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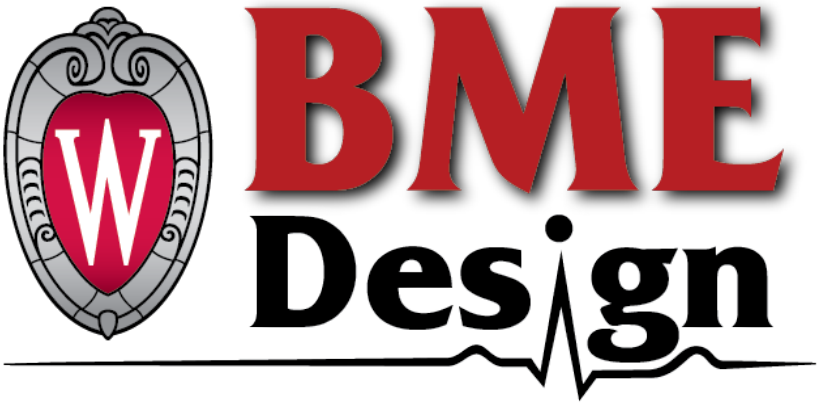

Tissue Biopsy Dissociation

BME 200/300

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Abstract

Patients with asthma have shown eosinophils present in the lumen of their blood, airways, and lung tissue. It is believed that these eosinophils have different surface markers depending on which area of the body they are taken from [1]. To study the eosinophils from the lung tissue, they must first be dissociated from the surrounding tissue. The project investigates how to successfully dissociate the eosinophils from the lung tissue in an environment similar to *in vivo* conditions. By dissociating the eosinophils from the tissue, the surface markers on the cells can be analyzed and compared to surface marks on the eosinophils from the blood lumen and airways. The design for the dissociation method should minimize the damage to the cells while yielding enough eosinophils for examination through flow cytometry.

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Introduction

Motivation and Client Background

Our client, Dr. Sameer Mathur, is an immunologist that conducts research regarding asthma and eosinophil immunoregulatory activity at the University of Wisconsin-Madison. The lab focuses on studying the effects of allergens on tissue resident eosinophils located in the the lungs as well as the lumen of the blood vessel. Currently, Dr. Sameer Mathur recruits asthma patients for bronchoscopies and lung tissue biopsies in order to determine mechanisms by which eosinophils can regulate epithelial responses to innate immune stimuli and viruses. To perform these studies, eosinophils must be isolated from the tissue samples, while still retaining cell viability and producing satisfactory yield, which ultimately prompts the need for the hydrogel-based tissue dissociator.

Asthma is a common condition. It affects at least 3 million people in the United States. Like any disease, there are varying degrees to which asthma can affect daily life. For some, asthma can be a minor annoyance, for others, it can be life threatening. Behind all cases is the mechanism by which asthma operates. During an asthmatic reaction, airways are constricted making it challenging to breath. As air pollution is increasing for a larger portion of the population, the prevalence of asthma will only increase. Asthma is often initiated by an irritant or an allergen, but can become chronic if the tissues of the airways maintain an inflamed state. Current medical dogma instructs treatment of asthma with a host of bronchodilator and anti-inflammatory drugs, as well as steroids. These treatments typically control the symptoms of asthma well, but are currently ineffective at curing the disease [1].

The main subject of our client's study is the tissue resident eosinophil. Eosinophils are a cell type that are considered a variety of white blood cells. As such, they are a part of the innate immune system and are typically associated with their role in fighting viral and parasitic infections. Eosinophils contain large granules that contain the cell's peroxidase, RNase, DNase, lipase, and plasminogen. It is believed that the eosinophils are in part responsible for the asthmatic response; the release of eosinophil granular contents will damage both the allergen irritating the lung tissue as well as the tissue around the allergen.

Current Methods of Dissociation

The two main procedures used to dissociate tissue samples use an enzymatic approach or a tissue dissociator machine such as the gentleMACs dissociator. A large number of research papers discussing tissue dissociation involve one or more combinations of an enzymatic dissociation. The majority of current dissociation methods follow the same basic steps: use a mixture of enzymes in conjugation with agitation at 37° Celsius, followed by various washing and straining techniques. These steps are then repeated until the tissue is properly dissociated.

