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Authorship Page

All members of the team worked equally in all aspects of this project. Each member's input was essential to the success of the final result.

Chapter 1: Introduction

Almost 3% of Americans suffered from heart failure (HF) in 2010, and this figure has only increased since, showing that HF is becoming an increasingly common issue in the United States [1]. HF is often a consequence of myocardial infarction, during which the heart's myocardial tissue undergoes necrosis as a result of oxygen deprivation. The necrotic region of the myocardium is replaced with collagenous scar tissue, which lacks the contractile mechanical properties of the lost muscle fibers, or cardiomyocytes. Thus, the heart's ability to conduct its life-sustaining function is severely hampered. Returning the heart to its former functional state is nearly impossible.

The commonality of myocardial infarction has given rise to an abundance of research pertaining to cardiac therapies. This research attempts to restore lost cardiac function through a variety of methods. First among these methods is heart transplantation from a deceased donor. Sadly, that is not always possible, since the demand for donor hearts far exceeds the amount of hearts available for transplantation [2]. Transplantation surgery can also result in rather severe health complications due to infection, organ rejection, etc.

Although alternatives to heart transplantation exist, such alternatives are still in their infancy and are under investigation. In the foreground of these new solutions is cell therapy. Cell therapy attempts to regenerate diseased myocardial tissue, rather than replace it through transplantation. There exists a variety of cells that could potentially restore myocardial tissue lost during myocardial infarction, ranging from various stem cells to skeletal myoblasts. For example, in the laboratory of Professor Glenn Gaudette (Ph.D., Associate Professor of

Biomedical Engineering at Worcester Polytechnic Institute), researchers work to study the use of human mesenchymal stem cells (hMSCs) in promoting cardiomyocyte proliferation.

In order to conduct research, Gaudette Lab requires a large supply of cardiomyocytes for in vitro testing. While animal testing is essential for all emerging cell therapies, prior in vitro testing provides important benefits. By testing individual cardiomyocytes in a confined environment, it is possible to limit experimentation to clearly defined variables to be tested, rather than conduct an experiment on an entire organism. Furthermore, as in vitro testing is faster and more economical than other models, it is possible to test potential ideas before any investment is needed, and to discontinue those ideas that are not viable. Cardiomyocytes used for in vitro testing are collected using a process called isolation.

The isolation process begins by extracting the heart from a specimen and placing it in a cold preservative. The extracellular matrix (ECM) must then be broken down. Two popular methods for doing this are the Langendorff method, which involves perfusing a digestive enzyme solution through the heart, and the manual method, which involves incubating minced cardiac tissue in a digestive enzyme solution. Once the ECM is broken down, the tissue is teased apart and the cells are mechanically agitated through trituration. The tissue is allowed to settle and the supernatant is extracted for centrifugation. Subsequent to centrifugation, the cells are collected and analyzed for yield and viability. Cell yield can vary based on the isolation method of choice. For example, Valamarthi et al., who employ the Langendorff isolation method, yield approximately 1.5 to 1.7 million cells [3]. On the other hand, Gaudette Lab, which uses the manual method, yields roughly 0.25 to 2 million cells with 10% viability (percentage of cardiomyocytes that are striated).

Current isolation methods are not ideal. Both methods of deteriorating the ECM have clear limitations. The Langendorff method requires an expensive apparatus and has a high learning curve to use. The manual method can be inconsistent since it depends on several factors, such as how well the user manually minces and teases the tissue. Lastly, the overall teasing and trituration is very time consuming and tedious for the user. As such, an alternate method of isolation is needed.

The goal of this project was to design and build a device that will automate the cardiomyocyte isolation process. This device should be more efficient than previous models. This means that the device should reduce the total isolation time, as well as have a greater, more viable final cell yield. The device must also be safe for both the user and the cells. To allow for repeat usage, the device must be durable. Furthermore, the results must be reproducible, as this device will be used many times in order to consistently provide cells for experimentation. The device should limit the amount of human interaction needed for proper functioning to occur. Finally, manufacturability of the device must be considered.

In order to design and create this device, the engineering design process was strictly followed. After establishing the objectives, functions, and constraints of the project, brainstorming began. We then explored and developed conceptual and preliminary designs. Then we produced prototypes that were tested until a final design that best fits all of the predetermined constraints, objectives, and functions was achieved.

Prior to brainstorming, the manual method, our gold standard, was broken up into four different steps: mincing, teasing, mechanical agitation, and collection. Brainstorming focused around changing teasing and mechanical agitation since they were the most time consuming

steps. For mincing, we chose to cut the heart tissue by using surgical scissors. For collection, we chose to collect the supernatant by using a syringe.

The two steps of the manual isolation process that the device sought to facilitate were the teasing and mechanical agitation steps. In the manual method, tissue teasing is done by tediously teasing apart individual pieces of tissue until they are no more than a couple millimeters in diameter. Through design and laboratory testing, it was chosen that the interaction an abrasive, rotating stainless steel brush and hook-and-loop fabric provided the best teasing effect.

For the mechanical agitation step, which is accomplished by pipetting fluid up and down a pipettor repeatedly in the manual method, a variety of different techniques were tested. These techniques cause shear stress on the cell to cause their release from the extracellular matrix. Through experimentation, a ribbed tube system was selected. This system was implemented in the form of an Allihn condenser, a glass condenser with a water jacket. The Allihn condenser is easy to sterilize and allow for temperature control for peak enzymatic degradation.

After months of material testing and research we were able to build our device, which can be seen in the figure below. Each part is sterilizable through either autoclave or ethylene oxide sterilization. Furthermore, the device in its entirety is able to fit in a biosafery hood to maintain sterility.

The device takes roughly 5 minutes to set up. First the custom made polypropylene base is secured to the ring stand base. Next the stainless steel cup is allowed to rest inside the polypropylene base and is secured to the ring stand. A hook-and-loop attachment is secured to the stainless steel cup. Minced cardiac tissue and kb solution is then placed inside the cup, on top of the hook-and-loop. The brush and motor complex is then brought down onto the tissue and secured to the ring stand. The brush motor is turned on for 7 minutes and the tissue is left to be

teased. Once the 7 minutes are done, the brush and motor complex are removed. The stainless steel cup is then lifted and the teased tissue is allowed to drain through the holes in the cup. An Allihn condenser is then attached to the polypropylene base and the attached pump is intermittently turned on and off every 3 seconds to aspirate and expel the tissue mixture in a turbulent manner. This process is automatically controlled by the use of a timer circuit. After 7 minutes of mechanical agitation the Allihn condenser is removed and the supernatant is collected. After centrifugation, the cells are fully isolated.

After final testing, it was found that the device successfully accomplished all of the goals that were set at the beginning of the project. The cell yield was found to be 700,000 cells. Furthermore, the entire process took thirty minutes; far less than the two hours required for manual isolation. The process automates all the tedious and user-intensive processes. The device is fully autoclaveable and produces consistent results

Chapter 2: Background

Clinical Need

The heart is essential in the transport of blood, oxygen, and nutrients through the body. The ability of the heart to achieve this function is due largely to its muscular architecture. A layer of cardiac muscle tissue can be found within the heart's outer wall. This sheet of muscle is called the myocardium. As the myocardium contracts, the heart acts as a pump, forcing blood through both the pulmonary and systemic portions of the cardiovascular system. The origin of the heart's contractions lies within the cells that constitute the myocardium, which are called cardiomyocytes. During cardiac contractions, cardiomyocytes throughout the myocardium are asynchronously and rhythmically stimulated by action potentials that originate within the heart itself. These electrochemical impulses induce intracellular interactions between myosin and actin filaments that ultimately lead to sarcomeric contractions. The sarcomeric contractions can be cumulatively observed with every heartbeat.

Cardiomyocytes play an integral role in normal heart function, and this role can be jeopardized by the onset of coronary heart disease (CHD), which is characterized by atherosclerosis [4]. As of 2010, the American Heart Association (AHA) estimated that 8.0% of Americans have CHD, and this figure is expected to reach 9.3% by the year 2030 [1]. Due to arterial plaque accumulation, CHD can inhibit the flow of oxygen-rich blood to certain regions of the myocardium, causing myocardial ischemia. If cardiomyocytes do not receive sufficient oxygen to carry out metabolic processes, myocardial ischemia will eventually lead to myocardial infarction (MI), which results in necrotic myocardial tissue, as shown in Figure 1 below.
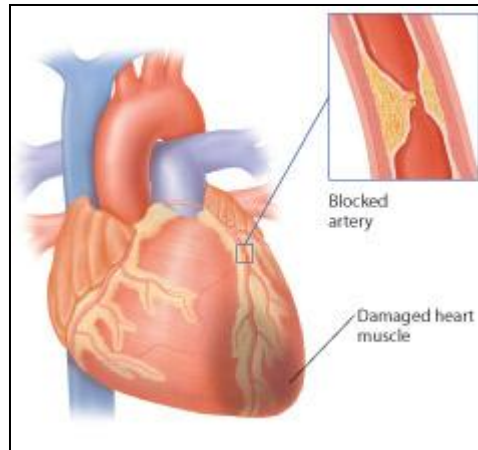
The limited mitotic capacity of cardiomyocytes prevents cell proliferation and myocardial regeneration; instead, lost cardiomyocytes are replaced with stiff, collagenous scar tissue [5]. This scar tissue lacks the mechanical and contractile properties of the remaining myocardial tissue and therefore impairs cardiac mechanical performance, possibly causing heart failure (HF). With MI as a key contributor, 2.8% of Americans experienced HF in 2010; by 2030, HF is expected to affect 3.5% of the U.S. population. Thus, HF is becoming an increasingly common medical issue [1].

In preventing HF, the current state of cardiology provides only two effective treatment options for patients post-infarct: pharmaceutical drugs and heart transplantation. Angiotensin-converting enzyme (ACE) inhibitors and beta-blockers are the two most commonly used pharmaceutical drugs in the prevention of HF [6]. Both drugs work by essentially reducing the work load on the infarct region. ACE inhibitors function as antihypertensive agents. They promote vasodilation by suppressing the activity of ACE, which functions in the production of a peptide that is active in vascular constriction (angiotensin II) [7]. Beta-blockers work in a similar fashion by decreasing the kidney's secretion of renin, which is a peptide hormone that is active in the production of angiotensin I (precursor to angiotensin II) and is thus active in

vasoconstriction and blood pressure elevation [8]. Although both ACE inhibitors and beta-blockers facilitate the infracted heart, they serve merely as a crutch, not a remedy. At the moment, the only way to actually attempt to restore normal heart function is through heart transplantation, which involves the removal of a diseased heart and the implantation of a functional heart from a deceased donor. However, heart transplantation is far from a perfect solution to HF. Firstly, the number of people awaiting a donated heart greatly exceeds the number of donor hearts available; according to the U.S. Department of Health and Human Services, as of September 9, 2011, approximately 3,200 Americans are on the waitlist for a heart transplant, while only 1,159 donor hearts are available [2]. Secondly, a heart transplant may entail post-surgery medical issues. For example, the immunosuppressive medications given to patients in order to inhibit rejection of a donated organ increases patients susceptibility to infections, making antibiotics a necessity [9].

The shortcomings of heart transplantation create a need for an alternative method of restoring heart function post-infarct. Advancements in cell therapy may provide a way to regenerate diseased myocardial tissue and eliminate the need for heart replacement. Contrary to the belief that adult mammalian cardiomyocytes are unable to proliferate, a study conducted by Bergmann et al. concluded that adult cardiomyocytes do indeed undergo proliferation. However, the rate of proliferation is far too slow to replenish the population of cardiomyocytes lost during infarction. At 25 years of age 1% of cardiomyocytes in the heart are replaced per year, and this rate decreases to 0.45% by the age of 75 [10]. Therefore, the main goal of current cell therapy is to find a cell type that can be used to replace cellular elements lost during MI. To achieve this goal, a therapeutically-administered cell type must meet several requirements: it must be readily available, safe to administer, and effective in engraftment, differentiation, and, overall, cardiac

repair [11]. Over the past decade, a variety of cell therapies has been, and is still being, tested for effectiveness in myocardial regeneration. Among these cell therapies are autologous whole bone marrow, skeletal myoblasts, various cardiac stem cell preparations, and bone-marrow-derived mesenchymal stem cells (MSCs), as well as MSCs from other tissues and MSC precursors [11]. With so many cell types under investigation, it is crucial that there be an economical way in which they can be tested. Such ways include in vivo and in vitro testing with cardiomyocytes.

Cardiovascular Models

Animal models have long been considered crucial in conducting scientific research. In vivo testing offers a variety of advantages that other models (e.g., in vitro or computational) lack. For example, animal models allow for the observation of the body's physiological responses to treatments. The biological interactions occurring within different systems of the body are intricate and impossible to efficiently reproduce in vitro. It is difficult to conceive the overarching physiological impact of an experiment on the body without taking the entire biological system into account. Animal models also make it possible to measure immunological responses. Due to this, the FDA requires animal testing as a necessary step in the market release of any drugs. Without successful animal testing, it is impossible to move into the human testing phase [12].

However, animal models have clear limitations, the greatest of which is the ethical standpoint. It is never the goal to needlessly sac animals, and it is important that each animal has a legitimate purpose in experimentation. For this reason, obtaining permission for in vivo testing is a stringent process regulated by the Institutional Animal Care and Use Committee (IACUC) [13]. The WPI subdivision of IACUC routinely inspects all of the animal facilities on campus and evaluates research programs and animal-related activities. IACUC at WPI is the interface

between the national IACUC organizations and the research laboratories on campus. As part of its operation, IACUC at WPI requires that research laboratories complete a protocol declaration form before any animal testing commences. This results in a significant loss of time and money [13].

Another limitation of animal models is their complexity. Scientific research attempts to link a problem to a cause, but that is quite difficult when using animal models [14]. There are far too many uncontrollable variables that cannot be accounted for. Therefore, other research models must be used to test variables in a controllable environment.

In addressing the limitations of animal models, there has been growing support for alternative research models used in conjunction with animal models. This is not only to reduce the use of animal research, but also to provide important testing parameters that animal models do not possess. As stated by the NIH Plan for the Use of Animals in Research, "Biomedical research will be most effectively advanced by the continued application of a combination of models—mathematical, computer, physical, cell and tissue culture, and animal—in a complementary and interactive manner, rather than by concentrating on any one or a few kinds of model system" [15].

This is where in vitro testing using cardiomyocytes come in. While animal testing is likely to always be a requirement for MI research, it is possible to severely reduce its usage by implementing in vitro testing. Through in vitro models, we can examine the effects of a specific experiment on individual cells, rather than on an organism as complex as a rodent. For example, conducting experiments on individual heart cells in Petri dishes provides a completely controllable environment. In vitro models offer a closed system, in which undesirable variables

can be eliminated. There is then no confusion when it comes to establishing which variable could be responsible for a difference in result.

However, in order to conduct in vitro research, a large supply of isolated cardiomyocytes is necessary. The isolation process starts by extracting the heart from a specimen and placing it in a cold preservative. The extracellular matrix (ECM) is then broken down. Two popular methods for doing this are the Langendorff method and the manual method. Once the ECM is broken down, the tissue is teased apart and the cells are mechanically separated through trituration. The tissue is allowed to settle and the supernatant is extracted for centrifugation. Subsequent to centrifugation, the cells are collected.

Heart harvesting procedure:

The final yield of utilizable cardiomyocytes can be affected by the method of isolation. The isolation process starts with heart harvesting. Generally, the specimen is anesthetized and the heart is harvested, placed in a cold preservative solution, and transferred for isolation. The hearts obtained for this project were extracted from rats that were anesthetized with a ketamine (75 mg/kg)/xylazine (10 mg/kg) cocktail and sacrificed by injecting 100 mg/kg of beuthanasia (sodium pentobarbital) into the heart. Sacrifice is verified by flashback in syringe and stop of pulse or respiration. Subsequently, hearts are excised and placed in cold KB solution. Another method, described by Louch et al., first injects the rat subject with heparin (400-5000 U/kg b.w.) to prevent blood clotting or infarction[16]. The rat is then anesthetized with pentobarbital, ketamine/xylazyne, or isoflurane. The heart is excised and placed in a cold preservative solution.

