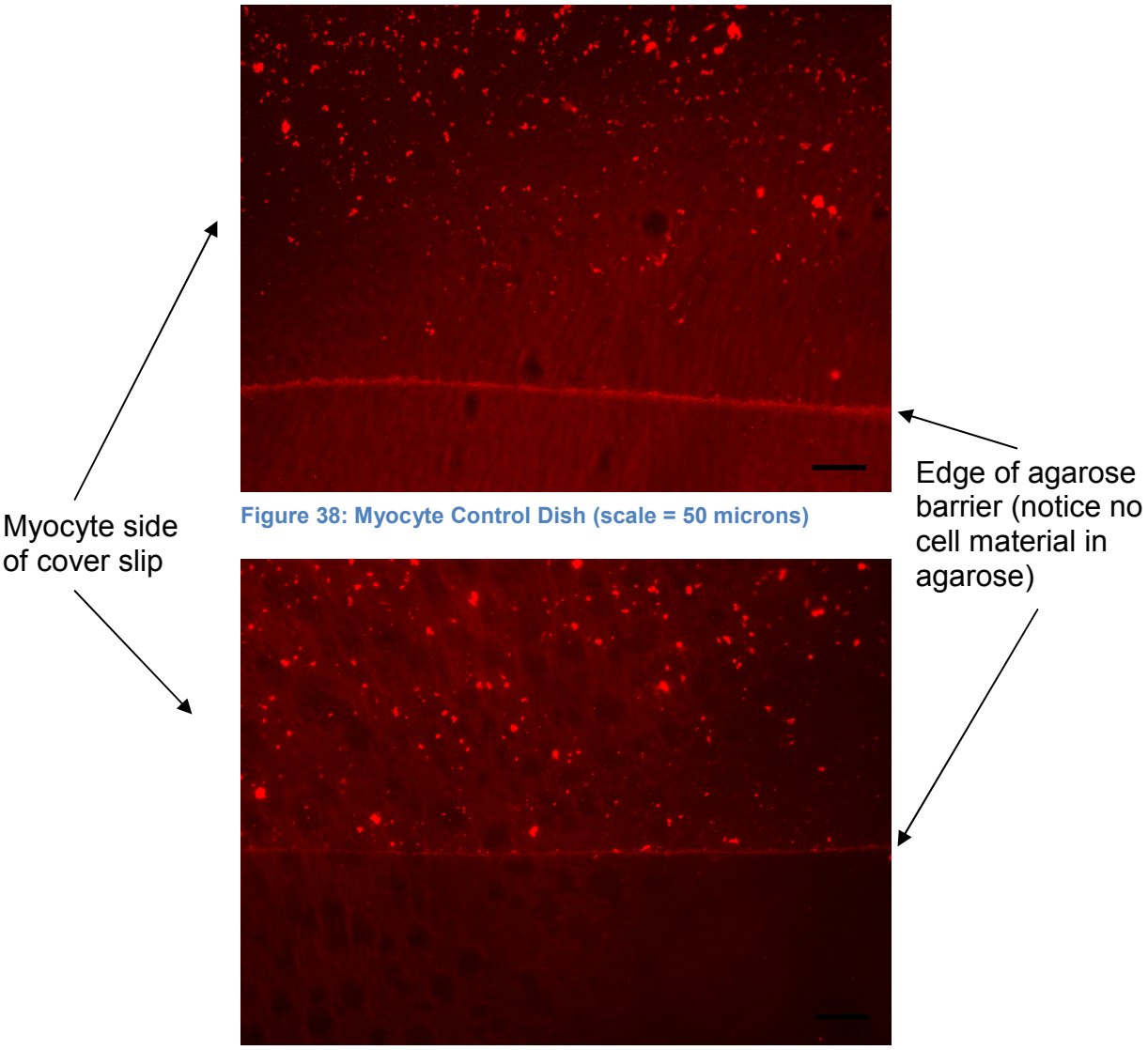


Figure 38: Myocyte Control Dish (scale = 50 microns)

Figure 39: Myocyte - hMSC Co-culture Dish (scale = 50 microns)

7. Analysis and Discussion

In this section, we will analyze the data that we obtained. We will discuss the control studies for migration and staining. The section also contains hypotheses related to the complications that arose during the testing of our system.

7.1 Myocyte Isolation

The project team had no prior experience working with primary tissue isolations. As such, Professor Gaudette and Joan Zuckerman (SUNY Stony Brook) were instrumental in answering questions and teaching the group how to effectively isolate cells from the mouse heart. This step was essential to the project as no co-culture experiments could have been performed without cardiac myocytes. Figure 28 shows an example of the myocytes that were isolated from mouse hearts. However, these particular cells were not able to be plated in the co-culture system as the final design was not yet developed.

The first several attempts to isolate cardiac myocytes were a valuable learning experience for the team. First, it was imperative that the team learn how to open the mouse and retrieve the heart. Through trial and error we learned that the heart could be removed along with excess tissue (i.e. lungs) attached, and that the tissue could be more easily removed once the heart was removed from the body. We also discovered that the minced heart tissue would yield more cells if the initial pieces were as small as possible. By cutting the tissue into very small sections, each piece can be exposed to the Blendzyme solution, thereby allowing more surface area for the enzyme to work. Additionally, we learned that it was important to use a 25ml pipet for the trituration process as anything smaller will cause heart tissue to become lodged in the end of the

pipet. Finally, we believe that time is a factor in isolating healthy myocytes. The first two or three isolations took a long amount of time to complete (~6 hours) and there were very few healthy myocytes and the end of the procedure. After a few isolations, we were able to cut the time in half (~3 hours or less). While we were still using the first Blendzyme batch, the isolated myocytes were healthier and more single cells were isolated as the isolation time decreased.

Once the team developed and implemented the final design using the agarose barrier, it was believed that myocyte / hMSC co-cultures would begin. After the protocol had been developed and proved successful, the following isolations were yielding little to no myocytes. However, the original batch of Blendzyme had been consumed by the experiments developing the cell isolation protocol and a new batch had to be purchased. Additionally, the hearts had to be transported from Gateway Park to Salisbury Labs, which added (roughly) an extra 30 minutes to the time between extraction of the heart and the start of the isolation process. The team believes that one or both of these changes in the process may have caused the myocyte isolation to yield little to no myocytes, thereby limiting the amount that could be used in the co-culture system.

Although we were faced with these challenges, we were able to develop a successful protocol for isolating myocytes when the isolation is done under optimal conditions. The protocol developed was far easier to complete than the original protocol the team attempted to use since it was less technically challenging. This protocol was proved successful at isolating single, healthy myocytes during several independent

isolation attempts. The healthy myocytes that are isolated using our protocol can be used in our co-culture system.

7.2 Migration Control Assay

The migration experiment was conducted in order to observe the migration of hMSCs up to agarose barrier, and to observe the hMSC behavior once the cells reached the barrier. The control migration study was conducted for 10 days. We plated 2 dishes for each time point (1 day, 2 days, 5 days, and 10 days). All four time points indicated no migration past the barrier, and images were taken of the day 10 time point because it is the longest culture time. In addition, the hMSC control for each isolation were observed for hMSC migrations. As the co-cultures were not cultured longer than 7 days, a 10 day migration assay was sufficient for the design to work effectively. By thoroughly scanning each dish and taking light microscope images, the team observed that the hMSCs did not grow past the agarose barrier at day 10. The cells did grow close to the barrier; however they did not grow through or over the agarose.