The gentleMACs dissociator is commonly used in cases where a higher throughput is required. It works on a similar principle as the standard enzymatic dissociation but includes a

specially formulated cap that adds an element of mechanical dissociation. The cap has small ridges that protrude to interact with tissues to dissociate the cells. While a tissue is in the dissociator, a proprietary enzyme solution is often included.

Problem Statement

Dr. Mathur's research group receives tissue samples from lung biopsies prior to and after the induction of asthma. He is specifically interested in the effect of tissue resident eosinophils in asthma. The biopsies he receives that are too small for current tissue dissociation methods. This device must be able to dissociate small pieces of tissue while still allowing for the interrogation of gene expression as well as cell surface markers. The time the tissue spends in the enzymes must also be considered. The cost of all materials must be around $10 if the device is disposable. If the device and be reused and sterilized, the total cost can be $300.

Background

Relevant Published Research

Tissue biopsies are extremely common, especially in the cancer field, as well as for the production of various cells for therapies. The extraction of cells through tissue dissociation will always be harmful to all cells, specifically those that require integrin bound attachment to the extracellular matrix. The application of enzymes and the intense change in physical surroundings for cells resident in tissues will be the most damaging aspects of this process. Cells, for example cardiomyocytes, that are typically extracellular matrix (ECM) bound are susceptible to programmed cell death within a short period of time if left suspended in biological conditions. This will be of lower concern due to the fact that eosinophils are not bound by integrin receptors to the ECM, although there is still debate as to whether there could be subtypes of these cells that act differently in respect to their interactions with the ECM. The eosinophils still interact with the ECM and are surrounded to it, but are not bound to it through integrin receptors like a variety of other cells are. While the lack of ECM binding of the eosinophils will aid in the dissociation, we must still be highly scrupulous in the choice of enzyme in order to ensure they do not affect surface markers. Additionally, the dissociation process will incorporate both enzymatic and mechanical dissociation. The desired process will have to limit mechanical dissociation to prevent mechanical cell damage while still providing enough mechanical stimulus to break apart the tissue. This should be of lesser concern due to the already small size of the tissue sample that our client will be using [2].

Our client is most concerned with the time frame that the dissociation process will take. It has been shown that the expression of different genes and surface markers can altered or damaged in conditions such as dissociation. These alterations and damages could be due to an actual change in the mRNA synthesis and associated protein production, surface marker internalization, or a combination of the two.

As previously mentioned, enzymatic dissociation is a commonly used technique to obtain single cell suspension. For example, Cooper et al. utilized a typical enzymatic dissociation in order to later analyze the tissue resident NK cells by flow cytometry [2]. They started by mincing the samples into 4-6mm^3 lung tissue fragments and putting them into cold RPMI, then transferred to "complete RPMI" for 16 hours to remove blood. The samples were then digested in collagenase and filtered through a 0.7-µm filter. Last, the group stained the cells and ran them through the flow cytometer.

The gentleMACs dissociator is also commonly used and a representative protocol is outlined in a publication by Bandyopadhyay et al. The samples are first dissected and then placed into a prewarmed buffer solution of DPBS, 10 mM HEPES, 150 mM NaCl, 5 mM KCL, 1 mM $MgCl_2$, 2 mg/mL collagenase type A, 1 mg/mL dispase II, 0.5 mg/mL porcine pancreas elastase, and 2 mg/mL bovine pancreas DNase-I. The cocktail was put into a gentleMACs C tube at 8 g of tissue/tube with 10 mL of cocktail. The sample tube and contents were then placed onto the gentleMACS tissue dissociator machine and run through a preset program. After the tissue dissociation cycle, the contents were strained through a 100-µm filter [3].

Competing Designs

There exist two products on the market for use in tissue dissociation or "deaggregation." One is the aforementioned gentleMACs dissociator. The other is the BD™ Medimachine which has disposable, specially formulated tubes with steel strainers and a rotating blade that works to dissociate the tissue in conjunction with an enzyme.

Interestingly, there also exist numerous products that utilize small beads made of stainless steel or glass to aid in homogenizing tissue samples. This process however, leaves the cells entirely nonviable because the stiffness of the steel and glass provide too much mechanical stimulus to the cells. This dissociation method demonstrates the need of a lower stiffness bead to maintain cell viability.

Figure 1: A schematic drawing and description of the gentleMACs C tube.

Figure 2: A cutout image of the BD Medimachine dissociation chamber.