Isolation Methods:

Two of the predominant procedures of isolation are the Langendorff and manual methods. In general, these cardiomyocyte isolation processes consist of two steps. The first step

involves breaking down the ECM surrounding the cells by using digestive enzymes such as trypsin, collagenases, hyaluronidase, elastinase, and protease [17]. The second step consists of mechanically breaking down intercellular connections between myocytes. One method of achieving this is trituration, which requires aspirating and expelling the heart tissue in a solution for several minutes.

The Langendorff method is usually used to isolate cells from larger hearts, such as those from rabbits [17]. The method was first used to model the physiological properties of a perfused heart by Oscar Langendorff in 1895 [18]. It was later adapted to also aid in the isolation process by perfusing digestive enzymes through the heart in order to break down the ECM. After harvesting, the aorta of the heart is anastomosed to a Langendorff apparatus, as shown in Figure2 below. Then, the heart is perfused by a non/low-calcium solution, which disrupts intercellular connections near the intercalated discs. To avoid the Calcium Paradox (the deterioration of heart tissue due to excessive perfusion of non/low-calcium solution followed by a high calcium concentration solution), the non/low-calcium solution is perfused for only three to five minutes[19]. Afterwards, the digestive enzyme is mixed with a low-calcium solution and perfused throughout the heart. Once the ECM is digested, the tissue becomes softer, paler, and more flaccid[16]. The tissue is then triturated with a pipette, and the cells are harvested by centrifugation. O'Connellet al. developed an isolation protocol that uses a Langendorff apparatus [20]. This protocol can be seen in Appendix II. On average, they obtained 1.5 to 1.7 million myocytes per heart, with 65% to 74% rod-shaped (viable) cells [20].
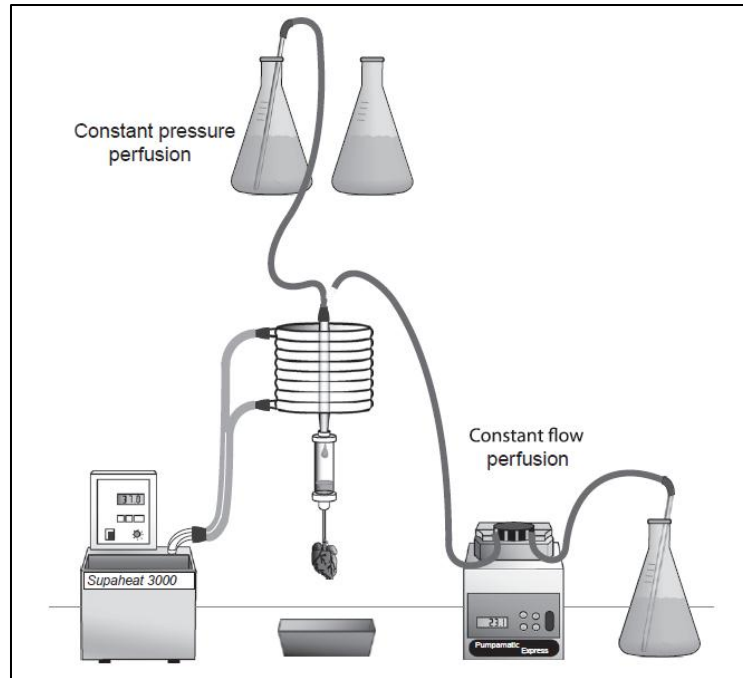
Figure 2: In the Langendorff method the heart is connected to an apparatus similar to that shown above. A heating unit maintains the perfused fluid at a constant temperature, which is usually 37 ºC. To maintain fluid motion throughout the apparatus, pressure can be provided by either gravity(constant pressure perfusion) or an apparatus (constant flow perfusion) [16]

The manual isolation method is more commonly used for small animal hearts. The heart is first minced into smaller portions of a couple millimeters in size. The minced tissue is then placed in an oxygenated, low-calcium (25 µmol/L), enzyme-containing, pH-buffered salt solution, and incubated briefly at 37ºC [17]. After incubation, the tissue is teased and triturated to separate cells. The supernatant is then aspirated and cells are collected via centrifugation. The enzyme solutions used in ECM digestion can differ from lab to lab depending on preference and type of study. For example, Valamarthi et al. used KRB-II-collagenase type II (Worthington) solution in a 37 ºC shaking water bath to digest the ECM[3], while Gaudette Lab currently uses liberase to digest the ECM. For the full description of the Gaudette Lab isolation protocol, see Appendix I. On average, Gaudette Lab is able to obtain 0.25 to 2 million cardiomyocytes per heart, with 10% striated cells (10% viability) [21]. Since our device functions similarly to the

manual isolation method, this yield was the gold standard to which our isolation device was compared.

There are numerous factors that may affect the yield percentage of isolated cardiomyocytes. Such factors include the conditions of glassware, the types of enzymes used, and the duration of enzyme exposure [17]. In order to obtain the best yield, the isolation workspace and tools must be sterile to avoid contamination. Furthermore, different labs use different isolation procedures depending on the animal model, the cells harvested, and the type of in vitro experimentation [16].

Advantages and Disadvantages of Current Methods:

As previously mentioned, the Langendorff apparatus is used for larger hearts that can easily be connected to the device. This method allows for uniform digestion of the ECM. However, even with larger hearts, it is very difficult to connect hearts to the apparatus. Poor connection between the heart and the Langendorff apparatus results in the perfused solution to be projected outwards, which causes a loss of enzymatic fluid and potential danger to the user. Furthermore, the learning curve for Langendorff assembly is much steeper when compared to the manual method.

The manual isolation method is used for smaller hearts and requires no apparatus. The learning curve for the manual method is less steep, since it does not involve the complicated apparatus that the Langendorff method employs. However, the manual method can be inconsistent, because the size of the minced heart pieces and the technique of mincing the heart differ from user to user.

There are also general disadvantages for both methods. Both the Langendorff and the manual isolation method require teasing the heart tissue, which can be very harsh for the cells if

done incorrectly and can decrease yield. The trituration process can also be damaging to cardiomyocytes due to shear stresses produced by pipette tips. Furthermore, the process can be very tedious for the person triturating, since they have to aspirate and expel the solution several times over a period of two hours.

Our Device

Overall, the current methods provide results that are merely adequate at the cost of the user's time and convenience. We propose that a safe, automatic cardiomyocyte-isolating apparatus be built to streamline the process of isolation. We have developed a machine that can break down the ECM of minced heart tissue, tease the tissue, triturate the tissue, and output the supernatant for centrifugation. Our device is able to automatically isolate cardiomyocytes efficiently with minimal human interaction and output consistent yields that match or exceed the standard.

Chapter 3: Project Strategy

After receiving the initial client statement, it was necessary to begin learning more about cardiomyocyte isolation and its clinical significance. We first became familiar with the manual isolation process through the mentorship of Evans Burford, who was a graduate student working in the Gaudette Lab. Next, we conducted research into the subjects of heart disease, the need for in vitro models, and current cardiomyocyte isolation methods. Through this research, as well as interviews with the client and the potential user, we defined the project objectives and constraints. We then constructed an objective tree and pairwise comparison charts to prioritize the design objectives. Finally, we revised the client statement and began to generate alternative designs.

Initial Client Statement

The initial client statement provided by client Glenn Gaudette (Ph.D., Associate Professor of Biomedical Engineering at Worcester Polytechnic Institute) called for a device that automated the cardiomyocyte isolation process. We elaborated upon the client's demands through research into current cardiomyocyte isolation methods and interviews with both the client, as well as a potential user, Evans Burford. The information obtained from the research and interviews helped us formulate our objectives and constraints, which shaped our revised client statement.

Main Objectives

The first step in expanding on the client statement involved establishing the project's main objectives. We concluded that these objectives were:

1. Cardiomyocyte isolation

2. Increased efficiency (in comparison to manual isolation)

3. Safety

4. User-friendliness

5. Durability

6. Reproducible results

7. Automation / limited human interaction

8. Manufacturability

Using a pairwise comparison chart, we determined the relative importance of each main object with respect to other main objectives. The pairwise comparison chart, which was completed not only by the group, but also by the potential user, can be found in Appendix IV part A1-A2.

Sub-objectives for Cardiomyocyte Isolation

In order to expand upon each main objective, we created more detailed sub-objectives. Therefore, the sub-objectives for cardiomyocyte isolation, which is a main objective, were:

1. Heart tissue input

2. Temperature maintenance

3. Increase in tissue surface area

4. Dissociation of cardiomyocytes from tissue

5. Transfer of isolation solution

Heart tissue input was a sub-objective, since it was important that a user be able to insert heart tissue into the device for cardiomyocyte isolation. It was also important that the solutions required for isolation (e.g., liberase) be maintained at an appropriate temperature and that the heart tissue was incubated in a digestive enzyme solution at an appropriate temperature as well. To increase the efficiency of digestive enzymes in breaking down the ECM of heart tissue, it was

necessary that tissue surface area be maximally augmented. The dissociation of cardiomyocytes

from other elements of heart tissue, such that cardiomyocytes could be collected, was also

another important sub-objective in cardiomyocyte isolation. Lastly, it was essential that there be

circulation of required solutions through the device during the intermediary steps of the

automated isolation process (e.g., the addition and removal of Kb solution, or the

cardiomyocytes in supernatant solution). The said objectives were prioritized using the pairwise

comparison chart found in Appendix IV part B1-B3.

Sub-objectives for Increased Efficiency

The sub-objectives for the main objective of increased efficiency were:

1. Faster isolation time than manual isolation method

2. Greater cardiomyocyte yield than manual isolation

3. Greater cardiomyocyte viability

Manual cardiomyocyte isolation can be a lengthy process, taking several hours. Thus, in

improving the efficiency of the isolation process, it was important that decreased isolation time

be an objective. According to the potential user, the device should complete isolation in no

longer than two hours. Improving upon the efficiency of manual isolation also called for an

improvement in cell yield and cell viability. With manual isolation, the cell yield is

approximately 0.25 to 2 million cells per heart, with average viability at approximately 10 %

(percentage of cardiomyocytes that are striated). Therefore, the cell yield and cell viability

achieved with the device must be greater than or equal to those achievable through manual

isolation. A pairwise comparison chart assessing the sub-objectives listed above can be found in

Appendix IV C1-C3.

Sub-objectives for Safety

In creating sub-objectives for safety, we focused not only on user safety, but also on the welfare of the cardiomyocytes being isolated. Thus, the sub-objectives are:

1. Cell sterility
2. User safety

Contamination poses a major threat during cell culturing. Not only can contamination result in the small- to large-scale loss of cells in culture, but it can also jeopardize the integrity of experimental results. Thus, maintaining cell sterility was an extremely important sub-objective and essentially entailed creating a closed system that prevents sample contamination from both chemical and biological sources. Of course, the device must not be harmful to the user. User safety was by far the most important objective. A pairwise comparison chart comparing cell sterility to user safety can be found in Appendix IV D1-D3.

Sub-objectives for User-friendliness

The sub-objectives that fall under device user-friendliness are:

1. Portability / light-weight
2. Ease of repair
3. Ease of maintenance and cleaning
4. Easy-to-adjust settings

User-friendliness was an important objective to consider, because if the device was difficult to use, then it would not be marketable. The device also had to be portable and light-weight; we and the potential user determined that the device should be able to fit inside a bio-safety hood, and it should therefore be easy to transport the device to a bio-safety hood, as well as to and from sterilization facilities. The device had to be able to be easily cleaned before and after isolation

sessions, and should the device become damaged, repairs had to be easy to make (e.g., easy-to-order machine parts). Lastly, as tissue conditions may change from isolation to isolation, the user should be able to easily adjust device settings in order to suit his/her needs. The sub-objectives listed above are compared in a pairwise comparison chart found in Appendix IV E1-E3.

Constraints

In addition to the aforementioned objectives, we also determined constraints for the device. These constraints are:

1. Device must be usable in a bio-safety hood
2. Device must be sterilizable
3. Device must not over-stress cells during dissociation
4. Device must complete isolation within two hours
5. Project costs must not exceed $468 ($156 per person)
6. Project must be completed within one academic year

Unlike the project objectives, which can be fulfilled to varying degrees and assessed using metrics, the constraints listed above must be completely satisfied. Failure to fully address all constraints results in an unsuccessful project.

Revised Client Statement and Project Approach

After determining all project objectives and constraints, we were able to create a more descriptive client statement, which read:

Develop a device that will expedite the isolation of cardiomyocytes from rodent hearts. The device should improve upon prior manual methods by teasing and dissociating heart tissue in an automated and adjustable manner. The device will result in a greater cell yield and viability, as well as reduced isolation time when compared to the standard.

Safety for both the cells and the user will be maintained. User-friendliness will be

accounted for by providing easy access for loading and unloading samples, adjustable

fluid aspiration, and simple device transportation. The device will be easy to repair,

maintain, and clean before and after each use. The device must be usable inside of a lab

safety hood and preserve sterility.

With this revised client statement, we were able to delve into the design process and set forth in

creating a device that successfully automates the isolation of cardiomyocytes.

Chapter 4 – Design Alternatives

After defining our objectives and constraints, we specified our functions and devised several preliminary designs that would fit our criteria. Our designs primarily focused on two functions, the increase of tissue surface area and cell dissociation. Through research of abrasive surfaces, dissociation techniques, and several brainstorming sessions, we were able to create several conceptual designs. Afterwards, we experimentally tested these designs to determine feasibility.

Functions / Morphological Chart

The main function of our device was to isolate cardiomyocyte cells. This function was broken down into the following functions:

- Receive heart tissue

- Increase tissue surface area

  o Minces

  o Tease

- Maintain temperature

  o Maintain Kb solution temperature

  o Maintain liberase temperature

- Dissociate cells from tissue

  o Allow for dissociation rate adjustment

  o Re-initiate teasing afterwards

- Transfer solutions

  o Transfer Kb solution

    ▪ Add Kb solution

- Add to minced tissue for washing

- Add to dissociated solution after supernatant extraction

  ▪ Remove Kb solution after washing of minced tissue

  o Add liberase

  o Extract supernatant

- Able to be sterilized

After defining our functions, we focused on means for our device to perform these functions by constructing a morphological chart, seen below. The functions and means will be explained further in the following sections.

Table 1: Morphological Chart showing different means for each function

Functions	Means						
Receive heart tissue	Whole heart	Pre-cleaned	Pre-cut				
Mince tissue	Wire	Blade	Flattening	Stretching			
Tease tissue	Forceps	Pins	Strainer	Grater	Blade	Abrasive surface	Flat surface
Maintain solution temperature	Hot plate	Fluid/bath	Wires	Fish tank heater			
Dissociate cells from tissue	Flow collision	Ribbed tube	Pipettor	Vortex			
Add liberase	Manual/user						
Add Kb solution	Manual/user	Automatic					
Remove Kb solution	Manual/user	Automatic					
Extract supernatant	Manual/user	Syringe	Strainer/filter				
Sterilizable	Autoclave	EtOH					

Receive Heart Tissue

Firstly, we needed to determine how users would insert heart tissue into the isolation device. Specifically, we needed to decide how processed the inserted tissue had to be. We decided on different forms heart tissue could take upon insertion into the device. We could place an entire heart into the device, or we could begin with the heart already cut into manageable pieces. Furthermore, we could cleanse the heart tissue of blood before placing it into the device, or we could have the machine clean the tissue instead.