As seen in Figure 29 in the results section, the hMSCs are confluent on the left side of the picture, and the cell density decreases as the cells move closer to the barrier. Based on observations of previous days, the cells had grown to the opposite edge of the cover slip (the one opposite the agarose barrier). Once the cells near the agarose reached the barrier, they did not grow directly up to the barrier. The first explanation that the team considered was that the cells had just begun to migrate into the area close to the agarose meaning that the space was just beginning to be filled. However, the region of confluent cells near the barrier ends abruptly, whereas during cell migration

there should be a region of cells at a lower density surrounding the confluent region. This region of lower density is seen surrounding the confluent region in all areas of the cover slip with the exception of the side parallel to the barrier. This suggests that there may be a factor that the agarose is emitting that prevents the cells from growing directly next to the agarose. However, the cells do not seem to be adversely affected by this possible factor, as the cells all looked attached and healthy from the team's observation.

The migration experiments proved that our co-culture system can be used to culture discrete populations for at least up to ten days. The system was more than efficient for our experiments since we only needed to co-culture the cells for 7 days. As mentioned, the agarose did not seem to have any negative effects on the hMSCs, since they were healthy and attached to the surface of the cover slip. This suggests that the system is biocompatible, but further testing would need to be conducted to confirm that finding.

Also, this co-culture system has a wider range of uses than simply myocyte / hMSC co-cultures; our system could be used to co-culture any two types of cells that need to remain separated. This means that our co-culture system could effectively replace insert co-cultures in many applications. Our system could be altered to meet the specific needs of other co-culture experiments since the agarose can be laid down in any conformation desired. It could be used to generate complex patterns in culture dishes, and so our system would be useful for a wide range of experiments.

7.3 Plating Cells in Wells and Dishes

During the preliminary stages of the agarose testing, the team tried many different variations of cell plating before finding a method that worked well. First, the team tried to use a 24-well plate with the agarose barrier to plate cells. As the cells were

plated, we noticed that the media seemed to be attracted to the edge of the well. When the media was placed in the well it would quickly move toward the wall, and then follow the shape of the wall. The media would follow at least halfway around each well, proving ineffective for hMSC seeding as the cells were to remain on only one side of the barrier.

In an attempt to develop a better system, the team moved up to a larger well size and experimented with a 12-well plate. This was done with the hypothesis that a larger well would allow more surface area for the media to be plated on the bottom of the well, where it would hopefully stay. Although we were able to place media in the bottom of the wells, it was always pulled to the wall and around the well by the time the team went back to change the media. At this point the team had to move to a larger well.

Based on the problems with the 24-well and 12-well plate, the team decided to try 35mm cell culture dishes. The 35mm dishes worked better than the well plates as the media was able to sit in the middle of one half of the well without being pulled to the wall. This was very important for effective hMSC seeding. However, the team did use caution when plating the cells as the media can very easily spill over the agarose if it is placed to close to the barrier. When great care was taken during plating and transferring, the plating process was successful at maintaining a pure population of myocytes.

7.4 Staining

The following is a discussion regarding the success of the staining protocols for control cells and tissue. There is also a discussion of possible reasons why the co-culture staining was not successful.

7.4.1 Cyclin D1 Control

The first attempt to stain control cells (fibroblasts) with cyclin D1 did not yield good results. Instead of seeing an effective stain that was localized in the nucleus, there was only general staining of all of the cells. After several more attempts with different combinations of primary and secondary antibodies, and with no effective stain found, it was necessary to look into other options. We now know that there are four isotypes of IgG antibody that are present in mice. The primary antibody we were using was specific to the IgG_{2b} isotype, while the secondary antibodies were for IgG_{2a}, or for general IgG isotypes. This combination was not specific enough for the primary being used, so the team purchased a different secondary antibody. The new secondary, specific for IgG_{2b}, worked well with the primary, providing the cyclin D1 staining that was present in the 3T3-J2 fibroblasts. The success with this stain indicates the protocol as an effective method to indicate the presence of cell cycle markers in cells.

7.4.2 α-actinin Control

The first attempt to stain control tissue for α-actinin went very well, as the procedure had already been established by other people in the Gaudette lab. When the team tried to stain more control tissue with the α-actinin stain, it worked once again without any problems. This result was expected, and welcomed by the team. The success with this stain shows the ability to stain cultured cells with α-actinin as an effective method to show the presence of cardiac markers in cells.

7.4.3 Experimental Data

One of the goals for the team at the beginning of this project was to promote the expression of cell cycle markers in adult cardiac myocytes. Unfortunately, this goal was not accomplished, mainly because the cells that were plated in the co-culture system did not attach particularly well to the culture surface.

In regards to the attachment of the cardiac myocytes, there are several possible explanations. As described previously, there may not have been a population of myocytes plated in the first place for the co-culture experiments due to the reduced effectiveness of the isolation procedure. This may have been due to the movement of hearts between buildings, the new batch of Blendzyme, or both. Also, it is important to consider the culture surface of the system. The cover slips used were not tissue culture treated, nor were they covered with any coatings or substrates. A protein coating of laminin or fibronectin may have enhanced the attachment of any available cardiac myocytes. Although tissue culture treated Petri dishes may have been more effective, the amount of antibody necessary to stain an entire dish would have been much more than that which is needed for cover slip staining. As mentioned in the design chapter, there were fourteen dishes that were plated during each experiment. It is not feasible to stain fourteen entire dishes for each experiment, so cover slips needed to be used in order to allow for proper staining to occur without using an excess of materials.

The attachment of the hMSCs may have been affected by several different factors. First, the hMSCs were at a high passage number (mid twenties). This could affect the attachment and proliferation of the hMSCs in culture. Also, the co-culture system used DMEM with only 5% fetal bovine serum in order to provide an acceptable environment

for cardiac myocyte culture. The team observed the hMSCs beginning to ball up in this media, and tried to remedy the situation. We tried to add DMEM + 8% FBS, however this proved to be ineffective. It is possible that the hMSCs were already too damaged to be brought back to a healthy state, or the late passage may not tolerate the lower amounts of FBS as well as an earlier passage. In any case, the hMSCs that were present in the co-culture were not healthy upon fixing, and therefore not well adhered to the cell culture surface. The team believes that this caused the hMSCs to wash off of the slide during fixing and/or staining procedures.

Although we were unsuccessful in gathering quantitative data from the co-culture system, we have demonstrated that the system has the potential for success. As seen in Figures 38 and 39, the top portion of the pictures shows that there is myocyte cell matter in the field of view. Although the Hoescht dye did not indicate the presence of nuclei, the fact that there is something on the dish after multiple media changes indicates that at least some cell matter can be found in the system. Also, the line at the bottom of the pictures is the edge of the agarose barrier. If cells were moving through the barrier, then the cells / cell matter would be present in the barrier. Because there is nothing visible in the barrier, this indicates that the system is effective in keeping the cell populations separate from each other, which was the specific goal of this project.

Although we were never able to conduct a successful experiment from beginning to end, we were successful at completing each individual component of the design during different phases of the design process. We were able to isolate healthy myocytes which could be used for co-culture. We were able to prove that hMSCs would not cross over the barrier and migrate into the myocyte population during the 7 day culturing period.

We were able to develop protocols for analysis of our system that would yield tangible results in a successful co-culture. Since the individual components of the system work, it is reasonable to believe that the overall system would work. We believe that had our successful trials of the individual components all occurred during the same experiment, the system would have yielded positive results.