Preliminary Designs

Components of All Designs

Each design varies in the amount of physical stimulation that would be used to dissociate the tissue. Each design has the small piece of tissues being degraded by enzymes prior to any dissociation occuring. Every design must be sterilizable and the combination of enzymes used for each of the designs will largely be the same. All of these designs also must show reproducible results. They all should prioritize duration of tissue dissociation and cell viability after dissociation.

Enzymatic and Gentle Agitation

The enzymatic and gentle agitation method is the simplest approach of the three design methods considered. This method involves soaking the small piece of tissue within a mixture of enzymes [3]. This enzyme mix would need to be both extremely gentle and quick thereby not harming any of the cell or it's vital cell markers. The enzyme needs to completely dissociate the tissue. The tissue will be soaked within the enzyme mix for approximately 4 hours while simultaneously undergoing gentle agitation. It is hoped that this process causes the tissue to break up completely allowing for it to be analyzed through flow cytometry.

Enzyme And MicroFluidic Device

The unique characteristic of this dissociation process is the incorporation of a microfluidic device which serves as a means of creating turbulent flow. Using a microfluidic device made of PDMS would allow a mechanical means of dissociation that is cheap, as well as biocompatible [4]. The small piece of tissue should be soaked within a collection of collagenase to begin to soften the tissue and begin this process. Then, the tissue would be passed into the fluidic device and by the differences between laminar and turbulent flow, the tissue would be

broken up into smaller and smaller pieces. This resulting tissue should in theory, be prepared to undergo flow cytometry in order to analyze the desired eosinophils from the surrounding tissues.

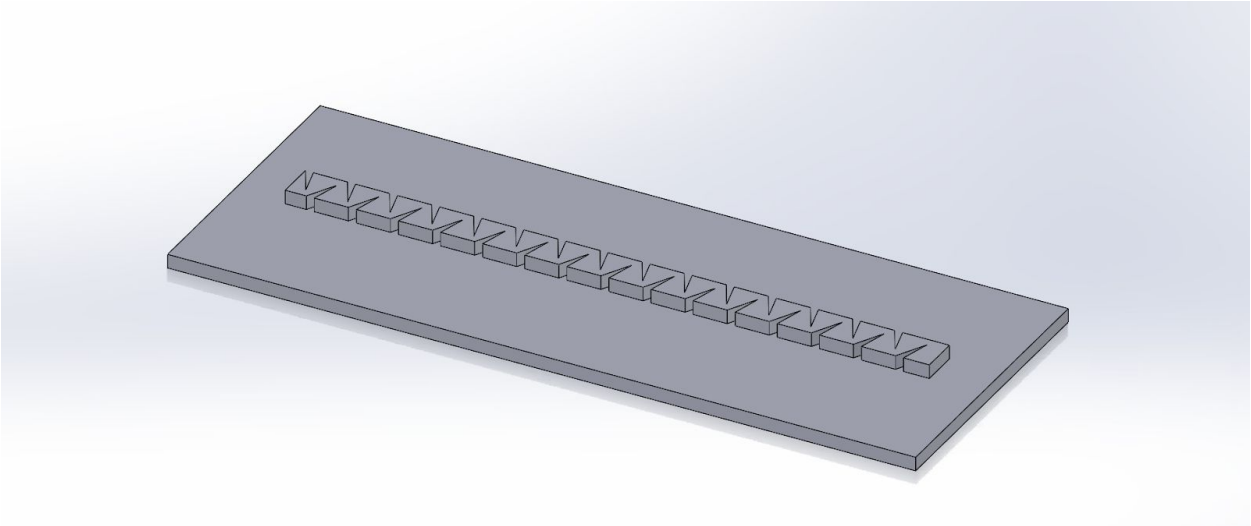


Figure 3: Microfluidic Device design to create turbulent flow to dissociate the tissue.

Hydrogel Beads and gentleMACS Dissociator

The main focus of this design is to limit the amount of time the tissue biopsy sample spends in solution with enzymes. The dissociation process is performed by placing the tissue sample in the gentleMACS dissociator with hydrogel beads and an enzymatic solution. The gentleMACS machine dissociates tissue by swirling the sample in a specially designed conical tube. As shown in Figure 1, the conical tube is able to dissociate samples using various ridges, a rotor, and spacers. Hydrogel beads are added in the conical tube with the tissue sample because the gentleMACS dissociator is designed to dissociate tissue samples larger than 1 mm^3. By placing the tissue sample in the dissociator with hydrogel beads, the volume and mass of the content being dissociated will be increased. A greater volume and mass will allow to gentleMACS dissociator to more effectively dissociate the tissue. Finally, the hydrogel beads surrounding the tissue sample will provide further mechanical stimulation which will aide in the dissociation process.