Increase Tissue Surface Area: Mincing and Teasing

Tissue surface area must be increased such that the digestive enzyme, liberase, can effectively degrade the ECM that holds individual cardiomyocytes together. We defined mincing as simply cutting heart tissue into manageable pieces of approximately .5 cm^3 in volume, while we defined teasing as meticulously breaking down heart tissue into pieces of approximately 1 mm^3 in area or less. In general, teasing was a more delicate process than mincing, since the tissue had to be broken down into smaller pieces, 1 mm or less in length.

We devised four different ways of mincing heart tissue, the first of which implemented a blade that would simply chop the tissue. We also thought of using a mesh of thin wires that could be pressed against heart tissue to cut it into cuboidal chunks. Applying a normal force to flatten and split tissue, as well as stretching and tearing the tissue with tensile forces, were also considered methods of mincing.

We produced even more ideas for teasing. Inspired by the protocol of manual isolation, we considered using forceps, or pins, to shred and pull apart minced tissue. Another idea was to press tissue through the miniscule holes of a strainer or to work tissue against a cheese grater. Lastly, we devised abrading the tissue between flat, textured surfaces, such as sandpaper.

Maintain Solution Temperature

The temperature of Kb solution and liberase must be maintained at the physiological temperature of 37°C [17 Liu and Melchert]. More importantly, this temperature must be maintained while the tissue is incubated with liberase. In order to achieve this, we considered warming the tissue solution with a hot plate, a heated water bath, wires heated by an electric current, or fish tank heater.

Dissociate Cells from Tissue

After incubation with liberase and teasing, the cardiomyocytes must be dissociated from the weakened ECM. Since the cells are suspended in a Kb/liberase solution, dissociation can be induced by taking advantage of fluid flow. We first considered implementing trituration with a pipettor; in manual isolation, trituration induces tissue dissociation as the pipette tip applies shear forces on the moving tissue solution. Another devised mean for dissociation called for opposing flows of solution, which would cause tissue collisions and the resultant liberation of individual cardiomyocytes. One of our most innovative ideas was to run the tissue solution through a ribbed tube; we hypothesized that the varying diameter of the tube would create eddies, which would dissociate cells. Lastly, we thought of swirling the tissue solution in a vortex as a means of dissociation.

Transfer Solutions

The device must allow for the movement of different solutions. These solutions include Kb solution, liberase, and the final supernatant. The addition of Kb solution is required to wash tissue several times prior to isolation. Furthermore, KB solution must be added to liberase to dilute the digestive enzyme, and the Kb-liberase solution must be added to the tissue for incubation. Upon the completion of dissociation, the supernatant product must be extracted from the device for centrifugation, and more Kb solution must be added to the remaining tissue prior to re-dissociating the remaining cardiomyocytes. We decided that solution movement would either be performed automatically or done manually by the user.

Sterilizable

Finally, we had to figure out how to sterilize our device after each use. Three safe and affordable means of sterilizing the device that are readily available are ethylene oxide gas sterilization, sterilization via autoclave, and cleaning with ethanol.

Ethylene oxide gas sterilization uses EtO gas to kill any microorganisms on the surface of materials. An ISO standard, ISO 11135, dictates the protocol for ethylene oxide gas sterilization [22]. Autoclave dry heat sterilization uses an oven in order to heat the device at a temperature where all foreign particles are eradicated. There exists an ISO standard, ISO 20857, which dictates proper procedure for dry heat sterilization [23]. The standard dictates that the oven should be set at 160 degree Celsius for two hours for proper sterilization [24].

Design Alternatives

After brainstorming means for various functions, several design alternatives were conceptualized. These designs focused specifically on increasing tissue surface area and dissociating cells from the tissue. For the designs that increased surface area, two main designs were conceptualized. These designs were the circular and horizontal drag designs, in which two surfaces are either rotated or dragged against each other, respectively. We also conceptualized alternative designs for the abrasive texture of the opposing surfacing and considered hook-and-loop fabric, stainless steel brushes, cheese graters, and sandpaper as potential materials to use in tissue teasing. For the cell dissociation designs, we conceptualized three main designs: a vortex, a double triturator, and a ribbed tube.

Horizontal Drag

One of the first designs to be conceptualized was the horizontal drag setup for tissue teasing. This design involved a textured plate that slid back and forth within a textured base.

Figure 3 shows one of the early computer-aided design (CAD) models of this design. With this design, a user would have to place heart tissue, along with liberase/Kb solution, into the device. The top surface would then rub against the bottom surface and abrade the tissue with shear forces. Once the tissue has been sufficiently teased, the user would have to aspirate the tissue solution from the bottom of the device.
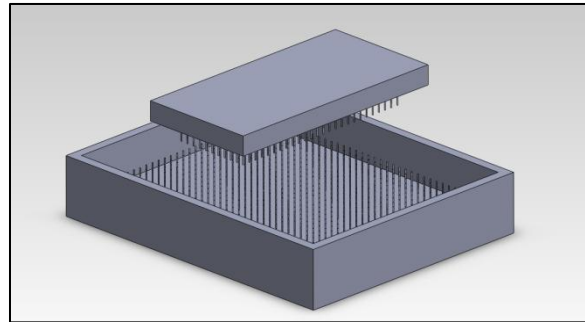
Figure 3: Early conceptual design of horizontal drag. This model shows a pin surface on the top and bottom.

A later design for the horizontal drag device, seen in Figure 4, consisted of some additional components. The textured surface of the base became detachable so it could be customized or replaced. Furthermore, we included input ports for solution to flow into the device and an output port so the user could tilt the base and expel the teased tissue for collection. The output port could be closed with a sliding component.
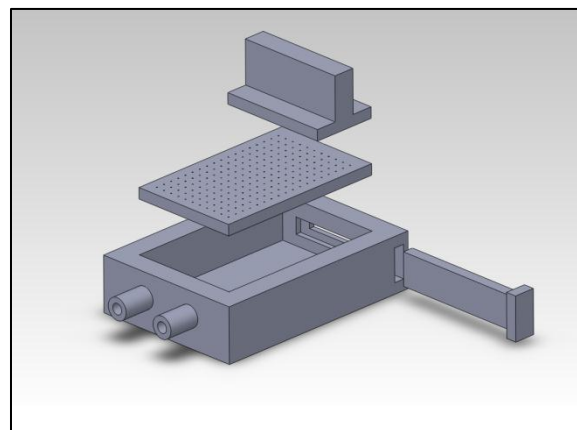
Figure 4: Developed conceptual design of horizontal drag

Circular Drag

The circular drag device for teasing can be seen in Figure 5. The device has three independent parts. The bottom piece, or the base, holds and locks into an attachable, textured plate. This attachment could have any abrasive surface topography, although the Figure 5 shows a pin configuration. The top piece also has an abrasive surface and rubs against the bottom plate. A bar on the upper plate allows for manual rotation about a central axis to break apart the tissue that is held in between the two plates. The diameter of this device is approximately three inches.
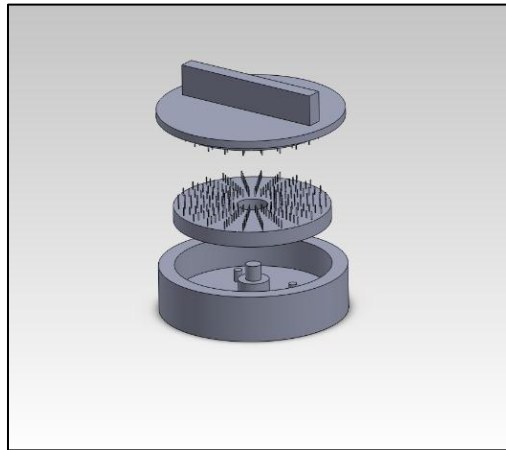
Figure 5: Early circular drag design. This model shows a pin surface on the top and bottom.

A more developed version of the circular drag device can be seen in Figure 6. The device works very similarly to the earlier design, since it has a base and an abrasive plate, which fits into the base and rubs against an abrasive top piece. During teasing, heart tissue is placed into the base-plate assembly. The base-plate assembly fits flush into a Petri dish below, such that liberase/Kb solution from the petri dish enters through the 24 holes in the base and plate and submerges the heart tissue. Once the heart tissue is sufficiently teased, the base is raised from the dish such that liquid and smaller pieces of tissue can fall through the perforations of the base-plate assembly and collect in the dish for dissociation. Important to note is that the holes in the base and plate are arranged in eight rows of three holes. The holes in the plate are concave to

provide teased tissue with a sloped surface for movement and reduce the likelihood of tissue getting stuck on the plate surface. Additionally, the design incorporates a post and plate to place the plate holder above the Petri dish. It is possible to regulate the height of the plate holder depending on the size of the collecting dish.
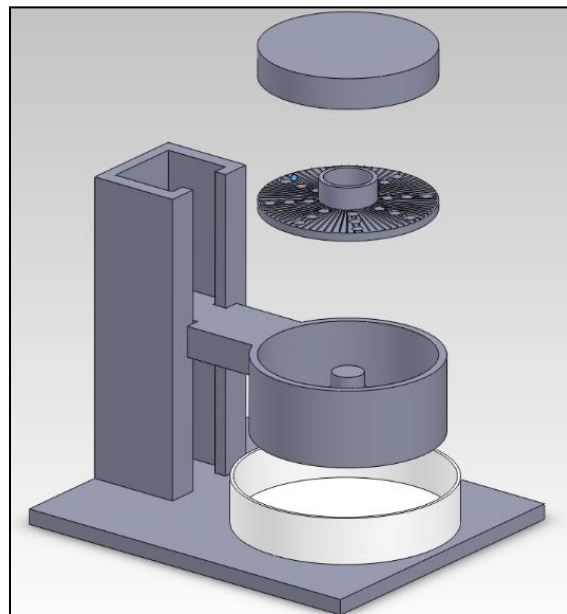
Figure 6: Developed conceptual design of circular drag design

Surfaces

For the two abrasive surfaces in both of the above design alternatives, several different surface materials have been considered. Six different combinations of abrasive surfaces were considered for the teasing of cardiac tissue.

Two Hook-an- loop Surfaces

We first considered abrading heart tissue between two hook-and-loop surfaces. Hook-and-loop fabric possesses a coarseness that originates from the hook-like protrusions. The team believed that moving two pieces of hook-and-loop fabric against one another would result in rapid degradation of heart tissue.

Two Stainless Steel Brushes

We also considered working heart tissue between two stainless steel brushes. We found stainless steel brushes to be suitable for our project for several reasons. Firstly, stainless steel brushes are sharp and extremely abrasive, as they are often used in paint and rust removal. Secondly, the bristles are very hard, meaning that they can withstand abrasion against another hard surface. Also, stainless steel does not corrode and can thus withstand interactions with fluids during cardiomyocyte isolation. In teasing heart tissue with two stainless steel brushes, a minimal amount of normal force is applied on the tissue, as the brushes rely on lateral movement to tear apart the heart.

A Stainless Steel Brush against Hook-and-loop Fabric.

Abrading heart tissue between a stainless steel brush and hook-and-loop fabric was also taken into consideration. We felt that the length and flexibility of the steel bristles would better cater to moving heart tissue back and forth against hook-and-loop fabric. Tissue may tend to roll in place during teasing between two pieces of hook-and-loop fabric, diminishing the frictional forces needed to tear the tissue apart.

A Stainless Steel Brush against a Cheese Grater

We saw potential in using a stainless steel brush in conjunction with a surface similar to that of a cheese grater. The sharp protrusions of a cheese grater would work in unison with the sharp steel bristles to shred heart tissue, and pieces of teased tissue could fall through the holes of the cheese grater for easy collection. The uniform perforation of a cheese grater would guarantee that all the teased tissue pieces are of the appropriate size.

Sandpaper against Sandpaper

When thinking of abrasive materials, sandpaper was the first to come to mind. By rubbing heart tissue between two pieces of sand paper, the tissue would be rolled, lengthened, and shredded into very small pieces. The primary concern with using sandpaper for teasing is the release of the particulate matter form the sandpaper surface.

Vortex

One devised method of cardiomyocyte dissociation is the use of a vortex. We thought that the acceleration of teased tissue masses around a vortex would induce tension in the tissue and pull individual cells apart. We also looked to the downdraft at the center of a vortex to agitate tissue and cause dissociation. Approximately 20 mL of Kb solution containing digested tissue and traces of liberase would be placed in a container. A magnetic stirrer would then be used to create a constant vortex. A magnetic stirrer would allow for the gradation of vortex intensity and tissue agitation. Furthermore, commercially available magnetic stirrers also have a hot plate incorporated into them, so the temperature of the spinning solution would also be controlled.

Double Triturator

The double triturator can be seen in Figure 7 and was inspired by the trituration of manual isolation. The double triturator was conceptualized as a tube of constant diameter with a segment of smaller diameter midway along the tube's length. Roughly 20 mL of Kb solution containing digested tissue and trace amounts of liberase would be placed in the tube and oscillated back and forth using pistons on either side of the tube. The sudden change in diameter would create turbulence in the solution flow and shear stress on the tissue (similar to the role of a pipette tip during the trituration of manual cell isolation) and cause cells to dissociate. The rate of

oscillation would be a change of flow direction approximately every two seconds, which is in accordance with the trituration rate used in manual isolation.
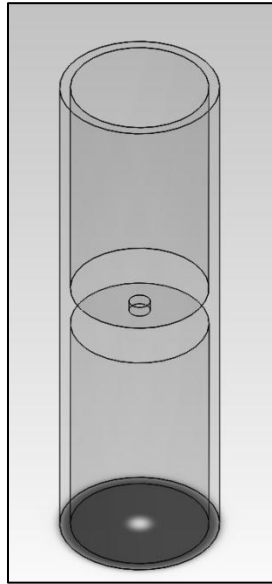
Figure 7: Double triturator conceptual design

Ribbed Tube

The ribbed tube involves a tube with an inner diameter that varies between 1 cm and 0.5 cm, as shown in Figure 8. This change in inner diameter creates disturbances in solution flow and results in eddies. These eddies would agitate the heart tissue in solution, allowing for the release of cardiomyocytes from the ECM. The tissue solution is passed through the ribbed tube repeatedly to provide maximum cell release. Similar to the double triturator, the direction of the flow would be changed every two seconds. A drawback of this design alternative is that ribbed tubing would have to be a molded, custom-made piece, complicating the manufacturing process.
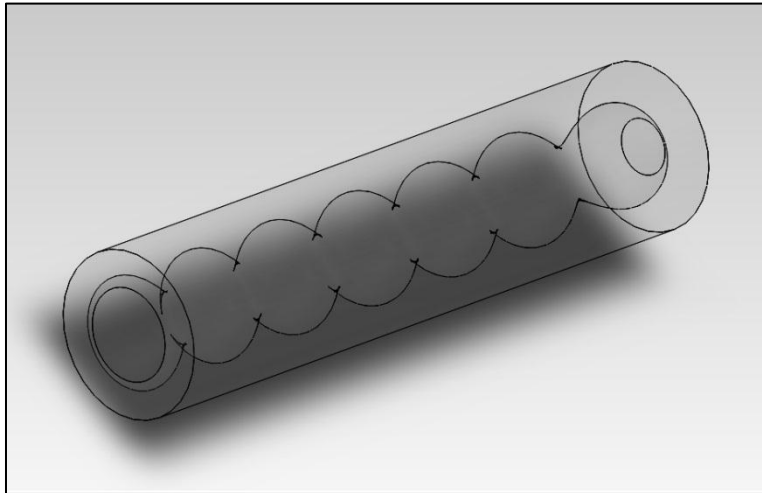
Alternative Design Simulation

Before constructing full-fledged prototypes of our conceptual designs, it was necessary to economically simulate our ideas in the lab to roughly determine design feasibility and efficacy. In order to conclude the best method of increasing tissue surface area, we bought inexpensive items to either tease rat heart tissue or simply mince it. After working the heart tissue with the various items, we examined the product both by eye and by microscopy to determine the degree of tissue separation as well as cardiomyocyte yield and viability. We also simulated methods of dissociating the bound cardiomyocytes; we focused on either spinning teased tissue in a vortex or triturating teased tissue through a normal pipette tip or a ribbed tube. We also examined the dissociation products via eye and via microscopy.