8. Conclusions

The project team has developed a system that allows for co-culture of two different cell populations. This system utilizes an agarose barrier that is effective against cell migration, while still allowing media to flow between the two different cell populations. This system allows for cells to be in closer contact than other systems previously used in co-culture research, allowing for cells to be less than 1mm away from each other, while still keeping the populations separate. The length and height of the agarose barrier can be altered for different applications, and the system itself could be used for other cell populations in the future. This system could be used in any scenario where Transwell® systems are currently used, while allowing the co-cultured cells to be closer to one another and allowing the user to analyze the effects of distance between the cell types. The team has also successfully developed a protocol for primary adult cardiac myocyte isolation, as well as staining procedures for cell cycle markers and cardiac tissue labeling. All of these results indicate that this system may one day be used for the co-culture of cardiac myocytes and human mesenchymal stem cells with the goal of promoting mitosis in cardiac myocytes for the treatment of myocardial infarction.

9. Future Recommendations

The project team believes that this design has great potential for use in the future as a system to promote mitosis in adult cardiac myocytes. The improvements recommended here are for both short term and long term consideration.

First, a reasonable short term recommendation is to reduce the size of the design to a smaller well size, perhaps a 12-well or 24-well plate. This would maximize the amount of space being used for co-culture. As the size of the design is decreased, the number of cells that can be co-cultured in close proximity (i.e. those that are close to the agarose barrier) can be increased. This may lead to a more efficient system for promoting cardiac myocytes to re-enter the cell cycle.

The team also recommends that the media formulation be re-evaluated from two directions. First, it would be imperative to find a medium / serum formulation that allows for the effective culture of cardiac myocytes and human mesenchymal stem cells. This may involve using different serum levels, or using only low passage hMSCs. Also, it may be possible to add other components to the media, such as proteins, that may help to induce myocyte proliferation.

Another recommendation includes re-evaluating the cell culture surface. The addition of a protein coating such as fibronectin or laminin may improve cell adhesion, thereby increasing the likelihood of cells being present on the slides for staining and microscopy.

The final recommendation for improvements over a short time span would be to identify paracrine factors that are secreted by the hMSCs to promote myocyte proliferation. If these factors can be identified and developed into a therapeutic form, it

may be possible to treat MI directly. This could be done by administering these paracrine factors to an MI patient to directly stimulate the existing myocytes to proliferate and regenerate the damaged myocardium.

The long-term goal of this type of research is to induce human adult cardiac myocytes to proliferate. If this research can be proven effective using human cells, then it may be used as an effective treatment for MI patients. This could be done by stimulating the myocytes to undergo mitosis *in vitro*, and then placing the cells back in the infracted region. The development of an effective treatment for regenerating damaged myocardium would enhance the lives of millions of people affected by myocardial infarction, and research can only help in the search for answers.

References

Ahuja, P., Sdek, P., & MacLellan, W. R. (2007). Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiological Reviews, 87*(2), 521-544.

Anversa, P., Cheng, W., Liu, Y., Leri, A., Redaelli, G., & Kajstura, J. (1998). Apoptosis and myocardial infarction. *Basic Research in Cardiology, 93 Suppl 3*, 8-12.

Barile, L., Messina, E., Giacomello, A., & Marban, E. (2007). Endogenous cardiac stem cells. *Progress in Cardiovascular Diseases, 50*(1), 31-48.

Beltrami, A. P. e. a. (2001). Evidence that human cardiac myocytes divide after myocardial infarction. *The New England Journal of Medicine, 344*(23), 1750-1757.

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., et al. (2003). Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell, 114*(6), 763-776.

Bockeria, L. A., Gorodkov, A. J., Dorofeev, A. V., Alshibaya, M. D., & RESTORE Group. (2006). Left ventricular geometry reconstruction in ischemic cardiomyopathy patients with predominantly hypokinetic left ventricle. *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery, 29 Suppl 1*, S251-8.

Cattoretti, G., Becker, M. H., Key, G., Duchrow, M., Schluter, C., Galle, J., et al. (1992). Monoclonal antibodies against recombinant parts of the ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *The Journal of Pathology, 168*(4), 357-363.

Collins, S. D., Baffour, R., & Waksman, R. (2007). Cell therapy in myocardial infarction. *Cardiovascular Revascularization Medicine : Including Molecular Interventions, 8*(1), 43-51.

Coqueret, O. (2002). Linking cyclins to transcriptional control. *Gene, 299*(1-2), 35-55.

Czyz, J., Wiese, C., Rolletschek, A., Blyszczuk, P., Cross, M., & Wobus, A. M. (2003). Potential of embryonic and adult stem cells in vitro. *Biological Chemistry, 384*(10-11), 1391-1409.

Doronin, S. V., Kochupura, P. V., Azeloglu, E. U., Kelly, D. J., Potapova, I. A., Zuckerman, J., et al. Canine heart regeneration. *Nature, Submitted,*

Dym, C., & Little, P. (2004). *Engineering design: A project-based introduction, second edition* John Wiley & Sons, Inc.

Engel, F. B. (2005). Cardiomyocyte proliferation: A platform for mammalian cardiac repair. *Cell Cycle (Georgetown, Tex.), 4*(10), 1360-1363.

Folch, A., & Toner, M. (2000). Microengineering of cellular interactions. *Annu. Rev. Biomed. Eng., 2*, 227-256.

Ford, L. E. (2000). Microscopic appearance, action potential duration. *Muscle physiology and cardiac function* (pp. 7, 198). USA: I. L. Cooper.

Freshney, R. I. (1994). *Culture of animal cells: A manual of basic technique, third edition*. New York: Wiley-Liss, Inc.

Gladden, A.B. and Diehl, J.A. (2005) Location, Location, Location: The Role of Cyclin D1
Nuclear Localization in Cancer. *Journal of Cellular Biochemistry.* 96:906–913.

Gutenwik, J. et al. (2004). Determination of protein diffusion coefficients in agarose gel with a diffusion cell. *Biochemical Engineering Journal, 19*, 1-7.

Heller, D. A., Garga, V., Kelleher, K. J., Lee, T. C., Mahbubani, S., Sigworth, L. A., et al. (2005). Patterned networks of mouse hippocampal neurons on peptide-coated gold surfaces. *Biomaterials, 26*(8), 883-889.

Ingber, D. E., & Levin, M. (2007). What lies at the interface of regenerative medicine and developmental biology? *Development (Cambridge, England), 134*(14), 2541-2547.

Isomura, T., Horii, T., Suma, H., Buckberg, G. D., & RESTORE Group. (2006). Septal anterior ventricular exclusion operation (pacopexy) for ischemic dilated cardiomyopathy: Treat form not disease. *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery, 29 Suppl 1*, S245-50.

Kim, H., Jo, C., Jang, B. G., Oh, U., & Jo, S. A. (2008). Oncostatin M induces growth arrest of skeletal muscle cells in G(1) phase by regulating cyclin D1 protein level. *Cellular Signalling, 20*(1), 120-129.

Kocica, M. J., Corno, A. F., Carreras-Costa, F., Ballester-Rodes, M., Moghbel, M. C., Cueva, C. N., et al. (2006). The helical ventricular myocardial band: Global, three-dimensional, functional architecture of the ventricular myocardium. *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery, 29 Suppl 1*, S21-40.

Lindberg K., Badylak, S.F. Porcine small intestinal submucosa (SIS): a bioscaffold supporting in vitro primary human epidermal cell differentiation and synthesis of basement membrane proteins. *Burns.* 27 (2001) 254–266.