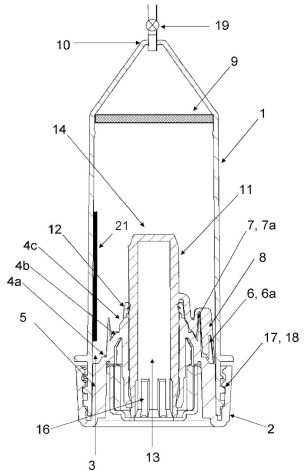


Figure 3:
Design schematic of the conical tubes used with the gentleMACS dissociator.

Preliminary Design Evaluation

Design Matrix

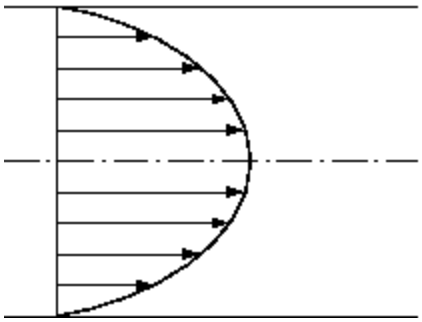
The following design matrix evaluates three methods to dissociate 1 mm^3 pieces of lung tissue. The evaluation criteria were derived from our client's need to obtain viable cells with minimal damage to cell surface markers. An increase in procedural duration will decrease cell viability and increase cell surface marker damage. Each procedure was also evaluated for ease of fabrication and use. While our client's lab is well equipped, our goal is to minimize time between bronchial extraction and single cell analysis by flow cytometry. Our client has requested that the average cost per procedural use would be less than $10.

Single cell viability surface marker integrity were the primary concern of each design. Our team anticipates that the gentleMACS dissociator with hydrogels would allow for dissociation of the most numerous viable single cells with the greatest cell surface marker integrity.

1) $$Re = \frac{\rho \upsilon L}{\mu}$$

1.1) $$L = \frac{4A}{P}$$

In order to achieve velocity profiles aid in dissociating our client's tissue sample, turbulent flow would need to be produced in a microfluidic device. In laminar flow, the sample would simply travel by convection along the flow of the liquid. In order to decrease resistance, the sample would preferentially travel in the vertical center of the included quadratic velocity profile. For this reason, the tissue sample would not experience anywhere close to the approximation of the previous group's estimation of shear force.



In order to induce turbulent flow in a newtonian fluid, a Reynolds number of ~2600 must be achieved. The previous group utilized channels with a 0.00082 m height and 0.00064 m width. Our group would use a channel of similar dimensions due to fabrication and

$$2)\ \upsilon = \frac{Re*\mu}{\rho L}$$

The microfluidic device would decrease cell viability due to the cells as they flow through the device. Using an enzyme and gentle agitation would have the greatest effect on the cell surface markers because this method would require the cells to be suspended in enzymes for the longest period of time [5]. Duration of cell dissociation procedure is an important factor to consider. Dr. Mathur explained that the tissue should be dissociated within four hours of the bronchoscopy or else the surface markers will have the time and ability to change, skewing Dr. Mathur's research. The gentleMACS dissociator will be the fastest approach so therefore it won that category. This was an important aspect to be considered due to the need for cell viability.

The first procedure to be considered for the dissociation of the small tissue sample is the usage of enzymes and gentle agitation. This procedure would be the least expensive because Dr. Mathur's lab contains most of the materials required for this process. Dr. Mathur's lab has already purchased the gentle MCAS device for dissociation that already comes with the required enzymes making this method the least expensive. The cost of the gentleMACS with the addition of hydrogel beads would depend on the materials needed for creation of the hydrogel. The microfluidic device would be the most expensive dissociation method because it would be made of PDMS which is not a material found in our clients lab.