In order to observe the efficacy of pins in abrading and teasing apart cardiac tissue, we first worked tissue back and forth between two stainless steel brushes. Tissue pieces that became stuck between bristles could be removed with relative ease by tapping the brushes against a hard surface. Furthermore, as shown in Figure 9 below, the stainless steel brushes produced extremely

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fine tissue pieces, and striated (viable) cardiomyocytes were observed in the tissue suspension. However, during the second teasing attempt, cleaning the brushes with bleach led to bristle corrosion and the contamination of the isolation solution with rust particles, as shown in Figure 9. Cell striation, however, was still observed.
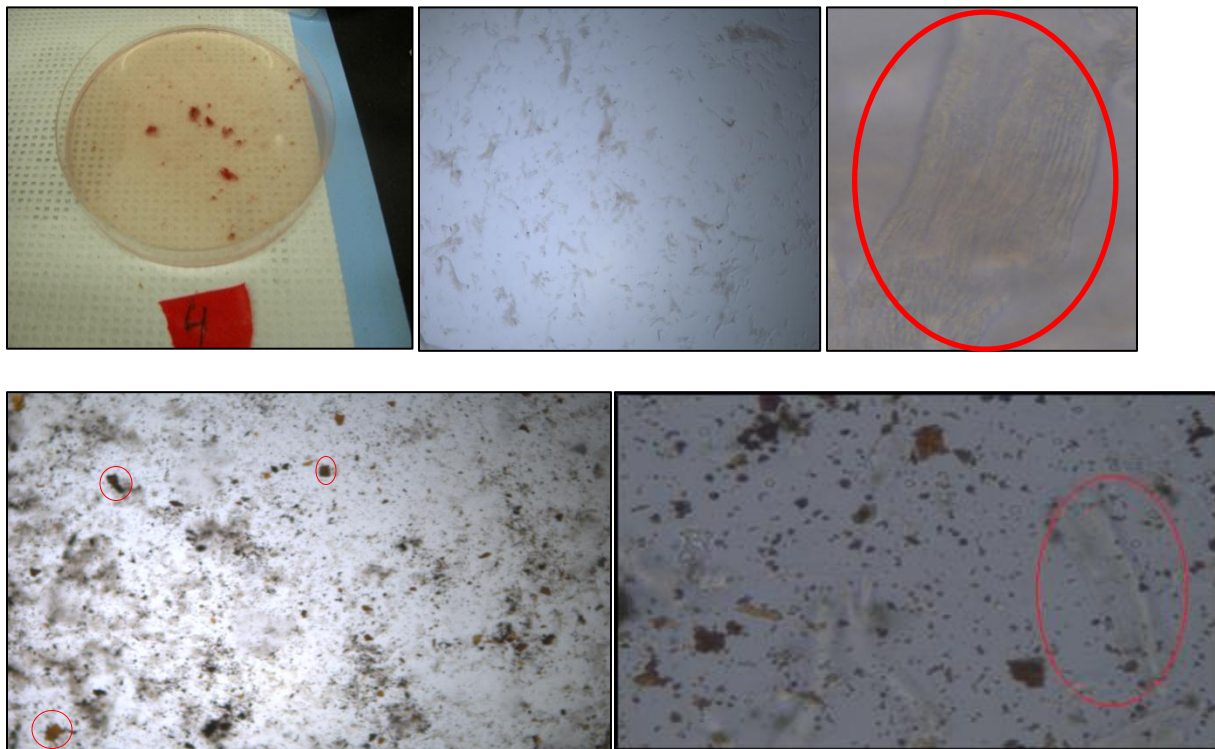
Figure 9: Fine tissue pieces remained in PBS solution after teasing with stainless steel brushes (left). Microscopy of isolation solution at 4xmagnification shows tissue separation (top middle). Microscopy at 40x magnification shows a striated cell circled in red (top right). Washing the stainless steel brushes led to corrosion (rust particles circled in red) and sample contamination (bottom left). However, teasing still yielded striated cells (bottom right, striated cell circled in red).

In attempting to tease heart tissue, we also tried abrading tissue between a steel brush and a cheese grater. The goal of this simulation was not only to tease apart the heart tissue, but also to have small tissue pieces fall through holes in the cheese grater and into a petri dish as a method of tissue collection. This simulation was very messy and the tissue did not fall through

the grater holes as hoped; however, we were able to produce very fine tissue pieces that contained striated cells, as shown in Figure 10.

Figure 10: Fine tissue pieces remained in PBS solution after teasing with a stainless steel brush and a cheese grater (left). Microscopy of isolation solution at 4xmagnification shows tissue separation (middle). Microscopy at 40x magnification shows a striated cell circled in red (right).

Working the heart tissue between two pieces of hook-and-loop fabric seemed to produce fine tissue pieces upon gross inspection. At 40x magnification, we observed striated cells as we did after teasing with the two aforementioned methods. However, attaining the teased tissue from the fabric proved to be problematic; a nylon brush was needed to release the tissue pieces into solution. The teased tissue obtained using hook-and-loop fabric is shown below in Figure 11.
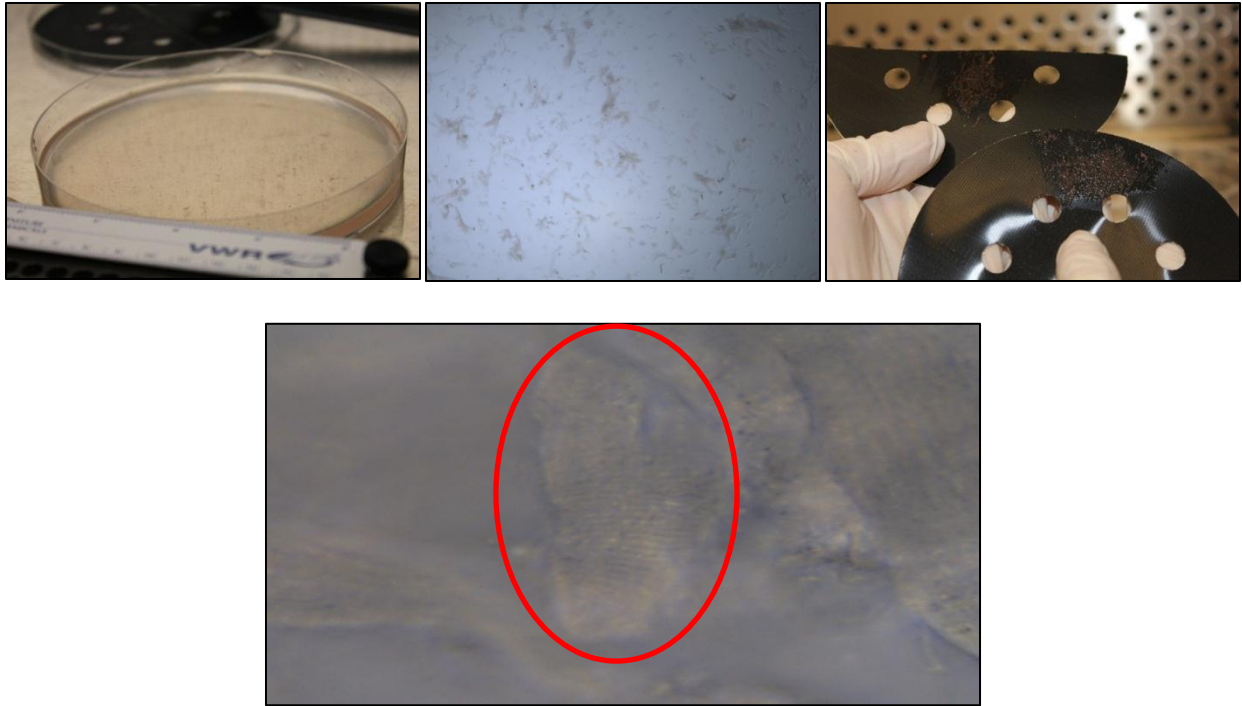
Figure 11: Fine tissue pieces remained in PBS solution after teasing with hook-and-loop fabric (left). Microscopy of isolation solution at 4x magnification shows tissue separation (top middle). Tissue pieces became stuck in hook-and-loop fabric and required removal via nylon brush (top right). Microscopy at 40x magnification shows individual striated cells (bottom, striated cell circled in red).

Abrading the tissue between two pieces of sandpaper produced poor results. The sandpaper left particulate matter in the tissue solution, and striated cells were absent from the solution. Images from sandpaper testing are shown below in Figure 12.

Figure 12: Upon gross inspection, particulate matter from the sandpaper surface was clearly visible in the tissue solution after teasing (left). At 4x magnification, sandpaper particles (circled in red) can be seen intermingled with teased tissue (middle). At 40x magnification, striated cells are not observed (right)..

The said tests have all focused on abrading tissue in order to tease it apart and increase surface area (to increase liberase efficacy). In attempting to increase tissue surface area, we also tested different methods of simply mincing heart tissue into manageable pieces, a process that would occur prior to teasing. We first tried simply chopping the heart tissue with a razor blade and found that, while the blade easily cuts through the muscular apex of the heart, it is not useful in chopping the heart at the base. We then tried working heart tissue through a garlic press, with hopes that cylindrical strands of heart tissue could be produced and collected. However, the heart tissue failed to cleanly exit the holes of the garlic press and had to be manually removed from the inside of the press. This yielded large pieces of heart tissue with a punctate pattern from the holes of the garlic press. These results are shown below in Figure 13.
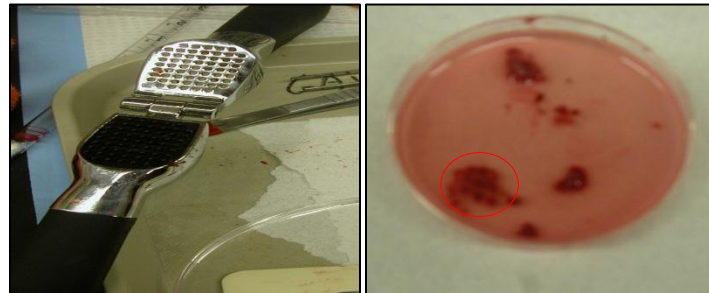
Figure 13: Working the heart tissue through the holes of a garlic press (left) failed to produce the desired cylindrical strands of heart tissue. Instead, the heart tissue had to be removed from the inside of the press and displayed punctate patterns (circled in red) from the holes of the press (right).

After testing the garlic press, we tried to dice the heart with a makeshift wire mesh that was made using a switch plate and stainless steel wire. The mesh was unable to fully penetrate and dice the heart tissue. The fabricated wire mesh is shown below in Figure 14.
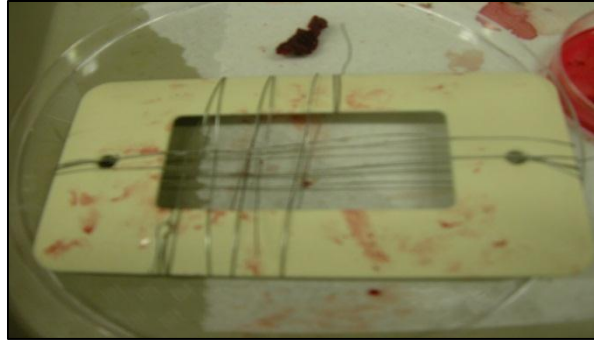
Figure 14: The makeshift wire mesh failed to cut the heart tissue (shown next to the mesh).

In addition to the tests mentioned above, which all focused on increasing tissue surface area, we also conducted tests in cell dissociation, which is necessary in actually obtaining isolated cells from teased tissue. After receiving a solution of tissue that had been teased using hook-and-loop fabric, we divided the solution into three equal quantities and subjected each solution to a different dissociation treatment. The dissociation treatments were conventional trituration, trituration through a ribbed tube, and tissue spinning a vortex. Microscope images of the tissue solution prior to dissociation are shown below in Figure 15.
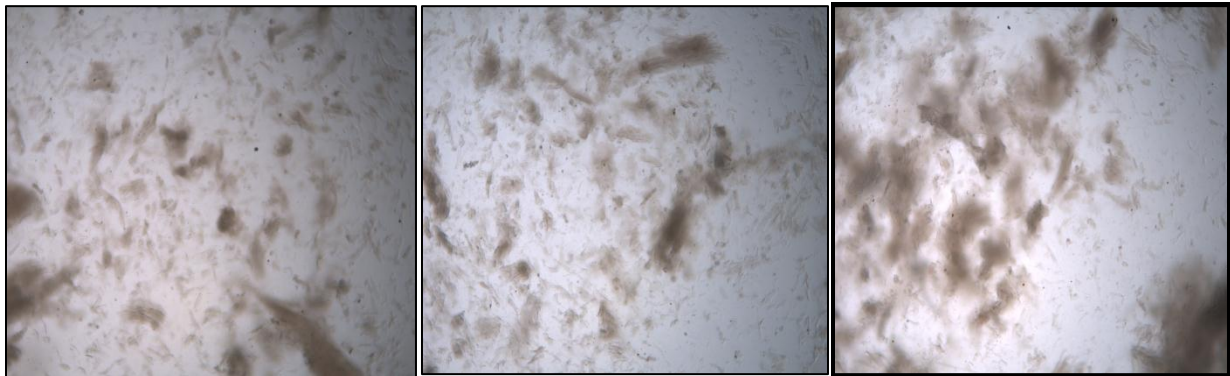
Figure 15: 4x magnification microscope images of hook-and-loop-teased tissue prior to dissociation.

Conventionally triturating the tissue consisted of simply aspirating and expelling the tissue solution with a pipettor (shown below in Figure 16), allowing the bore of the pipette tip to exert shear forces on the passing solution and dissociate the cells. This was done for seven minutes (the time taken to triturate tissue solution during manual isolation), with an

aspiration/expulsion duration of two seconds. The triturated solution was examined via microscopy. When compared the triturated solution to the tissue solution prior to dissociation (shown in Figure 15), we observed noticeable cell dissociation. The results of trituration are shown below in Figure 16.
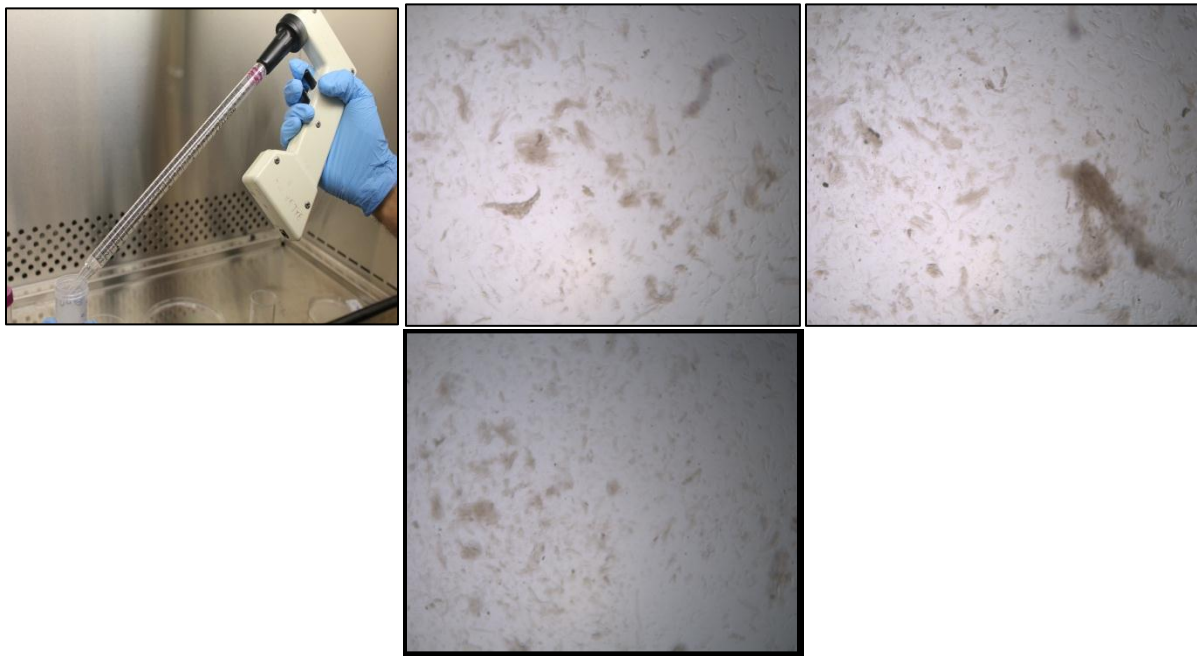
Figure 16: Trituration consisted of simply aspirating and expelling solution through a pipette tip (top left). Microscope images of triturated tissue at 4x magnification (top middle, top right, bottom) show noticeable cell dissociation when compared to images of tissue prior to trituration.