Marieb, E. N., & Hoehn, K. (2007). *Human anatomy and physiology, seventh edition*Pearson Education, Inc.

Matsui, Y., Fukada, Y., Suto, Y., Yamauchi, H., Luo, B., Miyama, M., et al. (2002). Overlapping cardiac volume reduction operation. *The Journal of Thoracic and Cardiovascular Surgery, 124*(2), 395-397.

McFarland, D. (2000). Preparation of pure cell cultures by cloning. *Methods in Cell Science, 22*, 63-66.

Mensink, A., Mul, F. P. J., van der Wijk, Thea, Brouwer, A., & Koeman, J. H. (1998). Macrophages influence gap junctional intercelluar communication between smooth muscle cells in a co-culture model. *Environmental Toxicology and Pharmacology*, *5*, 197-203.

Myocardial infarction redefined--a consensus document of the joint european society of Cardiology/American college of cardiology committee for the redefinition of myocardial infarction.(2000). *European Heart Journal, 21*(18), 1502-1513.

Ogawa, E. et al. (2000). Outside-in signalling of fibronectin stimulates cardiomyocyte hypertrophy in cultured neonatal rat ventricular myocytes. *J Mol Cell Cardiol, 32*, 765-776.

Oh, H., Bradfute, S. B., Gallardo, T. D., Nakamura, T., Gaussin, V., Mishina, Y., et al. (2003). Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction. *Proceedings of the National Academy of Sciences of the United States of America, 100*(21), 12313-12318.

Principles and techniques of practical biochemistry: Fifth edition(2000). In Wilson, Keith and John Walker (Ed.), . Cambridge, UK: The Press Syndicate of the University of Cambridge.

Rangappa, S., Entwistle, J. W., Wechsler, A. S., & Kresh, J. Y. (2003). Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. *The Journal of Thoracic and Cardiovascular Surgery, 126*(1), 124-132.

Ribeiro, G. A., da Costa, C. E., Lopes, M. M., Albuquerque, A. N., Antoniali, F., Reinert, G. A., et al. (2006). Left ventricular reconstruction benefits patients with ischemic cardiomyopathy and non-viable myocardium. *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery, 29*(2), 196-201.

Rosamond, Wayne, et al. (2007).
Heart disease and stroke Statistics—2007 update: A report from the american

heart association statistics committee and stroke statistics subcommittee. *Circulation, 115*, 69-171.

Tang, Y. L., Shen, L., Qian, K., & Phillips, M. I. (2007). A novel two-step procedure to expand cardiac sca-1+ cells clonally. *Biochemical and Biophysical Research Communications, 359*(4), 877-883.

Ueno, T., Sakata, R., Iguro, Y., Yamamoto, H., Ueno, M., Ueno, T., et al. (2007). Mid-term changes of left ventricular geometry and function after dor, SAVE, and overlapping procedures. *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery, 32*(1), 52-57.

Vats, A., Bielby, R. C., Tolley, N. S., Nerem, R., & Polak, J. M. (2005). Stem cells. *Lancet, 366*(9485), 592-602.

Wang, T., Xu, Z., Jiang, W., & Ma, A. (2006). Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. *International Journal of Cardiology, 109*(1), 74-81.

Xu, W., Zhang, X., Qian, H., Zhu, W., Sun, X., Hu, J., et al. (2004). Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Experimental Biology and Medicine (Maywood, N.J.), 229*(7), 623-631.

Appendix

Appendix A: Cell Cycle Regulator Proteins

Table 16: Cell Cycle Regulator Proteins

Group	Name	Type	Phase	Function	Expression
Cyclin	Cyc A	Mediator	S, G2, M	Partners with Cdk2	Fetal and Neonatal
	Cyc B	Mediator	G2, M	Partners with Cdc2	Fetal and Neonatal
	Cyc D1	Mediator	G1, S	Critical for ventricular development, Partners with Cdk4, Cdk6	Fetal and Neonatal
	Cyc D2	Mediator	G1, S	Critical for ventricular development, Partners with Cdk4, Cdk6	Fetal and Neonatal
	Cyc D3	Mediator	G1, S	Critical for ventricular development, Partners with Cdk4, Cdk6	Fetal and Neonatal
	Cyc E	Mediator	G1, S	Partners with Cdk2	Fetal and Neonatal
Cyclin D-dependent Kinases	Cdc2	Mediator	G2, M	Aids mitotic phase initiation	Fetal and Neonatal
	Cdk2	Mediator	G1, S	Phosphorylates Rb family	Fetal and Neonatal
	Cdk4	Mediator	G1, S	Phosphorylates Rb family	Fetal and Neonatal
	Cdk6	Mediator	G1, S	Phosphorylates Rb family	Fetal and Neonatal
Proteins	p16	Inhibitor	G1, S	Impede Cdk4, Cdk6 activity	unknown
	p18	Inhibitor	G1, S	Impede Cdk4, Cdk6 activity	Fetal and Neonatal
	p21	Inhibitor	G1, S	Impede Cdk2, Cdk4, Cdk6 activity	Fetal, Neonatal, Adult
	p27	Inhibitor	G1, S	Impede Cdk2, Cdk4, Cdk6 activity	Fetal, Neonatal, Adult
	p38	Inhibitor		Induces differentiation	
	p53	Inhibitor	all	Regulates cell cycle checkpoint	unknown
	p57	Inhibitor	G1, S	Impede Cdk2, Cdk4, Cdk6 activity	Fetal
	p193	Inhibitor	G1, S	Regulates G1/S checkpoint	Fetal and Neonatal

Pocket Proteins	Rb	Inhibitor	G1, S	Critical for cell cycle exit and cardiac muscle differentiation, Impede E2F activity	Neonatal and Adult
	p107	Inhibitor	G1, S	Impede E2F activity	Fetal and Neonatal
	p130	Inhibitor	G1, S	Impede E2F activity	Fetal, Neonatal, Adult
TSC	TSC2	Inhibitor	G1, S	Regulates p27	Fetal, Neonatal, Adult
E2F	E2F1	Transcription factor	all	Activator, expressed in proliferating cells, interact with Rb	Fetal and Neonatal
	E2F2	Transcription factor		Activator, expressed in proliferating cells, interact with Rb	Fetal and Neonatal
	E2F3	Transcription factor	all	Activator, expressed in proliferating cells, interact with Rb	Fetal and Neonatal
	E2F4	Transcription factor		Repressor, expressed during differentiation, interact with pocket proteins	Fetal, Neonatal, Adult
	E2F5	Transcription factor		Repressor, expressed during differentiation, interact with p130	Neonatal and Adult
	E2F6	Transcription factor	G1, S	Repressor	Fetal and Neonatal
	E2F7	Transcription factor		Repressor	
	E2F8	Transcription factor		Repressor	
Myc	c-Myc	Transcription factor	all	Critical for cardiac myocyte division	Fetal and Neonatal
	L-Myc	Transcription factor		Critical for cardiac myocyte division	
	N-Myc	Transcription factor	all	Critical for cardiac myocyte division	Fetal and Neonatal

Information for chart from Source (Ahuja et al, 2007)

Appendix B: Interview with the Client and the User

Interview with Professor Gaudette and Jacques Guyette October 5, 2007

1. What would you like our project to accomplish?
   We would like to increase the presence of cell cycle markers within myocytes

2. How would you be using this apparatus/procedure in your own research?
   The apparatus/procedure would be used to isolate cells and grow them in culture with a method to allow myocytes to proliferate

3. How important is it to keep the cells sterile (for your purposes)?
   Have to be sterile…is a constraint

4. Is cost an issue?
   Cost is not a significant issue.

5. How long would you like the culture process to take?
   We would prefer a maximum culture time of 30 days from isolation to proliferation, but two weeks is more realistic.