The ease of use and fabrication also are won by the enzymatic and gentle aggiation approach. These techniques are frequently done in the lab and would require minimal training. The hydrogel with gentleMACS method came in second for the ease of use and ease of fabrication because the lab has already purchased and used the gentleMACS device for larger tissue dissociation. The only thing the client and his staff would need to learn would be the

fabrication of the hydrogels. The microfluidic device came in last in these categories because the creation of the PDMS mold and future devices using the molds would require training and would have an increased chance for error. Overall, the ease of use and fabrication was not the most important criteria, but was still something to consider when making the final design decision.

With all of these criteria in mind, the final preliminary design was chosen.

	Weight	Enzyme and Gentle Agitation		Enzyme and Microfluidic Device		Hydrogel Matrix with the gentleMACS Dissociator	
Cost	10	5/5	10	3/5	6	4/5	8
Ease of Fabrication	15	5/5	15	3/5	9	4/5	12
Duration of Dissociation Process	20	3/5	12	4/5	16	5/5	20
Cell Viability	20	2/5	8	4/5	16	5/5	20
Effect on Cell Surface Markers	25	4/5	20	3/5	15	5/5	25
Ease of Use	10	5/5	10	3/5	6	4/5	8
TOTAL	100		75		68		**93**

Figure 4: Design Matrix of the three designs discussed above. Criteria is outlined on the left, evaluations of that criteria for each design is highlighted in grey.

Proposed Final Design

Based on the criteria discussed in the former section, the hydrogel matrix with the gentleMACS Dissociator design scored the highest on the design matrix. This design out performed the other designs in effect on cell surface markers, cell viability, and duration of dissociation process.

A major consideration at this point that helped us make this decision was the fact that this design outperformed the others on its duration of dissociation and its effect on the cell viability markers. As previously mentioned, it is of paramount importance that whatever mechanism is chosen does not have an impact upon cell viability so that when flow cytometry is done on the resulting tissue, the cells that are isolated are as close to *in vivo* conditions as possible. The two other designs that have been proposed involve placing cells in solutions in high concentrations of enzymes for long periods of time or exposing them to large amounts of mechanical stress causing them to experience larger amounts of stress leading to poorer flow cytometry results.

Similarly, it was also considered within the design matrix how various designs would affect the cell surface markers and once again, it was found that the hydrogel design was the best among all of the possible ones. For the hydrogel design, the dissociation process takes the least

amount of time. This allows the tissue to undergo the least amount of stress possible. Having cell surface markers remain intact is important because Dr. Mathur's research predicates highly upon looking at the differences between the surface markers of esophinel's of cells found with the lumen of the blood, lumen of the lungs, and those present within the tissue itself. For this reason, the final design that was picked had the least amount of interference present to these vital surface markers.

Fabrication/Development Process

Materials

Moving forward, the team will propose to the client a chosen kind of hydrogel for design purposes. Depending on the kind of hydrogel the team chooses, the team will either purchase the hydrogel directly from a online vendor or purchase materials needed to synthesize the hydrogel that fits design specifications. Enzymes that complement the hydrogels during the dissociation of the eosinophils will also need to be purchased.

Methods

Once materials have been purchased, different hydrogels will be fabricated. These will vary depending on cross-linkage, swellability, and size. Testing will be discussed in the following section. After the fabrication of the hydrogels and testing to determine the ideal size, cross-linkage, and swellability the procedure will be further analyzed for consistency. To prove that this procedure works for Dr. Mathur's research, further analysis via flow cytometry will be performed.

Testing

During the creation of the hydrogel beads, several factors will be considered, most important of which is which type of material will be used to synthesize the hydrogels. Natural materials like alginate, chitosan, and agarose or synthetic materials such as PEG+Monomers and PHEMA are all possibilities. The material must not be harmful to the tissue or alter any of the tissues surface markers. The chosen material also must be able to withstand the enzymes used in dissociation without falling apart. Different levels of stiffness, swellability, and size are also factors that could affect the dissociation of the tissue. Cross linking will affect the stiffness and swellability of the hydrogel [6]. Different materials as well as combinations of size and stiffness will be tested to find the optimal conditions for dissociation.

Conclusion

The team will conduct additional research to select the material needed for hydrogel fabrication. Additionally, research will be done on enzymes and the various settings on the gentleMACS machine to decide which will yield the most viable cells with the least damage to cell surface markers. Further steps include finalizing a materials order list, fabricating hydrogels, and performing trials to ensure that all product design requirements have been met.