As we conducted traditional trituration with a normal pipette tip, we simultaneously performed trituration using a pipette tip modified with a ribbed tube. To create this modified pipette tip, a 1-cm diameter rubber tube was maximally constricted along its length at 1.5-cm intervals with zip ties and attached with masking tape to the end of a pipette tip, as shown below in Figure 17. The goal of this test was to induce cell dissociation through eddies created by the undulations in the tube diameter. Using this construct, tissue solution was triturated for seven minutes with an aspiration/expulsion duration of two seconds. Similar to normal trituration, this

modified trituration produced noticeable cell dissociation, but to a lesser extent, as shown in

Figure 17.

Figure 17: Modified trituration consisted of simply aspirating and expelling solution through a ribbed tube (top left). Microscopy at 4x magnification shows that this form of dissociation produced noticeable cell dissociation (top middle, top right, bottom). However, this method of dissociation appears to be less effective than normal trituration.

As the two trituration tests described above proceeded, we also simultaneously conducted tissue dissociation using a vortex. To create the vortex, we simply mixed the teased tissue solution using a magnetic stirrer. Similar to the trituration tests, the vortex test was allowed to proceed for seven minutes. As displayed below in Figure 18, while the vortex did cause some cell dissociation, it appears to be the least effective of the tested dissociation methods.
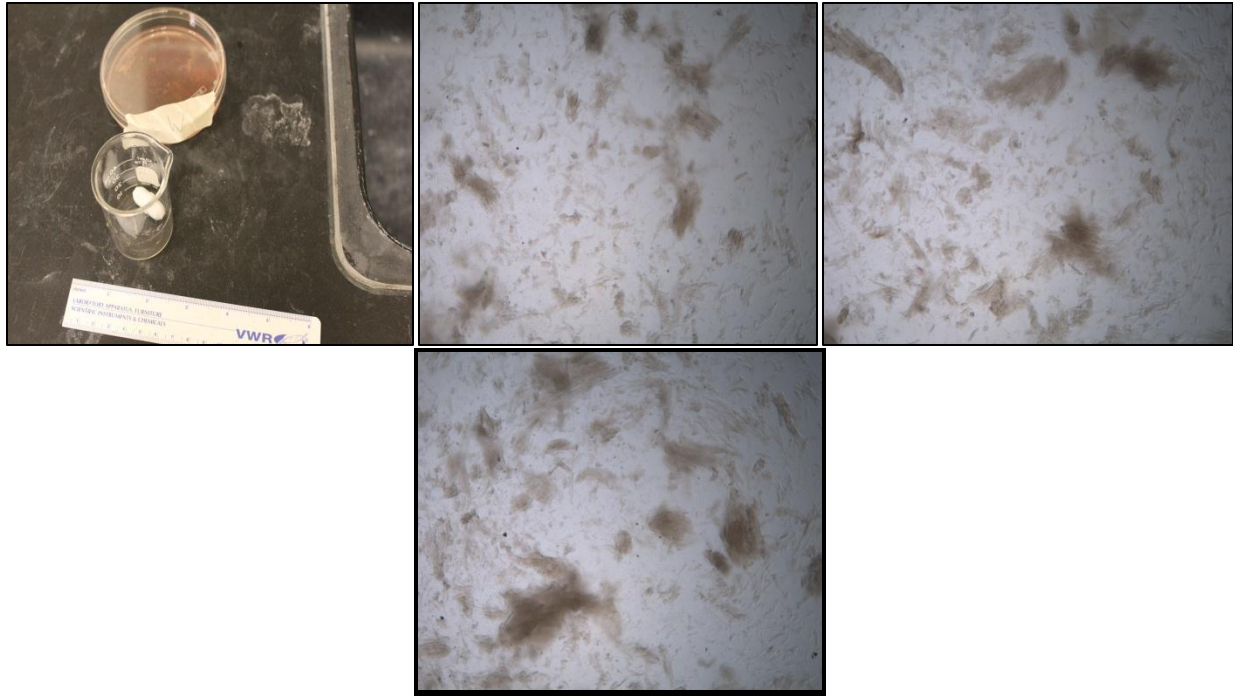
Figure 18: Vortex dissociation consisted of stirring the teased tissue solution with a magnetic stir bar (top left). Microscope images of tissue dissociated via vortex at 4x magnification (top middle, top right, bottom) show minimal cell dissociation when compared to images of tissue prior to trituration.

Quantitative Hemocytometry

While microscopy results were useful in assessing the different methods of tissue mincing/teasing and cell dissociation, we required quantitative data pertaining to cell yield. To obtain this data, we used a hemocytometer in conjunction with trypan blue staining (see Appendix V) to estimate the number of viable cells present after dissociation simulation. As mentioned above, we first teased heart tissue using hook-and-loop fabric and then assigned a third of the teased tissue solution to each of the three dissociation methods. Dissociation was allowed to proceed for seven minutes, which is equal to the duration of trituration during manual isolation. We then mixed 10 µL of each dissociated solution with 10 µL of trypan blue to stain dead cardiomyocytes blue. Using a hemocytometer, we found that the three means of dissociation all produced similar cell yield: approximately 911,111 living cells remained after

trituration, approximately 1,066,666 living cells remained after trituration through the ribbed tube, and approximately 933,333 living cells remained after the vortex treatment. At the beginning of the project, our client asked that our device obtain at least 50,000 viable cells from a heart, and our simulations showed that our device may possibly exceed this expectation by a factor of 20.

Assessment of Alternative Designs

After reviewing the results of the aforementioned design simulations, we were able to determine which design alternatives would best achieve the functions of our device. However, in assessing our designs, we reflected on more than just the findings from microscopy and trypan blue. We also had to consider the feasibility of the different designs, in terms of both sterility and manufacturing.

Assessment of Design Alternatives for Increasing Tissue Surface Area

In assessing the design alternatives for increasing tissue surface area, we automatically excluded sandpaper from our project. As can be seen in Figure 12, sandpaper contaminated tissue solution with particulate matter and thus posed a great risk to cell sterility and viability. We also dismissed using the cheese grater; the majority of tissue became lodged in the holes of the grater, and the pieces that did manage to fall through the holes were too large to be considered thoroughly teased. Furthermore, the designs in which we aimed to simply mince tissue (i.e., the garlic press, the wire mesh, and the razor blade) were rejected since they failed to completely cut through the heart tissue. After setting all of these design alternatives aside, we were left with the stainless steel brush and hook-and-loop fabric. Although both of these materials seemed to tease heart tissue equally well, they each had their imperfections. We found that, when hook-and-loop material alone is used to abrade heart tissue, tissue becomes stuck in

the textured surface; this leftover tissue could threaten the sterility of future isolations.

Additionally, we feared that extensively using two opposing stainless steel brushes would lead to

the degradation of bristles, which could lead to isolation contamination as well. Thus, we

decided to abrade heart tissue between a stainless steel brush and hook-and-loop fabric. Using a

stainless steel brush against hook-and-loop fabric seemed to put less wear on the stainless steel

bristles, and the steel bristles seemed effective in somewhat loosening tissue from the hook-and-

loop surface. With these two materials, manufacturing is not an issue since stainless steel brushes

and hook-and-loop fabric are readily available in many stores. Also, after teasing heart tissue

between hook-and-loop fabric and a stainless steel brush, viable striated cells were observed in

solution. The results from teasing tissue with a stainless steel brush and hook-and-loop fabric can

be seen below in Figure 19.
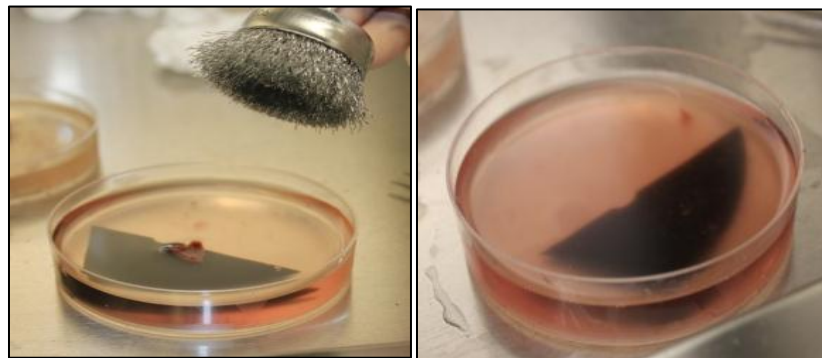
Figure 19: The hook-and-loop and stainless steel brush was able to tease cardiac tissue into very fine pieces.

Assessment of Design Alternatives for Cell Dissociation

In assessing the design alternatives for cell dissociation, we first considered the results of

the trypan blue stain. As mentioned above, we found the highest cell yield after dissociation with

the ribbed tube (1,066,666 living cells). Although we initially perceived the manufacturing of

ribbed tubes as a problem, this worry was quickly extinguished when we thought of

implementing an Allihn condenser as a ribbed tube. As shown below in Figure 20, Allihn condensers, which are used extensively in the cooling of hot vapors and liquids, feature a flexuous diameter for eddy production and a water jacket, which can be used to conveniently warm solution during dissociation.
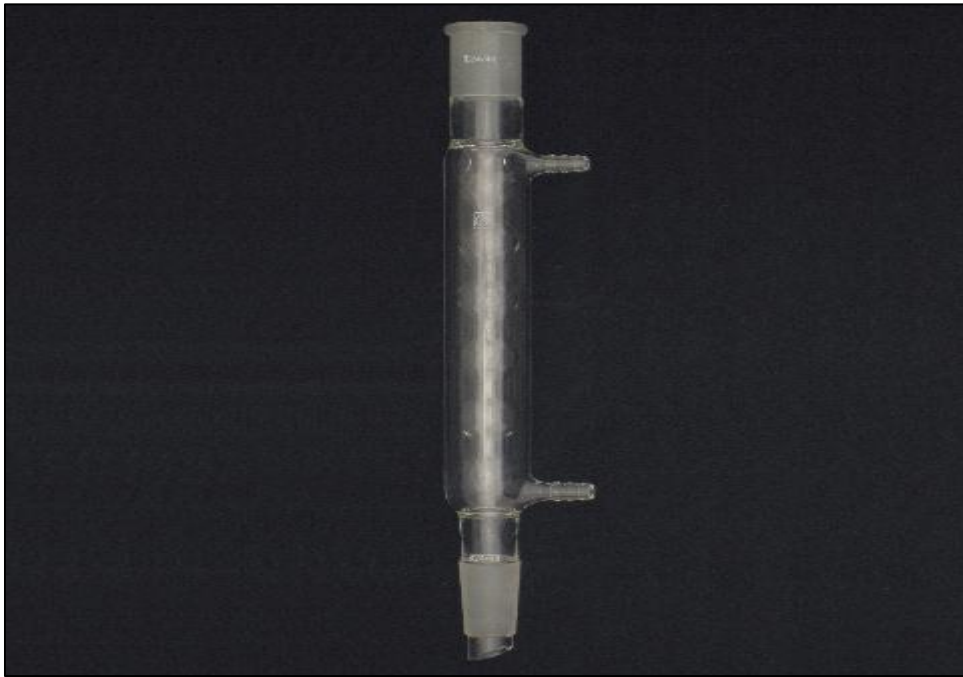
Figure 20: An Allihn condenser was the perfect substitution for a ribbed tube [25].

Using an Allihn condenser is more advantageous than using a magnetic stirrer to create a vortex or using a double triturator. Our client felt that the magnetic stir bar needed for vortex dissociation is unsanitary, and the double triturator would be extremely difficult to manufacture. Thus, the team agreed to use an Allihn condenser in cell dissociation.

Chapter 5

In order to determine whether the device accomplished everything that was required of it. Several experimental protocols were established. These protocols gave us decisive benchmarks to determine success of device.

Experiment 1: Time Efficiency

There are two different ways to establish if the device is efficient in time. First, is the overall process faster than the manual method? This is separated into the duration of teasing and trituration. Teasing takes about 10-15 minutes. The device will be compared in 5 or more trials to determine whether teasing is now faster. Time efficiency for teasing is dependent on rotation speed of the brush. However, as yield is more important the rotation is set to 20 rpm to prevent cell lose.

For trituration, the duration is not constant because it is dependent on the number of cycles completed before the process no longer yields many cells. This cycle number is variable, and has been found to be between 3 and 7 cycles, and takes about 1.5 hours. The goal of this experiment, then is to see the amount of cycles before it is no longer beneficial to continue as very few cells are isolated.

Experiment 2: Cell Count

This experiment is the basis of the device, and must thus be repeated several times to standardize outliers. This experiment is repeated 5 different times. This experiment can be performed at the same time as experiment 1. The final live, striated cell count is recorded and compared to the benchmarks which were previously set. In order for the device to be successful, the device must have a higher result than 25,000 cells, which is the minimum amount of cells

that is obtained through the manual methods. In order to compete with the gold standard—the

Langendorff method--- the device must be able to consistently produce 600,000 cells.

Experiment 3: Sterilization

Testing sterilization is a simple test. Because all the materials, except for the attachment

piece, were selected for their ability to be autoclaved, each individual must be placed in the

autoclave and the autoclave must be run according to laboratory recommended settings for full

sterility. These settings are:

For the attachment, it must be placed in the ethylene oxide sterilization machine, and the

machine must be run according to laboratory recommended settings. The device must then

appear ready for usage.

Chapter 6: Discussion.

In order to assess the success of this device, it is essential to revisit the objectives set at the beginning of the project and to establish how well each objective has been fulfilled. The main objectives were cardiomyocyte isolation, increased isolation efficiency, safety, user-friendliness, durability, reproducible results, automation/limited human interaction, and manufacturability.

Cardiomyocyte Isolation

The first objective, cardiomyocyte isolation, describes the overall goal of this project. Our device has been found to successfully isolate cardiomyocytes by partially automating tissue teasing and cell dissociation processes.

Improved Efficiency:

To improve the efficiency of the isolation process, we must increase cell yield, increase cell viability, and reduce isolation time. The performance of our isolation device is compared to that of the manual isolation method currently used in the Gaudette Lab. As previously stated, this method isolates 0.25-2 million cardiomyocytes per heart. Approximately 10% of these cells are striated and thus living [21]. As such, the minimal requirement for our device is that it obtains approximately 25,000 striated cells per heart. With an average cell count of 700,000, our device exceeds the performance of the manual method. This test was done on atrial tissue alone because of availability problems. Because atria tissue is harder to isolate, and contains far less cells than the ventricle, this test indicates that far more cells would be produced from a normal isolation. Furthermore, no liberase solution was used as it was not available at the time of the testing. The use of liberase further increases the cell count. The result of this test is supported by the testing done on individual components of the device during preliminary testing, where very good results

were obtained. These results can be found in Chapter 4. While the Langendorff method is not used in the Gaudette Lab, there is value in comparing it to our device, as it is the existing gold standard in the industry. On average, the Langendorff method yields 1.5 to 1.7 million cardiomyocytes with an accepted viability of 40%, resulting in a minimum of 600,000 viable cells [26]. Despite the higher benchmark set by the Langendorff isolation method, one must consider the problems presented by the Langendorff apparatus (discussed in Chapter 2), many of which result from operational difficulties. As a result, there must be a compromise between our device's performance and its ease of use.