6. Is lab space an issue (can we take up as much space as necessary, or are we limited)?
   Lab space is not an issue, but culture space is. The system must fit within the incubator and cell culture hood.

7. Have you noticed any specific problems in the past with this type of procedure?
   Contamination is always an issue during cell culture.

8. Do you have a cutoff for the efficiency of the process (ie. 40% must express markers) or is it more about the number of cells?
   There is no specific cutoff, but the efficiency should be somewhere between 5% and 10%.

9. What equipment/materials are available?
   Cell culture lab in Salisbury Labs which consist of an incubator and cell culture hood.

10. How often would it be used?
    If the system works it will be used often.

11. Would they working alone or on a team?
    Plan to have this be a one-person procedure.

12. How many viable end product cells would be necessary to make the investment worthwhile?
    1 million cells would be the goal, finding cell factors

Interview with Professor Pins October 4, 2007

1. What would you like our project to accomplish?
   Design a bioreactor or cell culture system to enhance the efficiency of cardiac myocyte proliferation.

2. How would you be using this apparatus/procedure in your own research?
   I would use these cells to engineer scaffolds and tissues to promote myocardial regeneration.

3. How important is it to keep the cells sterile (for your purposes)?
   This is essential as non-sterile cells are useless!

4. Is cost an issue?
   Always, the cheaper the better.

5. How long would you like the culture process to take?
   As little time as possible. Ultimately, cell culture time directly correlates with patient survival probability.

6. Is lab space an issue (can we take up as much space as necessary, or are we limited)?
   Space is limited. Unless you build a bench-top bioreactor that precisely monitors temp, pH, and humidity, you are limited to a device that will fit into a bio-safety cabinet and a TC incubator.

Appendix C: Pairwise Comparison Charts

Client Charts:

Will Increase the Presence of Cell Cycle Markers	Express Cell Cycle Markers	Efficient	Cost	Safe	Ease of Use	Total	Total + 1	Percent of Objective	Percent Overall
Express Cell Cycle Markers		1	1	1	1	4	5	33	33
Efficient	0		1	0	1	2	3	20	20
Cost	0	0		0	1	1	2	13	13
Safe	0	1	1		1	3	4	27	27
Ease of Use	0	0	0	0		0	1	7	7
						Column Totals:	15	100	100

Express Cell Cycle Markers	Repeatable	Effective	Total	Total + 1	Percent of Objective	Percent Overall
Repeatable		0	0	1	33	11
Effective	1		1	2	67	22
			Column Totals:	3	100	33

Safe	For Cells	For Users	Total	Total + 1	Percent of Objective	Percent Overall
For Cells		0	0	1	33	9
For Users	1		1	2	67	18
			Column Totals:	3	100	27

Ease of Use	Size	Time	Interface	Simplicity	Total	Total + 1	Percent of Objective	Percent Overall
Size		1	0	0	1	2	20	1.4
Time	0		0	0	0	1	10	0.7
Interface	1	1		1	3	4	40	2.8
Simplicity	1	1	0		2	3	30	2.1
					Column Totals:	10	100	7

Minimize Materials	Cells	Media	Equipment	Total	Total + 1	Percent of Objective	Percent Overall
Cells		0	0	0	1	17	3
Media	1		1	2	3	50	10
Equipment	1	0		1	2	33	7
				Column Totals:	6	100	20

User Charts:

Will Increase the Presence of Cell Cycle Markers	Express Cell Cycle Markers	Efficient	Cost	Safe	Ease of Use	Total	Total + 1	Percent of Objective	Percent Overall
Express Cell Cycle Markers		1	1	1	1	4	5	33	33
Efficient	0		1	0.5	1	2.5	3.5	23	23
Cost	0	0		0	1	1	2	13	13
Safe	0	0.5	1		0.5	2	3	20	20
Ease of Use	0	0	0	0.5		0.5	1.5	10	10
						Column Totals:	15	100	100

Express Cell Cycle Markers	Repeatable	Effective	Total	Total + 1	Percent of Objective	Percent Overall
Repeatable		0.5	0.5	1.5	50	16.5
Effective	0.5		0.5	1.5	50	16.5
			Column Totals:	3	100	33

Safe	For Cells	For Users	Total	Total + 1	Percent of Objective	Percent Overall
For Cells		1	1	2	67	13
For Users	0		0	1	33	7
			Column Totals:	3	100	20

Ease of Use	Size	Time	Interface	Simplicity	Total	Total + 1	Percent of Objective	Percent Overall
Size		0	0.5	0	0.5	1.5	15	1.5
Time	1		1	0.5	2.5	3.5	35	3.5
Interface	0.5	0		0.5	1	2	20	2
Simplicity	1	0.5	0.5		2	3	30	3
					Column Totals:	10	100	10

Minimize Materials	Cells	Media	Equipment	Total	Total + 1	Percent of Objective	Percent Overall
Cells		1	1	2	3	50	12
Media	0		1	1	2	33	8
Equipment	0	0		0	1	17	4
				Column Totals:	6	100	23

Appendix D: Mouse Myocyte Isolation Protocol

Adult Mouse Myocyte Isolation

Basic Solution(BS, Ca^{2+}-free)

Compound	Concentration (mM)	Amount (g/L)	Amount (g/2L)
NaCl	112	6.54	13.1
KCl	5.4	0.40	0.8
NaH_2PO_4	1.7	0.20	0.4
$MgCl_2$ 1M Sigma M-1028	1.63	1.63 ml	3.26 ml
$NaHCO_3$	4.2	0.35	0.7
HEPES	20.04	4.78	9.56
Glucose	5.4	0.97	1.94
L-Glutamine[1] (200mM, Gibco Cat.no.25030-081)	4.1	20.5 ml	41 ml
Taurine	10	1.25	2.5
MEM Vitamins (Gibco, Cat.no. 11120-052)	100X	10 ml	20 ml
MEM Aminoacids (Gibco, Cat.no. 11130-051)	50X	20 ml	40 ml

pH 7.4 with NaOH, osmolarity should be ~ 292 mosm.
Sterile filter (0.22 μm). Store at +4°C.

On the day of experiment, make solutions

A. BS 50 ml+ 50ul heparin (Sigma H3393 dilute to 5000units/ml)
B. Perfusion solution: BS 50 ml+BDM (2,3-Butanedione monoxime Sigma B0753) 50 mg
C. Plain BS bubbled with O2 (or 95/5 O2/CO2)- about 100ml for perfusion of tissue bath for cannulating aorta.
D. Digestion solution: BS 50 ml + Blendzyme 4(Roche) 200ul of stock solution (Reconstitute 90mg vial as directed and aliquot 200ul/tube. Do not freeze and thaw.) Add collagenase immediately before perfusion.
E. KB+ ATP- bubble about 50 ml with O2. (Can be done while the heart is digesting).

Procedure

1 Rinse the isolation apparatus with filtered H2O before (and after) use.

2 Bubble A with 100%O2. Pour B into a Langendorf syringe on the apparatus and bubble.

3 Prepare to euthanize the mouse using 0.1ml of euthanasia pentobarb in a 1ml syringe with a 25 gauge needle. Set up three 60mm petri dishes with bubbled A and fill a 5cc syringe with A to use at the tissue bath. Place a small lump of clay on the edge of the tissue bath, fill the cannulation needle with A and wrap parafilm around the end so the needle does not empty. Place the needle in the clay with the end in the bath to cannulate the aorta.