It is anticipated that several challenges will be encountered along the way. An area of concern is whether or not the tissue sample is fully dissociated. In order to ensure that that full dissociation, the amount of detached cells will be counted. Varying the stiffness, swellability, and size of the hydrogels will hopefully allow for full dissociation. Another challenge the team anticipates is the retainment of the surface markers of the eosinophil. The surface markers on the cells must be maintained so Dr. Sameer Mathur can conduct accurate flow cytometry. In order to overcome this problem, hydrogels that produce mild chemical effects upon the tissue sample must be fabricated.

If the synthesized hydrogels do not provide sufficient volume and mass for mechanical stimulation to properly dissociate the tissue, another dissociation approach will be adopted.

Acknowledgements

The Tissue Biopsy Dissociator design team thanks Dr. Sameer Mathur for providing the opportunity and necessary equipment to work on the project. The team also thanks Dr. Wan-Ju Li for providing insightful guidance and advice as well as helping at every stage of this project.

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Appendices

Appendix I - Preliminary Design Specifications

Function:

Extract Eosinophils from a small (1-2mm^3), human lung sample to study the correlation between the presence of eosinophils in lung tissue and asthma. The device should extract approximately 10,000 cells for examination with flow cytometry.

Client Requirements:

The device must have minimal disruption to the eosinophils to allow proper flow cytometry to be performed while still producing a sufficient sample of eosinophil separated from the remainder of cells in the tissue sample. The eosinophils must also be able to be properly identified using flow cytometry. The eosinophils are of primary concern and the other cell types can remain intact in the tissue as long as all the eosinophils are dissociated.

Design Requirements:

1. Physical and Operational Characteristics:

A. *Performance Requirements:* 50% (+/- 10%) of the total cellular mass must be recovered in a single cell suspension in order to be analyzed by flow cytometry. This will require that the cells are not lysed during the dissociation procedure. The device needs to be able to extract roughly 10,000 eosinophils through tissue dissociation of the lung sample. The device will need to be operational daily and subject to common sterilization techniques.

B. *Accuracy and Reliability:* The device needs to extract enough cells for flow cytometry while also not altering cell surface markers or causing cell lysis. At least 10,000 eosinophils but be obtained after the used of the device.

C. *Life in Service:* Non reusable option only need to last for one tissue dissociation. A reusable tissue dissociator will need to be reusable for approximately three years.

D. *Operating Environment:* The device will be used in a common lab benchtop setting and will be subjected to various enzymes and sterilization products. The product may also be subject to a variety of temperature depending on whether it is reusable or not.

E. *Ergonomics:* Simplicity is the main goal of the ergonomic aspect of the device.

F. *Size:* The device must be able to fit on a lab bench and be able to dissociate a tissue sample of an approximate size of 1-2 mm^3.

G. *Materials:* The materials used in the tissue dissociator must be biocompatible as not to interact with the tissue sample. A large component of the materials is that they must be cost effective as a disposable devices must be less than $10 per unit if they are to be considered disposable.

H. *Aesthetics, Appearance, Finish:* Aesthetics and appearance are not a large factor in the making of this device. The device should generally be simple to cause as little confusion as possible as the functionality of the device is the main goal.

2. Production Characteristics

A. *Quantity:* In initial testing only one prototype is required for testing. One device should be used per patient if it is not reusable. If the device is reusable, a fewer quantity will be needed. If chemical dissociation is used, each container should be used once.

B. *Target Product Cost:* A budget for the full project is not defined. The target price of production for a disposable device is around $10. The target price of production for a reusable device would be higher as the client can get more uses out of it. The exact number isn't established as it would depend on how many times the client would be able to reuse it.

3. Miscellaneous

A. *Standards and Specifications:* This is a custom device being used in a specific research setting; there are no international or national standards to abide by.

B. *Customer:* The client desires a way to recover any valuable human tissue should the device not be able to completely dissociate it as it is extremely difficult to procure these samples.

C. *Patient-Related Concerns:* This device will be used in a research setting and the patient will not have contact with the device. No patient information will be retained in the device. The device will be sterilized after each use if reusable, or if device is one time use it will be disposed.

D. *Competition:* As of now, there exists other devices that allow for tissue dissociation. However, these devices often require a large amount of tissue to be passed through them in order for the process to occur. The current Miltenyi tissue dissociator costs $6.40 per sample tube [1]. Currently there are no devices on the market that are capable of taking in such a limited quantity of tissue and being able to completely dissociate it.

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