Isolation time is another aspect in the overall efficiency of the isolation process. The isolation time when using the device was found to be no more than thirty minutes. This time was divided across several sections. The brush/hook & loop teasing took approximately seven minutes. Likewise, the mechanical agitation also took seven minutes. The rest of the time was used during set up, switching out the cup system, and collecting the supernatant. When compared to the two or three hours that manual isolation requires, the device exceeds all expectations for this objective. It is also important that our device reduce the level of human involvement in the isolation process. This was successfully completed by removing the need for manual teasing, and manual trituration. The only tasks that the user must perform during isolation is turning the brush motor on and off, removing the cup system, turning the mechanigcal agitation switch on and off, remoing the Allihn condenser, and aspirating the tissue. All of these actions are brief and simple to perform. Therefore, we consider this objective to have been properly met. .

Safety:

As with any project, safety was a major objective to consider during the development of our isolation device. Specifically, we had to consider both cardiomyocyte sterility and user

safety. In creating our device we took several measures to maintain cell sterility. Firstly, we made sure that cell isolation occurs within a closed system: cell dissociation takes place within the confines of an Allihn condenser, and tissue teasing is enclosed by a cover that comes down over the stainless steel brush and interfaces with the perforated cup. Also, materials have been selected to improve the ease of sterilization after isolation. The device's base and the perforated plate for hook-and-loop fabric are both composed of polypropylene, which is a plastic that can withstand sterilization via autoclave. An autoclave can also safely sterilize the stainless steel perforated cup for the hook-and-loop plate, the stainless steel brush, and the glass Allihn condenser. Although the hook-and-loop fabric cannot be autoclaved, it can be sterilized with ethylene oxide. To simplify the sterilization of our device, we designed our device such that it is easily decomposable into its constitutive components. Furthermore, the dimensions of our device allow for usage in a biosafety hood, which effectively maintains cell sterility. The device can be taken apart to allow it to be used in a biosafety hood.

      User safety is of the utmost importance. The greatest danger presented by our device is the rotating stainless steel brush used for tissue teasing; the bristles of this brush are sharp and can harm the user when the brush is in rotation. However, we used a weak motor to ensure that brush rotation is fast enough to tease tissue, but slow enough not to pose a threat to users. This velocity was found to be 2.4 hertz. Also, during device operation, the brush is protected by the stainless steel cup such that heart tissue is protected in a closed system and the user is shield from the brush.

User-Friendliness:

      The objective of user-friendliness drove this project, since we were aiming to ease the burden of cardiomyocyte isolation. Our device simplifies the main steps of the isolation process.

During teasing, the user must simply turn a motor on or off to regulate the duration of stainless steel brush rotation; this is extremely simple when compared to manual isolation, during which one must meticulously tease away at pieces of heart tissue. Our device makes cell dissociation just as simple; rather than continuously triturating a tissue solution with a pipettor, users need only turn a switch that alternatively turns a motor on or off to control the duration of fluid flow in the Allihn condenser. Collecting the tissue solution after dissociation requires simply removing the Allihn condenser and collecting the solution with a pipette.

Durability:

Due to the high demand for cardiomyocytes for in vitro experimentation, cardiomyocyte isolation is a process that is conducted quite frequently. Thus, we expect our device to undergo much usage and have designed it accordingly. All parts of the device that will likely need replacement, such as the stainless steel brush and the hook-and-loop fabric, are removable and readily available.

Reproducible results:

Unlike the manual method of isolation, our device achieves cell isolation primarily through the use of motors. Thus, the element of human inconsistency has been eliminated from the isolation process. Cell yield and viability are therefore more consistent between isolations.

Manufacturability:

Our device is fairly easy to manufacture, as the majority of its components are easily obtainable items with simple modifications. The perforated cup is simply a measuring cup with drill-made holes. Nails were inserted through three of these holes and soldered into place to create the pegs that stabilize the plate for the hook-and-loop fabric. The plate for the hook-and-loop fabric is composed of polypropylene, which is relatively easy to manufacture and cut. The

Allihn condenser is simply used as purchased. The only complicated piece in our device is the polypropylene base, which was custom-built for this project. However, this piece can be reproduced using the CAD drawings included in this report.

Device limitations

After testing our finished device, we found that our product has some limitations. Firstly, the device is not fully automated and some user input is still required. As we were the first team to build such a device for an MQP project, complete automation was deemed an objective for future projects in this area. The team also lacked the expertise in robotics that would be required to achieve full automation. Furthermore, while sterilizing our device is relatively simple, the fact that the hook-and-loop piece must be sterilized by ethylene dioxide rather than by autoclave can be seen as a limitation. Finally, the inevitable deterioration of the stainless steel brush and hook-and-loop fabric is a limitation and calls for the eventual purchase of replacement parts.

Design For

When first designing a new device, many consequences may not seem obvious at first. In order to conceive a successful device the forthcoming impacts must be theorized and considered. Some important considerations that must be explored include: economic impact, environmental impact/sustainability, societal influence, political impact, ethical concerns, health/safety issues, and manufacturability.

Economic Impact

Were our device to be mass produced, it would have several beneficial effects on the health care industry. Firstly, our device would eliminate the numerous hours spent manually isolating cardiomyocytes from heart tissue, providing more time for actual in vitro experimentation and the development of more regenerative therapeutics. Also, with our device

on the market, labs would not have to devote as many costly lab materials to isolation (e.g., petri dishes and conical tubes), allowing funds to be directed towards other purchases. Furthermore, it is probable that our device could be implemented in the isolation of cells other than cardiomyocytes, meaning that a single purchase of our isolation device could fulfill an abundance of laboratory tasks. Lastly, the production and future development of our device could call for a startup company, which would provide jobs for Americans and thus boost the economy.

Environmental/Sustainability Impact

Our device is generally quite eco-friendly. Many of the pieces are able to be sterilized and are thus reusable, eliminating the disposal of countless petri dishes, conical tubes, and volumetric pipette tubes normally used in manual isolation. The only components of our device that may need to be replaced are the stainless steel brush and the hook-and-loop fabric, both of which may deteriorate after extensive use. Also, the manufacture of polypropylene pieces may produce byproducts that are hazardous to the environment,

Societal Influence

Our device will positively affect society by expediting the development of cardiac therapeutics. As mentioned earlier, the occurrence of coronary heart disease and myocardial infarction is projected to increase over the next two decades. Our device will decrease the time needed to isolate cardiomyocytes for in vitro experimentation and provide researchers with more time to develop regenerative medicine for ailing American hearts, increasing the standard of living in the U.S.

Political Impact

We do not imagine our device having any political implications. Although it is unlikely, the device may be criticized for associations with stem cells research, which is quite controversial in the U.S. and many foreign countries.

Ethical Concerns

Some individuals may not accept our device for ethical reasons. Our device isolates cardiomyocytes from rat hearts, meaning that rats must be sacrificed prior to isolation. Despite the protocols that explicitly outline the humane treatment of animal models, there will inevitably be people who oppose the use of animals in medical research and thus oppose our device. However, compared to manual isolation methods, our device allows for the acquisition of more viable cardiomyocytes per rat heart, meaning that fewer rats will have to be sacrificed to obtain sufficient cells for in vitro research. Furthermore, it is likely that our device would be used to aid stem cell research, which is quite controversial in the U.S. and many foreign countries.

Health and Safety Issues

We have created an isolation device that is generally safe for users. There are only a few precautions users must take when operating our device. Firstly, users should avoid directly touching the stainless steel brush bristles, especially while the brush is rotating, as the bristles are very sharp. Users should also handle the Allihn condenser with care since it is composed of glass and can pose a hazard is broken. Lastly, our device is electrical, and users should therefore be careful when operating the device in the presence of fluids such as Kb solution.

Manufacturability

We designed our device such that it is relatively easy to manufacture. The device consists of materials that are economically priced and readily available, such as a measuring cup, an Allihn condenser, and a stainless steel cup brush. Any modifications made to these materials are

quite feasible; for example, creating the device's perforated cup piece simply required drilling

holes into the bottom of a measuring cup. Also, the number of parts needed for our device was

kept to a minimum in order to decrease the burden of sterilizing each part. Furthermore, the parts

that would inevitably need to be replaced due to wear were made for replacement.

Chapter 7 – Final Design and Validation

As coronary heart disease and resultant myocardial infarctions become increasingly prevalent in the U.S., it is essential that we remain diligent in advancing regenerative cardiac therapy. In making such advancements, it is necessary that potential therapies be assessed in vitro. By creating a device that automatically isolates cardiomyocytes, the team has eliminated the time-consuming, and tedious, process of manual isolation and allowed for more time to be invested into in vitro research with isolated cells.

Fulfilling the objectives of the project required a sequential task-based approach. As with any design project, the first task was to expand on the received client statement. The team held group brainstorming sessions and interviewed both the client and a potential user of the designed device. Brainstorming and interviews allowed us to create a more detailed problem statement, which gave us greater insight into the scope of the project. Specifically, our revised client statement provided us with the objectives, functions, and constraints that our final design must fulfill.

We then used several tools to give clarify the relative importance of various project goals. Using an objectives tree (Appendix III), we were able to distinguish general objectives from the more specific sub-objectives. We also created such a hierarchy for the device functions. Pairwise comparison charts (Appendix IV) were used by both the team and the potential product user to assess the importance of each objective and sub-objective through a points system; the scores from both parties were then averaged to give a general consensus of how much weight each objective and sub-objective carried in the execution of this project. Also, a morphological chart (Table 1) was very useful in organizing ideas regarding potential means that could be used to execute device functions.

Much of the aforementioned work was preparatory and helped to organize our ideas so that we could translate them into design alternatives. We generated several CAD models depicting our ideas for tissue teasing and cell dissociation. After creating these models, we devised ways of economically testing them to assess their efficiency. As described in Chapter 4, we tested our ideas in a very crude manner; but the qualitative and quantitative results from our simulations were enough to give us a general idea of what would best isolate cardiomyocytes from heart tissue. We tested various methods of mincing heart tissue. However, successfully mincing heart tissue required more force than we could foresee our device applying, and we thus decided to leave heart mincing to the user. As a result, we chose to begin the automated isolation process with tissue teasing and tested various teasing methods. This essentially consisted of abrading tissue between various combinations of surface types. We assessed the degree of teasing through gross inspection and determined cardiomyocyte viability through microscopic inspection at 4x and 40x magnifications. We then tested our three devised dissociation methods through simulations described in Chapter 4. Cell dissociation was assessed qualitatively through microscopy at 4x magnification, and cell yield was assessed via hemocytometry with trypan blue staining.

After evaluating the various means for tissue teasing and cell dissociation, we chose the most promising design alternatives, which were teasing via stainless steel brush and hook-and-loop fabric and cell dissociation via Allihn condenser. We then developed these design alternatives into a full-fledged isolation device, as shown in the CAD drawings in Figure 21.
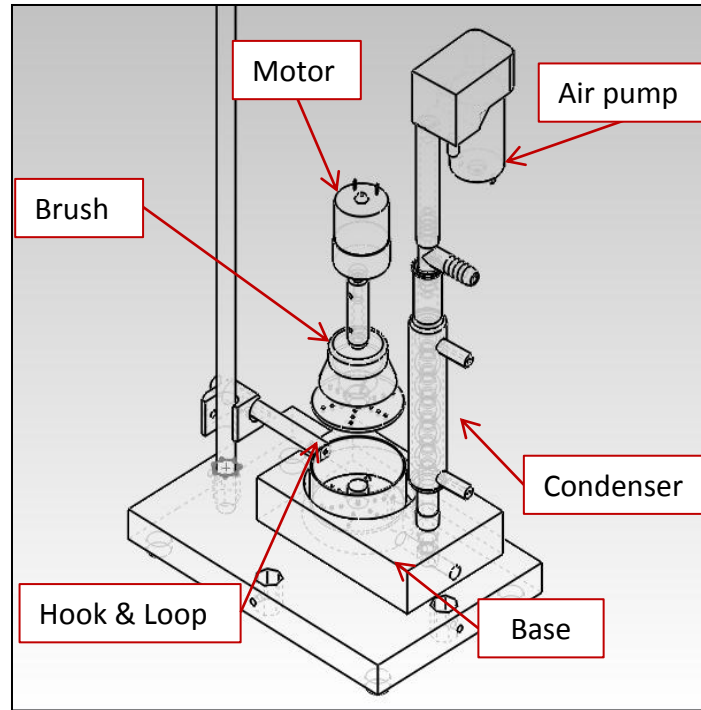
Figure 21: CAD assembly of the final device

We took the CAD drawings seen in Appendix V to a machinist for manufacturing. Although we

did not manufacture the device parts ourselves, the manufacturing process proved to be quite

involved. Due to machining limitations, we had to be very resourceful when finding materials for

different device components. For example, for the perforated cup that holds the perforated hook-

and-loop plate, we implemented an appropriately sized, stainless steel measuring cup in order to

avoid the complicated task of cutting a cup out of bulk metal.

The device was assembled and tested in the lab. At the time of testing, only atrial tissue

was available. This was an acceptable substitute as the atria has far less cardiomyocytes and is

harder to isolate than ventricular tissue. If a high cell count can be collected from atrial tissue

alone, it can be reasonably predicted that far more cells will be collected from ventricular tissue.

Furthermore, at the time of the experiment, no liberase was available. The isolation was

completed in approximately thirty minutes in total. This includes seven minutes of teasing and

seven minutes of mechanical agitation. The rest of the time was allotted to setting up the

experiment. Trypan blue staining was performed for viability analysis. The cells were inspected for striation via microscopy at 40x magnification. Cell yield was measured by hemocytometry and trypan blue staining. It was found that approximately 700,000 living cardiomyocytes were isolated from the atrial tissue. A photograph of the resultant, viable cardiomyocytes at 40x magnification can be viewed below in Figure 22.

Figure 22: Microscope image of viable, striated cardiomyocytes at 40x magnification. The cardiomyocytes of interest are circled in red.

Chapter 8: Conclusion and Recommendation

Conclusion

Over the course of this MQP, we transformed our received client statement into a finalized and tested cell isolation device. Our finished product performs all of the main tasks conducted during manual isolation in a partially automated fashion. With our device, users must only activate a motor to tease tissue between hook-and-loop fabric and a rotating stainless steel brush; this requires much less time and patience than meticulously teasing tissue with forceps. After teasing, the user can activate the motor to an air pump, which moves the teased tissue up and down within a vertical Allihn condenser to achieve cell dissociation; this replaces the tedious and time-consuming process of trituration and proves to be more effective than trituration. Lastly, the user can collect the tissue solution from a small out-port in the base of the device, and aspirate the supernatant for centrifugation.

Foremost among the requirements for this device was the fact that this device needed to produce an equal number of live cells as the manual method currently used in the lab. Through experimentation, we have determined that the device repeatedly exceeds expectation, and provides over 700,000 cardiomyocytes to be used for in vitro testing. Not only does this device provide a high number of cardiomyocytes, these results are highly reproducible. This allows for a dependable source of cells that will minimize the amount of rat hearts needed by the lab, and will assure no delays due to unsuccessful trituration.