4 Euthanize the mouse by IP injection. Pump C through the tissue bath.

5 Open chest and remove the heart-thymus-lungs complex and rinse in the first dish. Cut away the lungs and rinse in the second dish. Squeeze the heart very gently to expel blood. Transfer to the third dish.

6. Place the heart in the tissue bath

7 Pull away the fat residues from the distal part of aorta (cut again exactly below the branches if necessary) and cannulate it, tie it with 5-0 silk suture between heart and the

first branch, the tip could be seen via the aorta wall. Tie twice. Put the heart on the 5cc syringe and gently expel blood.

8 Transfer the heart to the Langendorf apparatus and perfuse with B for 10 minutes. During this time, prepare D and place in second Langendorf syringe and bubble.

9 Perfuse with D for 6 minutes. Examine heart. If the heart has become lighter in color, perfuse for an additional minute. If not, perfuse for a maximum of 3 more minutes.

10 Remove the heart from the Langendorf apparatus and perfuse with KB in a 5 cc syringe (about 3 ml). Place the heart a petri dish of KB and cut away the atria with scissors, cut the ventricles into small pieces. Transfer the pieces to a 15ml tube and gently pipet up and down with a Pasteur pipet for several minutes. Filter the solution through 210 micron nylon mesh, let the cells settle in the frig for about 20 minutes and resuspend in fresh KB.

Appendix E: Making PDMS Molds

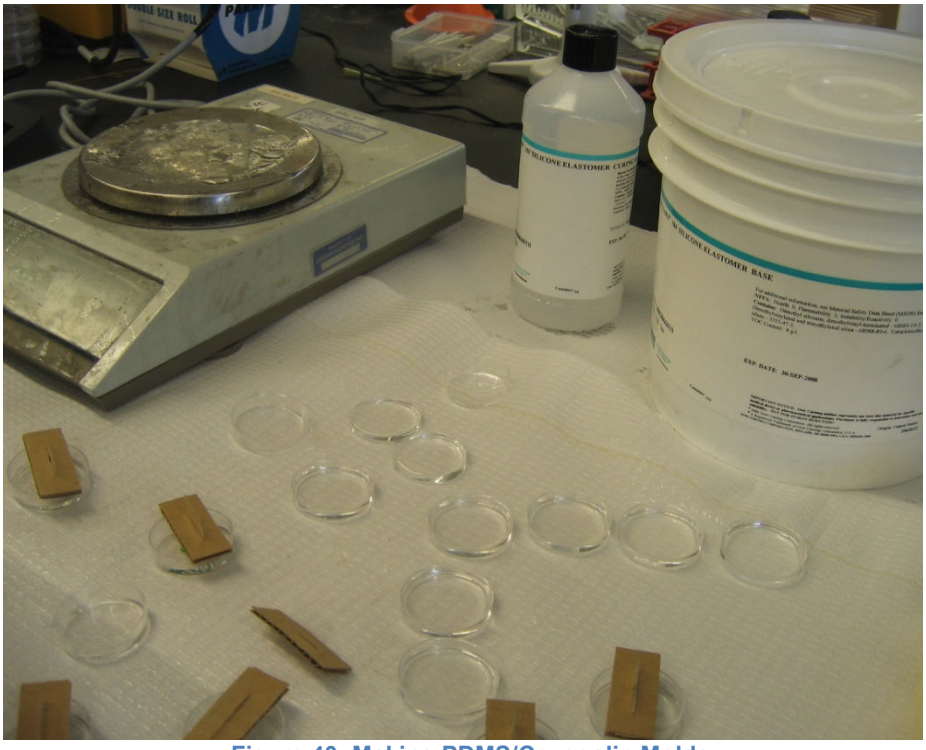


Figure 40: Making PDMS/Cover slip Molds

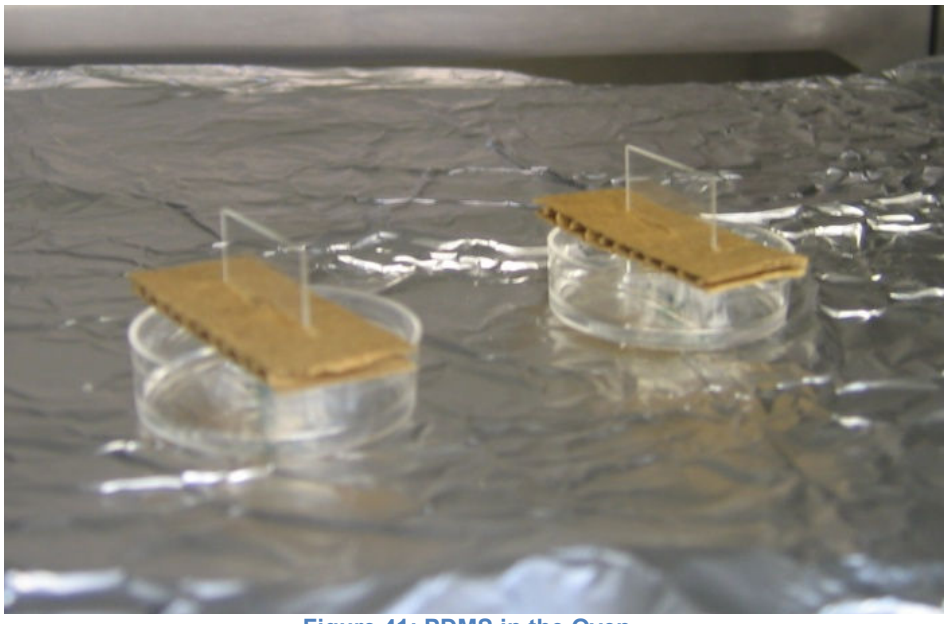


Figure 41: PDMS in the Oven

Appendix F: Making Agarose

After allowing the agarose to cool in the mold (Figure 42), the agarose was placed in Petri dishes and housed in a cell incubator overnight. This was done to ensure that the concentration used would retain its shape when heated to 37°C, as it would be during cell culture.