The device is also partially automated, as minimizing user activity was an important aspect of this project. This is the main reason why this process provides such repeatable results, as user experience and error is removed from the equation. Of equal importance, the manual process is a long and arduous process. Using the device, isolation was performed in thirty

minutes, as opposed to the two hours required for manual importance. More importantly, constant actions are not required, and the graduate students can focus their energy elsewhere while waiting for isolation to be completed..

Recommendations

As with all devices, there are always various changes that can be developed to further improve overall quality. While many of the recommendations listed below have been partially explored, they were deemed beyond the scope of what could be accomplished within the time frame of this project. Alternatively, some of these recommendations were seen as a secondary priority.

Additional Testing

Because of time constraints and availability of heart tissue, not all testing was possible. It is this team's belief that further testing would greatly benefit the authenticity of this project. While eight hearts were used during testing of individual components of the device, full isolation was only performed once. Ideally, full isolation should be performed ten times in order to guarantee good results and reproducibility.

Furthermore, trypan blue staining was performed to determine cell viability. However, actinin staining, which determines cell striation was not performed. It has been found that cardiomyocytes often lose their striation during manual isolation. Actinin staining would verify that the cells resulting from manual isolation are fully functional.

Alternative Materials for the Teasing Method.

The current method for teasing the tissue involves a metallic brush against an attachment piece which has a layer of Hook-and-loop material. The heart is placed between the two parts of the device, and a motor causes the brush to slowly rotate, while to hooks of the bottom

attachment cause the heart to get stuck, causing a shredding effect similar to the teasing method used in the manual process, which relies on pulling tissue apart with two tweezers.

While several different methods were tested for these materials, as seen in chapter 5, it is likely that better materials can be used for this method that have not been previously investigated. There are several small problems that exist with the current materials, which provide some difficulty to the user.

First, the Hook-and-Loop surface is made of a plastic which is not known to do well in the autoclave. It must be sterilized using the Ethylene Oxide method instead, while all other parts of the devices are made from materials that are full able to be autoclaved. Switching this surface with a coarse surface that can be autoclaved would simplify the sterilization process.

Although the Hook-and-Loop and brush surface interactions provided the best results during preliminary experimentation, it is likely that other interactions would provide better cell viability. More thorough experimentation covering a large variety of materials may provide a combination which results in a higher final cell count.

Finally, it has been observed that the materials used for the teasing process degrade over time. Due to the nature of the task of teasing, this is normal. Teasing the tissue apart requires two surfaces to brush against each other to cause enough shear to tear the extracellular matrix of the heart apart. Our device has been designed to minimize the amount of particulates that result from the two materials rubbing against each other. It has also been designed for easy replacement of the parts that are likely to wear down. However, further research and experimentation into different material combination could result in materials that are far more durable than the existing method.

Full Automation of Device

The current device is only partially automated in that the brush of the teasing process has an on/off switch, and tissue dissociation in the Allihn condenser is also motorized. However, several tasks remain manual. These include, addition of the liberase, stopping brush rotation, beginning pumping of the solution through the Allihn condenser, and collection of the supernatant for centrifugation. Most of these processes could be automated so that the user would only have to input the heart at the beginning and collect a finished product. This objective was not viewed as a priority to the project, but complete automation would greatly simplify the user's tasks.

Facilitate Input and Output.

The current device requires the user to cut the atria apart from the ventricles, remove the blood from the ventricles, and dice the heart in a few pieces. It may be possible to automate this process, or at the very least facilitate it through the use of a mechanical device. While this was not seen as a priority of the project, it would further simplify the process for the user by reducing prepping time.

The current output of the device requires the removal of the Allihn condenser. A pipet is used to aspirate the solution from the condenser opening. While this is a fairly simple process, it is likely that there is some tissue left behind. It would be beneficial to devise a method of fully collecting the supernatant and tissue without having to disassemble portions of the device.

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Appendix I: Manual Isolation Protocol

KB Solution Mixing

Table 2: Kb Solution Mixing

	g/500ml	g/l
KCl	.93125	1.8625
KH2PO4	.6805	1.361
MgCl2	.14265	.2853
Glucose	.9	1.8
EGTA	.095	.19
Taurine	1.25	2.5
L-glutamic acid	5.145	10.29
HEPES	1.18	2.38
ATP	.138	.276

- Mixed ingredients except ATP, warmed to 30C then mixed

- Filtered with filter bottle

Isolation Procedure

1. Removed 1 whole heart from euthanized rat

   i. Heart still beating after removal

2. Placed heart in 20ml KB and gently squeezed to remove blood

3. Placed heart in 20ml of fresh Kb and removed atria with surgical scissors

4. Squeezed heart gently to clean

5. Places heart in 20ml fresh KB and minced with scissors

6. Added 200ml stock liberase DH to 20ml KB and placed in water bath at 32C

7. Triturate 2 min

8. Minced tissue in petri dish

9. Repeated mince triturate cycle for 7 trituration

10. Following trituration allowed tissue to settle in 50ml conical tube and pipette off the supernatant

11. Centrifuged cells 2x at 400 rpm for 5 minutes

12. Plated Slides with cells for yield measurement.

Appendix II: Langendorff Isolation Protocol[27]

Isolation of Adult Mouse Cardiac Myocytes from One Heart

AfCS Procedure Protocol PP00000125

Version 1, 11/05/02

This procedure describes the isolation and culture of adult mouse cardiac

myocytes from one heart. The isolation routinely yields approximately 1 million rod-shaped

myocytes per heart.

Procedure Setup

1. This procedure uses male C57BL/6 mice, 8 to 10 weeks old (20 to 30 g). Record

strain, sex, and weight.

2. Prepare perfusion buffer; myocyte digestion buffer (MC digestion buffer); myocyte

stopping buffers (MC stop 1 and MC stop 2); myocyte plating medium (MC plating

medium); myocyte culture medium (MC culture medium); and laminin-coated

dishes (see "Laminin Coating of Culture Dishes" below) fresh daily. Perfusion

buffer and MC digestion buffer should be warmed to 37 °C prior to use.

Equilibrate MC plating medium and MC culture medium at 37 °C in a 2% CO_2

incubator (for at least 2 hr. to adjust temperature and pH). Sterilize instruments in

hot bead sterilizer.

3. Prepare the perfusion apparatus. Set the circulating water bath so that the outflow

from the tip of the cannula is 37 °C. Check the flow rate of the pump and adjust to 3 ml/min. (Note: the temperature of the perfusate and the flow rate of the pump should be checked routinely. Over time, peristaltic pump tubing will fail and will need to be replaced every 2 to 3 months to maintain consistent flow rates. These routine checks are essential to maintain consistency in preparations

4. Run 100 ml of purified water through the perfusion system; then run perfusion buffer through the system for at least 5 min.

5. Add perfusion buffer and MC digestion buffer to the correct reservoirs, prime the perfusion system with buffers, eliminate air bubbles, and allow time to achieve temperature (about 15 min). Note: no prewarming is required for perfusion system B.

6. Add 10 ml of room temperature perfusion buffer to a 60-mm culture dish for heart collection. Add 20 ml of room temperature perfusion buffer to a 100-mm culture dish for heart cannulation, and place on an adjustable stage under the perfusion apparatus.

7. Position the cannula with the tip close to the surface of perfusion buffer in the 100-mm dish. The pump can be set to a very slow rate (0.4 ml/min, for example) during the cannulation, but this is not necessary.

8. Cut several small pieces of 6-0 surgical silk (10 to 15 cm), knot loosely, and place on the adjustable stage (this will be used to secure the aorta to the cannula).

Isolation of Adult Mouse Cardiac Myocytes from One Heart

AfCS Procedure Protocol ID PP00000125 Page 2

Removal and Cannulation of the Heart

9. Inject the mouse i.p. with 0.5 cc heparin diluted in phosphate buffered saline (PBS) to 100 IU/ml.

10. Anesthetize the mouse with isoflurane and 100% O2. Set the isoflurane atomizer dial to 3% (scale 1% to 5% of total flow), turn the O2 valve to 0.5 L/min, and place animal inside the induction chamber. When the mouse is anesthetized, it will lose consciousness and roll over on its side. Check with a toe pinch to ensure that the mouse is fully anesthetized. Transfer the mouse to the surgery/perfusion area and place under a nose cone connected to the anesthesia system.

11. Once the animal is on the surgery area, wipe the chest with 70% ethanol and adjust the isoflurane (usually 1.5%) as necessary to ensure proper level of anesthesia (movement indicates that the anesthesia is too shallow, whereas irregular respiration indicates that it is too deep). Check with a toe pinch to ensure that the mouse is fully anesthetized.

12. Open the peritoneal cavity and chest with small scissors and use forceps to peel back the rib cage to expose the heart. Lift the heart gently using forceps. Identify and cut the pulmonary vessels, which will make it easier to identify and cut the aorta. Cut the aorta at about 2-mm from its entry into the heart and immediately place the heart in a 60-mm dish containing 10 ml of perfusion buffer at room temperature. Too long a section of aorta will make the aorta harder to identify and lift onto the cannula. Conversely, too short a section of aorta will make it harder to tie off the aorta on the cannula and increase the likelihood of pushing the cannula through the aortic valve, preventing good perfusion.

13. Remove extraneous tissues (thymus and lungs), if necessary, and transfer heart

to the 100-mm dish with perfusion buffer at room temperature.

14. Cannulate the heart using fine-tip forceps to slide the aorta onto the cannula so that the tip of the cannula is just above the aortic valve (check the 1-mm notch on the cannula to ensure proper cannulation; see description of cannula below). Attach a small brass clip at the end of the cannula to prevent the heart from falling. Start the perfusion immediately (3 ml/min). Tie the aorta to the cannula with 6-0 silk thread. Total time to cannulate the heart should be less than 1 min. (Note: using magnifying lenses or a dissecting microscope will make the heart and aorta easier to visualize and cannulate.)

Heart Perfusion and Enzyme Digestion

15. Perfuse the heart with perfusion buffer for 4 min at 3 ml/min (this flushes blood from the vasculature and removes extracellular calcium to stop contractions). Measure the temperature of the heart with an insulated wire probe attached to a digital thermometer, placing temperature probe into ventricle to ensure temperature is 37 °C. (Note: this does not need to be done each time, but should be done periodically to ensure reproducibility).

16. Switch to the MC digestion buffer and perfuse for 8 to 10 min at 3 ml/min (digestion times can vary from heart to heart). If the heart is well perfused during the enzyme digestion, the heart will become swollen and turn slightly pale, and separation of muscle fibers on the surface of the heart may become apparent.

Isolation of Adult Mouse Cardiac Myocytes from One Heart

AfCS Procedure Protocol ID PP00000125 Page 3

Myocyte Dissociation

17. Once enzyme digestion of the heart is complete (heart appears swollen, pale, and flaccid), cut the heart from the cannula just below the atria using sterile, fine scissors. Place the ventricles in a 60-mm dish containing 2.5 ml of MC digestion buffer. From this point forward, sterile techniques should be maintained, and all subsequent steps are performed under a laminar flow culture hood.

18. Cut the heart in half and begin to gently tease the ventricles into several small pieces with fine forceps. Pipette gently several times with a sterile plastic transfer pipette (2-mm opening). This process takes 60 to 90 sec. (Note: the tissue should be very flaccid, almost falling apart on its own, and require very little force to pull apart, which will indicate a good digestion.)

19. Transfer the cell suspension to a 15-ml polypropylene conical tube. Rinse the plate with 2.5 ml of room temperature myocyte stopping buffer 1 (MC stop 1), and combine with the cell suspension for a final volume of 5 ml. (Note: MC stop 1 contains serum to inactivate proteases; the final calf serum concentration is 5%, and the final calcium concentration is 12.5 μM).

20. Continue to dissociate the heart tissue gently, using sterile plastic transfer pipettes with different sized openings (2-mm, 1.5-mm, and then 1-mm diameters), until all the large pieces of heart tissue are dispersed in the cell suspension. Avoid vigorous agitation to minimize shearing of the cells. This process should take 3 to 5 min.

21. Count rod-shaped and round myocytes using a hemacytometer (see "Counting Myocytes with a Hemacytometer" below). Calculate the total number of myocytes, the number of rod-shaped myocytes, and the percent of rod-shaped myocytes.

Record these values as the initial number of cells obtained. (Note: the important number for comparison between labs is the total number of rod-shaped myocytes, as the method for counting the number of round myocytes is often hard to standardize. However, the percent of rod-shaped myocytes is still a useful number as it can reflect the quality of the isolation. If at this point the total cell yield from a specific heart is low [less than 1 million], or the percent of rod-shaped myocytes is low [less than 60%], the cells prepared from this heart should be discarded.)

Calcium Reintroduction(All steps conducted at room temperature.)

22. Allow the myocytes to sediment by gravity for 8 to 10 min in the 15-ml tube(s) while counting the myocytes. Transfer the supernatant to a new 15-ml tube and centrifuge for 1 min at 180 x g.

23. Resuspend the new pellet in 5 ml of room temperature myocyte stopping buffer 2 (MC stop 2), combine with the original sedimentedmyocytes, and adjust to a total volume of 10 ml with MC stop 2. (Note: MC stop 2 contains 5% serum, and the final calcium concentration is 12.5 µM).

24. Transfer the combined pellets (in 10 ml) to a 60-mm nonstick Valmark dish. To evenly distribute the myocytes in the dish, move the dish forward and backward and side to side, but do not swirl the dish. (Note: the myocytes in the 60-mm Valmark dish can be monitored under a microscope during calcium reintroduction.

Isolation of Adult Mouse Cardiac Myocytes from One Heart

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If during the reintroduction period, the myocytes begin to round up, or become

very granular, the myocyte quality might be poor. A decision may be made as to whether to continue with the prep or to start over, depending on the judgment of the technician.)

25. Add 50 μl of calcium chloride, 10 mM (10 mM CaCl2); final concentration is increased to 62 μM. Mix well and incubate for 4 min at room temperature.

26. Add 50 μl of 10 mM CaCl2; final concentration is increased to 112 μM. Mix well and incubate for 4 min at room temperature.

27. Add 100 μl of 10 mM CaCl2; final concentration is increased to 212 μM. Mix well and incubate for 4 min at room temperature.

28. Add 30 μl of calcium chloride, 100 mM (100 mM CaCl2); final concentration is increased to approximately 500 μM. Mix well and incubate for 4 min at room temperature.

29. Add 50 μl of 100 mM CaCl2; final concentration is increased to approximately 1 mM. Mix well and incubate for 4 min at room temperature.

30. Transfer the myocytes to a new 15-ml tube and allow the myocytes to sediment by gravity (8 to 10 min). Transfer the supernatant to another new 15-ml tube and centrifuge for 1 min at 180 x g.

31. Resuspend and combine both pellets in 5 ml of MC plating medium (1.2 mM Ca2+) at 37 °C.

32. Count rod-shaped and round myocytes using a hemacytometer (four counts, or both sides of two separate hemacytometers). Calculate the total number of myocytes, the number of rod-shaped myocytes, and the percent of rod-shaped myocytes. **Record these values as the number of myocytes for plating.** The

most important number is the **number of rod-shaped myocytes.** If the myocyte

number is low or myocyte quality is poor, a decision may be made as to whether

to continue with the prep or to start over, depending on the judgment of the

technician.