Figure 42: Preliminary Agarose Testing - Mold

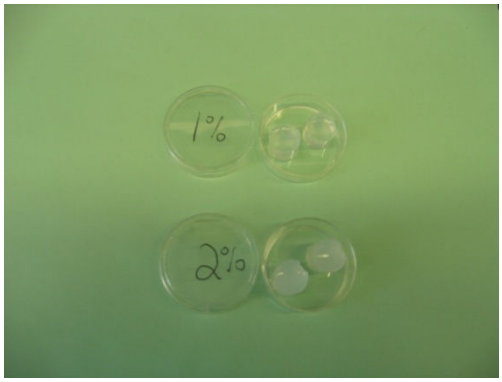


Figure 43: Agarose 1% and 2%

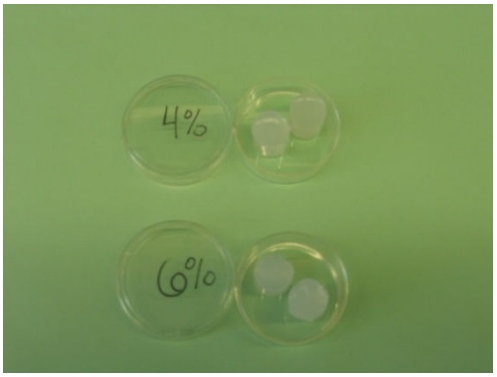


Figure 44: Agarose 4% and 6%

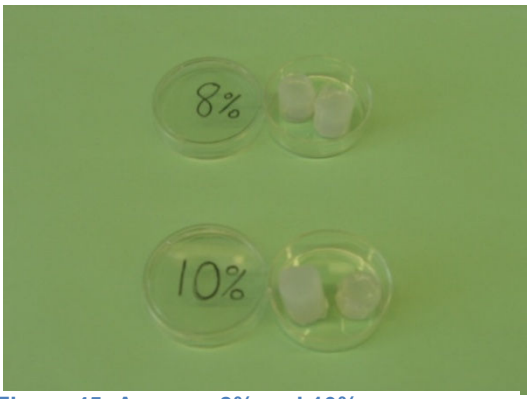


Figure 45: Agarose 8% and 10%

Appendix G: Cyclin D1 Stain: Positive vs. Negative Stain

Positive for Cyclin D1

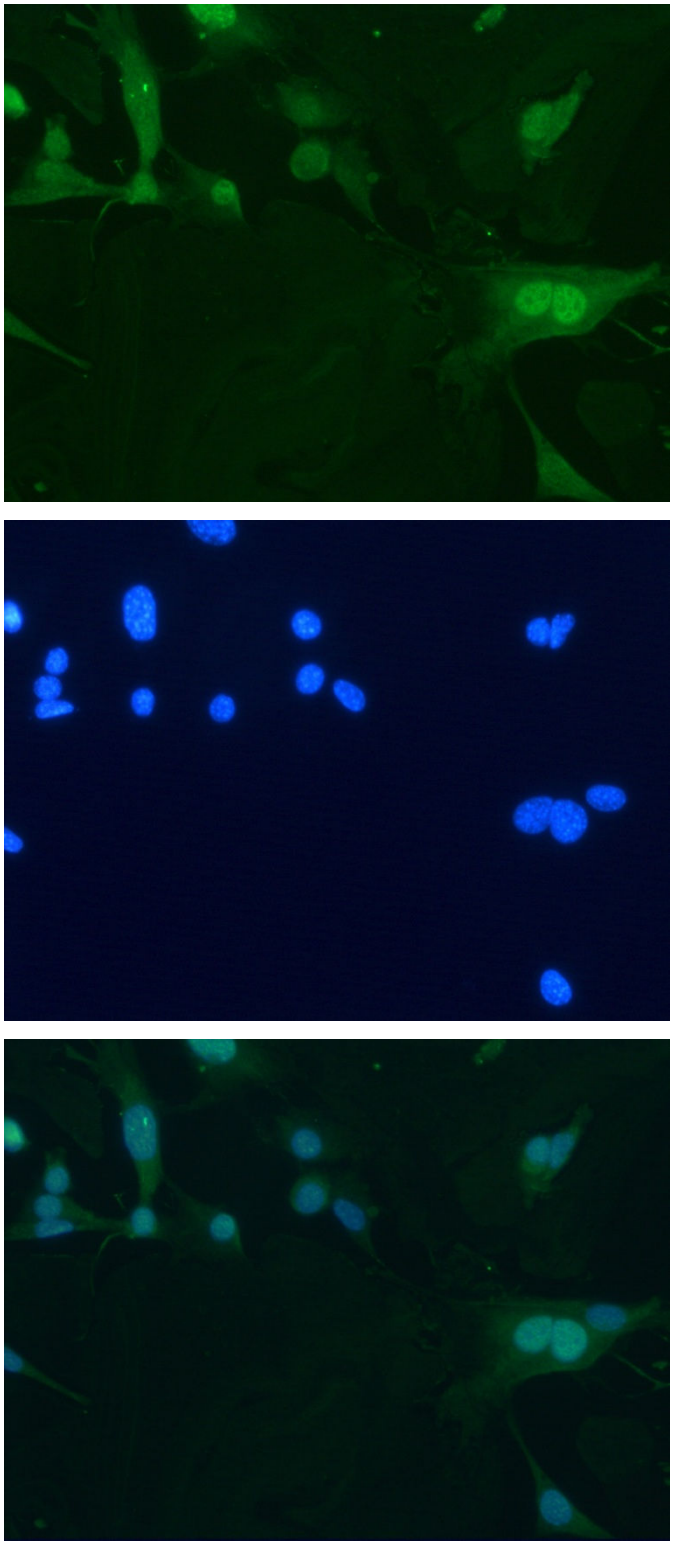