Appendix III: Objective Tree
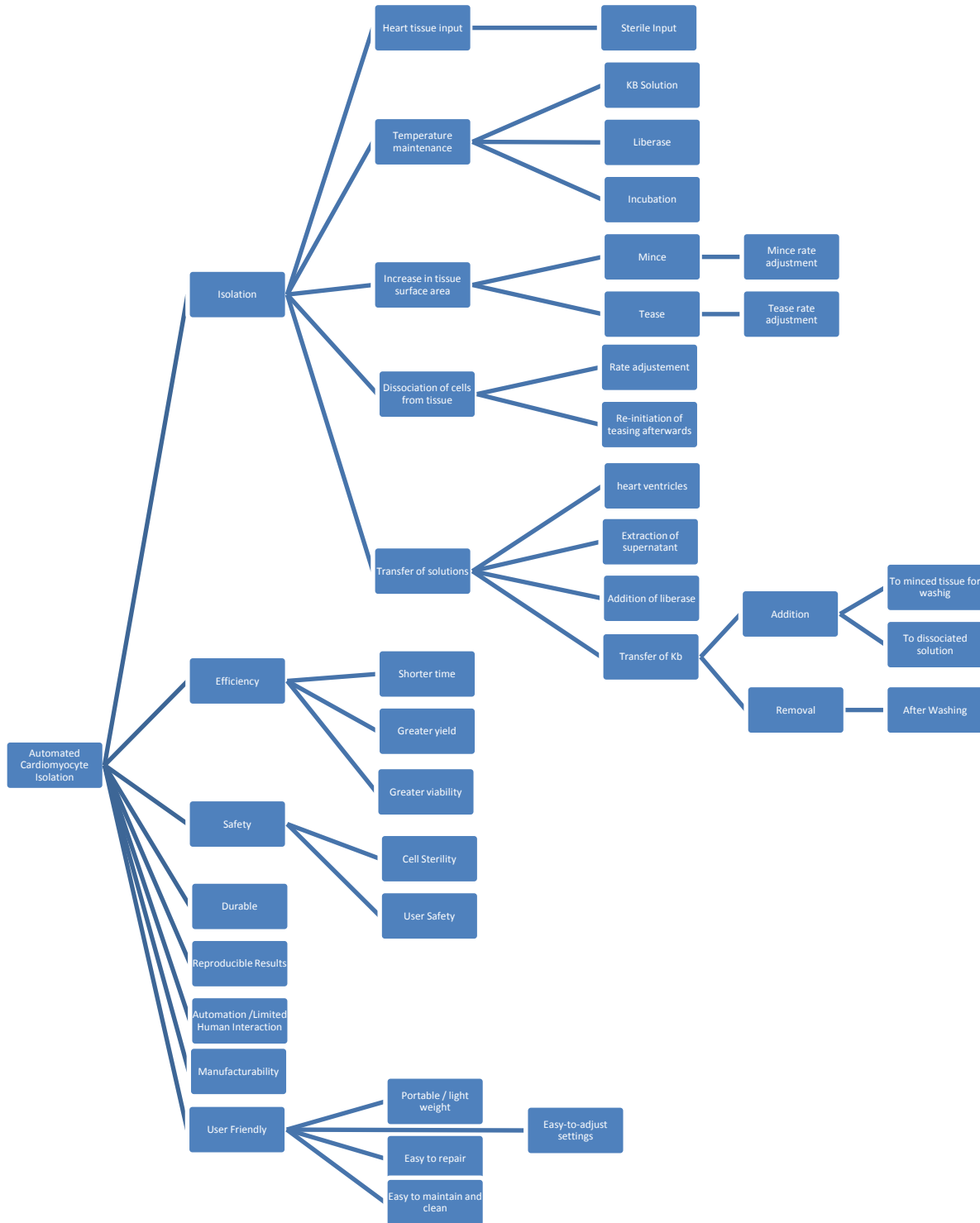
Figure 23: Objective Tree

Appendix IV: Pairwise Comparison Charts

A) Main Pairwise Comparison Chart

1) User

Table 3: User Pairwise Comparison Chart

Main	Cardiomyocyte isolation	Increased efficiency	Safety	User-friendly	Durable	Reproducible results	Limited human interaction	Manufacturability	Total
Cardiomyocyte isolation		1	0	1	1	1	1	1	6
Increased efficiency	0		0	1	1	0	1	1	4
Safety	1	1		1	1	1	1	1	7
User-friendly	0	0	0		0	0	1	1	2
Durable	0	0	0	1		0	1	1	3
Reproducible results	0	1	0	1	1		1	1	5
Limited human interaction	0	0	0	0	0	0		1	1
Manufacturability	0	0	0	0	0	0	0		0

2) Designer

Table 4: Designer Pairwise Comparison Chart

Main	Cardiomyocyte isolation	Increased efficiency	Safety	User-friendly	Durable	Reproducible results	Limited human interaction	Manufacturability	Total
Cardiomyocyte isolation		1	0	1	1	1	1	1	6
Increased efficiency	0		0	1	1	0	1	1	4
Safety	1	1		1	1	1	1	1	7
User-friendly	0	0	0		1	0	1	1	3
Durable	0	0	0	0		0	0	1	1
Reproducible results	0	1	0	1	1		1	1	5
Limited human interaction	0	0	0	0	1	0		1	2
Manufacturability	0	0	0	0	0	0	0		0

3) Comparison

Table 5: Main Objective Comparison

Main	User Total	Designer Total	Average %
Cardiomyocyte isolation	6	6	21.43%
Increased efficiency	4	4	14.29%
Safety	7	7	25%
User-friendly	2	3	8.93%
Durable	3	1	7.14%
Reproducible results	5	5	17.86%
Limited human interaction	1	2	5.36%
Manufacturability	0	0	0

B) Cardiomyocyte Isolation Pairwise Comparison Chart

1) User

Table 6: User Cardiomyocyte Isolation Pairwise Comparison Chart

Cardiomyocyte Isolation	Heart tissue input	Temperature maintenance	Increase in tissue surface area	Dissociation of cardiomyocytes	Transfer of isolation solutions	Total
Heart tissue input		1	1	1	1	4
Temperature maintenance	0		0	0	0	0
Increase in tissue surface area	0	1		1	1	3
Dissociation of cardiomyocytes	0	1	0		1	2
Transfer of isolation solutions	0	1	0	0		1

2) Designer

Table 7: Designer Cardiomyocyte Isolation Pairwise Comparison Chart

Cardiomyocyte Isolation	Heart tissue input	Temperature maintenance	Increase in tissue surface area	Dissociation of cardiomyocytes	Transfer of isolation solutions	Total
Heart tissue input	■	1	1	1	1	4
Temperature maintenance	0	■	0	0	0	0
Increase in tissue surface area	0	1	■	0	1	2
Dissociation of cardiomyocytes	0	1	1	■	1	3
Transfer of isolation solutions	0	1	0	0	■	1

3) Comparison

Table 8: Cardiomyocyte Isolation Objective Comparison

Cardiomyocyte Isolation	User Total	Designer Total	Average %
Heart tissue input	4	4	40%
Temperature maintenance	0	0	0%
Increase in tissue surface area	3	2	25%
Dissociation of cardiomyocytes	2	3	25%
Transfer of isolation solutions	1	1	10%

C) Increased Efficiency Pairwise Comparison Chart

1) User

Table 9: User Increased Efficiency Pairwise Comparison Chart

Efficiency	Faster	Greater yield	Greater viability	Total
Faster	■	0	0	0
Greater yield	1	■	0	1
Greater viability	1	1	■	2

2) Designer

Table 10: Designer Increased Efficiency Pairwise Comparison Chart

Efficiency	Faster	Greater yield	Greater viability	Total
Faster	■	0	0	0
Greater yield	1	■	1	2
Greater viability	1	0	■	1

3) Comparison

Table 11: Efficiency Objective Comparison

Efficiency	User Total	Designer Total	Average %
Faster	0	0	0%
Greater yield	1	2	50%
Greater viability	2	1	50%

D) Safety Pairwise Comparison Chart

1) User

Table 12: User Safety Pairwise Comparison Chart

Safety	Cell sterility	User safety	Total
Cell sterility	*	0	0
User safety	1	*	1

2) Designer

Table 13: Designer Safety Pairwise Comparison Chart

Safety	Cell sterility	User safety	Total
Cell sterility	*	0	0
User safety	1	*	1

3) Comparison

Table 14: Safety Objective Comparison

Safety	User Total	Designer Total	Average %
Cell sterility	0	0	0%
User safety	1	1	100%

E) User-friendly Pairwise Comparison Chart

1) User

Table 15: User-friendly Pairwise Comparison Chart

User-friendly	Portable/fits in hood	Easy to repair	Easy to maintain/clean	Easy to adjust settings	Total
Portable/ fits in hood		1	1	1	3
Easy to repair	0		0	0	0
Easy to maintain/clean	0	1		0	1
Easy to adjust settings	0	1	1		2

2) Designer

Table 16: Designer Friendly Pairwise Comparison Chart

User-friendly	Portable/fits in hood	Easy to repair	Easy to maintain/clean	Easy to adjust settings	Total
Portable/ fits in hood		1	1	1	3
Easy to repair	0		0	0	0
Easy to maintain/clean	0	1		1	2
Easy to adjust settings	0	1	0		1

3) Comparison

Table 17: User-friendly Objective Comparison

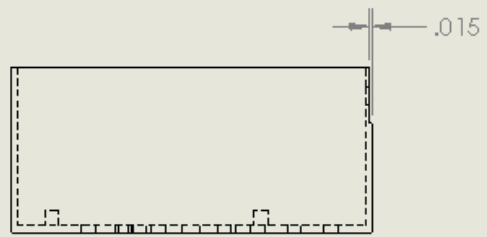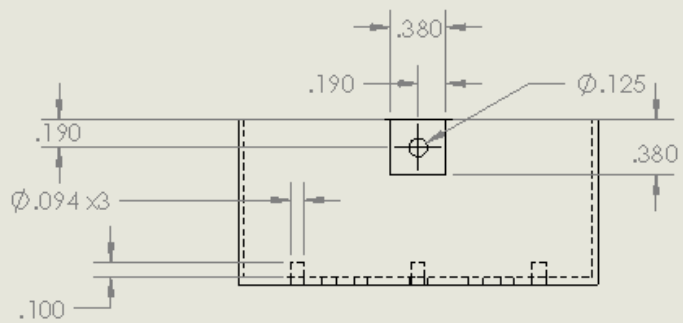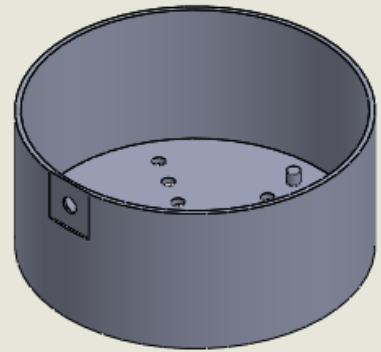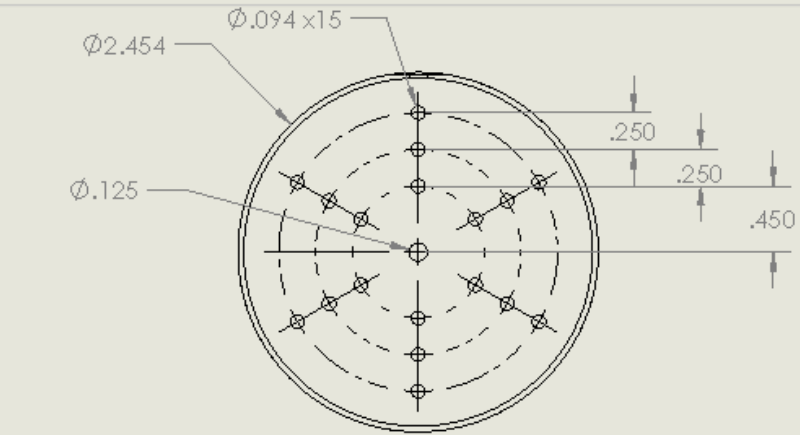| User-friendly	User Total	Design Total	Average %
Portable/ fits in hood	3	3	50%
Easy to repair	0	0	0%
Easy to maintain/clean	1	2	25%
Easy to adjust settings	2	1	25%

Appendix V: Trypan Blue Protocol

1. Pipette 10 µL of cell suspension into a 0.65 mL microcentrifuge tube.
2. Add 10 µL of trypan blue to the 10 µL of cell suspension.
3. Prepare the hemocytometer by placing the glass coverslip over the counting chamber.
4. Pipette 10 µL of the trypan blue / cell suspension mixture into one of the V-shaped wells located on either side of the counting chamber.
5. Place the hemocytometer under a light microscope.
6. Count the number of unstained (living) cells in the boxes of the counting chamber.
7. Be sure to keep track of the number of boxes in which living cells are found.
8. Use the quantity of living cells and the quantity of hemocytometer boxes containing living cells in the equation below to calculate the cell yield of the entire tissue solution.

$$Cell\ yield = \frac{\#\ living\ cells\ counted}{\#\ boxes\ used} \times 2 \times 10{,}000 \times quantity\ of\ cell\ suspension\ used\ (mL$$

Appendix V: CAD Drawings

Drawing callouts and dimensions:
- ∅.600
- .250
- .100
- ∅.094 x 18 through all
- Slip fit
- ∅.500 Slip Fit
- .250
- ∅2.375 Slip Fit
- .250
- .450
- Slip fit
- Slip fit
- 60.00°

UNLESS OTHERWISE SPECIFIED:		NAME	DATE		
DIMENSIONS ARE IN INCHES TOLERANCES: FRACTIONAL ± ANGULAR: MACH ± BEND ± TWO PLACE DECIMAL ± THREE PLACE DECIMAL ±	DRAWN	FO	2/24	TITLE:	
	CHECKED				
	ENG APPR.			HookLoopAttachment	
	MFG APPR.				
INTERPRET GEOMETRIC TOLERANCING PER:	Q.A.				
	COMMENTS:				
MATERIAL Stainless Steel				SIZE A	DWG. NO. HookLoopAttachment	REV
FINISH					
DO NOT SCALE DRAWING				SCALE: 1:1 WEIGHT: SHEET 1 OF 1	

NEXT ASSY   USED ON   APPLICATION

Ø2.454

Ø.094 x15

Ø.125

.250

.250

.450

.380

.190

Ø.125

.190

.380

Ø.094 x3

.100

.015

		UNLESS OTHERWISE SPECIFIED:		NAME	DATE		
		DIMENSIONS ARE IN INCHES TOLERANCES: FRACTIONAL ± ANGULAR: MACH ± BEND ± TWO PLACE DECIMAL ± THREE PLACE DECIMAL ±	DRAWN	FO	2/24	TITLE:	
			CHECKED				
			ENG APPR.				
			MFG APPR.			CupBody	
		INTERPRET GEOMETRIC TOLERANCING PER:	Q.A.				
			COMMENTS:				
		MATERIAL Stainless Steel				SIZE **A**	DWG. NO. CupBody	REV
NEXT ASSY	USED ON	FINISH						
APPLICATION		DO NOT SCALE DRAWING				SCALE: 1:1	WEIGHT:	SHEET 1 OF 1

I couldn't put a center mark. Dimensions are to the top surface center

Ø.551 Slip Fit
Ø.394 Slip Fit

2.079
1.750

R.198
.178

.049
1.750

.573
5.00°

Ø3.100
2.900

.706
.394

3.500
.338

1.250

5.500

Ø.375 Slip Fit
1.750

.150 Depth is Critical

			UNLESS OTHERWISE SPECIFIED:		NAME	DATE		
			DIMENSIONS ARE IN INCHES TOLERANCES: FRACTIONAL± ANGULAR: MACH± BEND ± TWO PLACE DECIMAL ± THREE PLACE DECIMAL ±	DRAWN	FO	2/26	TITLE:	
				CHECKED				
				ENG APPR.				
				MFG APPR.			SlantDish	
			INTERPRET GEOMETRIC TOLERANCING PER:	Q.A.				
				COMMENTS:				
			MATERIAL Polypropylene				SIZE A DWG. NO. SlantDish	REV
NEXT ASSY	USED ON		FINISH					
APPLICATION			DO NOT SCALE DRAWING				SCALE: 1:2 WEIGHT:	SHEET 1 OF 1

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