Figure 46: Positive Cyclin D1

Negative for Cyclin D1

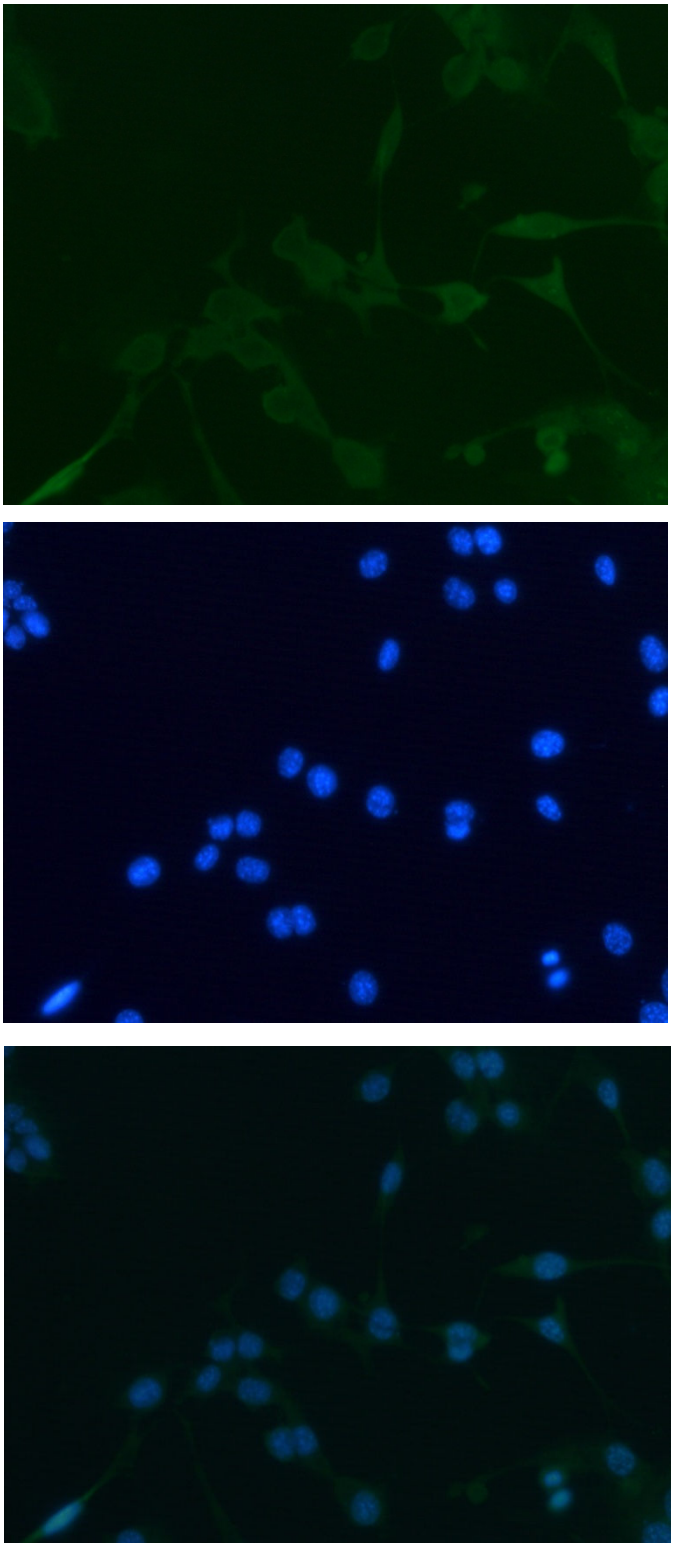


Figure 47: Negative Cyclin D1

Appendix H: α-actinin Stain: Positive vs. Negative Stain

Positive for α-actinin

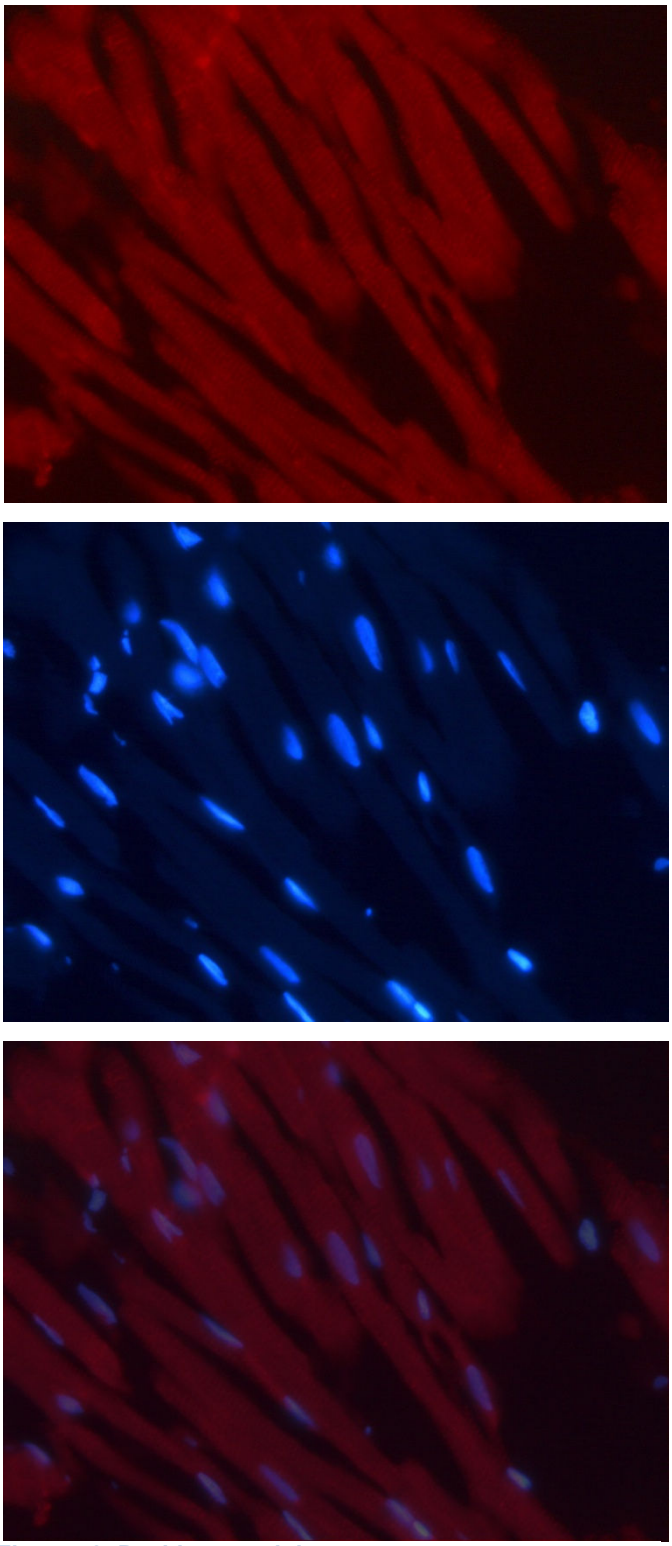


Figure 48: Positive α-actinin

Negative for α-actinin

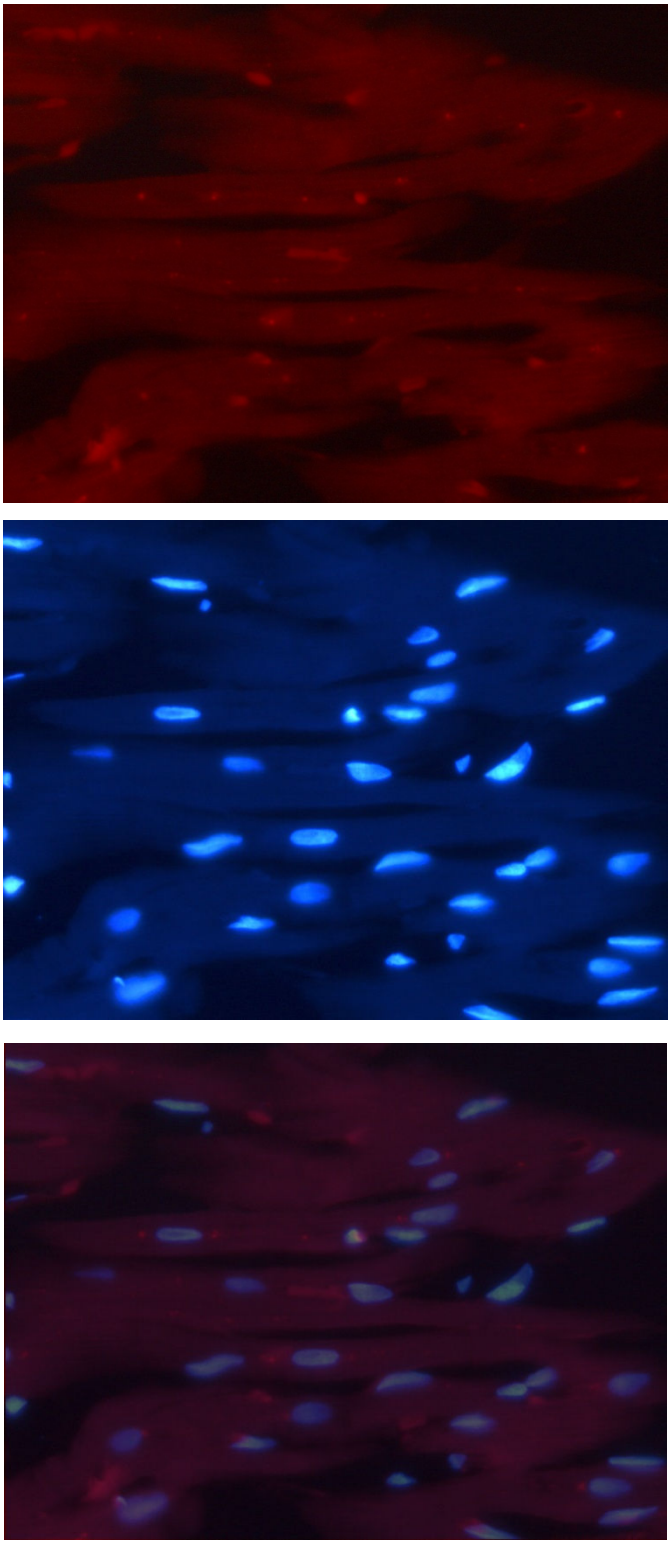


Figure 49: Negative α-actinin