Gompei's Lab-Grown Leather

A Major Qualifying Project Report

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Abstract

The current leather making process poses a detriment to society given the vast amount of waste and excess harmful chemicals that are produced from the process. The goal of this project is to transform the current process and diminish the negative effects associated with it. The solution is to grow leather within a laboratory through the establishment of a device capable of cell seeding on a plant scaffold while additionally allowing for the perfusion of media. The design team aimed to understand the dynamics of growing bovine dermis and its mechanical properties well enough to produce a device capable of growing a sheet of lab-grown leather. The results of the device were compared to the current leather making process.

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Chapter 1: Introduction

Leather is used in a variety of different applications and is prominent in people's day-to-day lives. The US alone produces approximately 719 million square feet of leather each year, but this only makes up 3% of the total global amount of leather produced (Global Industry Report, 2019). Unfortunately, the leather industry struggles with sustainability and toxicity given the vast amounts of solid and liquid waste produced from the process. Additionally, the industry needs to keep up with the increasing consumer demand and environmental standards for leather products.

In each step of the leather production process, there are factors that negatively impact the environment. Livestock farming, where the raw hide for leather originates, produces over 12% of the global greenhouse gas emissions (Jones, 2021). From there, the processes used to prepare the raw materials for leather production create both solid and liquid waste. Overall, there is 200 kg of water waste, 250 kg of tanning waste, a process that makes the raw hide a more stable material for leather production, and 350 kg of non-tanning waste produced for every 200 kg of finished leather (Li, 2019). Most of these resources are supplied from animals already used in meat production. The leather industry goes hand in hand with the meat industry and relies on certain resources.

Using chromium for tanning is another significant issue in the leather making process. Chromium ends up in the waste from tanning and is toxic and dangerous for the environment. Waste from tanneries, with no treatment, is directed into bodies of water and from all the years of this occurring, this water becomes unsuitable for public use (Li, Guo, Lu, Zhu, 2019). Tannery wastewater can surface, travel downstream, and contaminate freshwater deemed safe for use, as

well as aquatic life. Chromium also has several carcinogenic effects and can cause stomach cancer, asthma, and respiratory problems if ingested or inhaled (Shekhawat, 2015).

These problems demonstrate that there is a need for more sustainable practices. There are several companies already attempting to solve this problem. Modern Meadow utilizes biofabrication and fermentation to create a lab grown leather and Bolt Threads, a Material Innovation Initiative sponsored company, creates an animal-free mycelium leather.

This project aims at finding solutions that address the environmental impact, sustainability, and scalability issues raised in the current process. In order to solve these problems, our team looked into growing bovine dermal fibroblasts on a decellularized spinach leaf scaffold due to it's cost-efficient and environmentally friendly attributes. The team designed a device that would assist in the cell seeding and growth of these bovine dermal fibroblasts on the leaf scaffold to create a sheet of dermis that could later be treated to become leather. Overall, the broad goal of this project was to minimize the amount of solid and liquid waste in the leather production process.

Our device was created to be sterile and biocompatible to induce healthy cell growth. The final design aims to clamp the decellularized scaffold in place and offer a controlled, standardized space for cell growth. We used a cannula to perfuse media into the scaffold to allow for optimal cell growth and have a well underneath to collect any media runoff which was then tested and recycled through again. We also created a piece that attaches to the top of the device and applies 96 cell cloning wells to assist in the adherence of the seeded cells. Lastly, we coded an Arduino Uno to run multiple different sensors to detect changes in the media so we would know when it was no longer usable. We tracked changes in temperature, pressure, and pH of the media. The pressure and temperature sensors were used to further recommend enhancements to

media use such as the addition of a heat exchanger. Meanwhile, the pH sensor helped assess the viability of the media. For proper cell growth, we sought to keep the media between a pH of 7 to 8. If the media became too acidic or basic, then it would no longer be viable for proper cell growth conditions.

Overall, this design is more user-friendly, and is an environmentally conscious alternative to the current leather making processes as seen through a reduction in solid waste by 14% and a reduction in liquid waste by approximately 7%. Scaling up this process and implementing easy, large-scale production practices can lead to a significant decrease in wasted hide, excess water usage, and poor disposal of untreated chromium. While there may be adverse effects for global trade involving leather and cattle farmers that produce raw hide with the implementation of these processes, the impact this would have on the sustainability of leather production, our environment, and the safety of those working and living near leather production plants outweighs those concerns.

Chapter 2: Literature Review

This chapter will define the market for leather and the current methods for leather-making and evaluate the impacts those methods have on the environment. It will also discuss the different options we explored for methods of growing bovine skin in the lab and which options we decided on for our project.

2.1 The Leather Industry

Currently, the leather industry is worth approximately $40 trillion USD (Global Leather Goods, 2019). Over the last five years, the leather industry has thrived and is expected to continue growing in the next five years as well. Figure 2.1, shown below, represents this sentiment by outlining that the value of the leather market in all distribution categories has increased significantly and is estimated to continue well into the future.

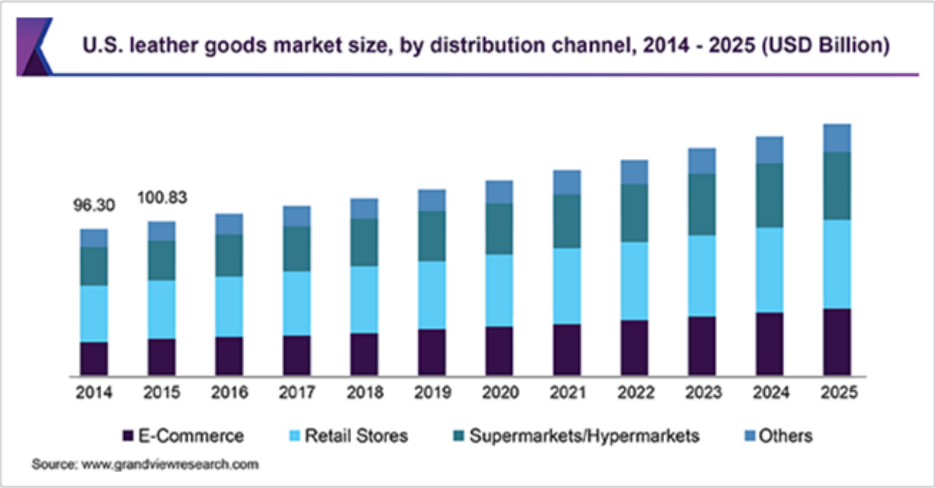


Figure 2.1: US Leather Goods Market Size Distribution over 9 Year Period (Leather Goods Market Size and Share, 2019).

Leather is predominantly made from cow hides but can be made from the hide of almost any animal including crocodiles, goats, pigs, sheep, and moles. The upbringing and environment of these animals will determine the quality and yield of how much leather can be produced from the hide. For instance, a good hide can yield about eighty percent of workable material. However, a hide that has been damaged from barbed wire, insect bites, or from being branded, etc. will most likely result in a sixty percent yield (Fowler, 2007). It is important to note that the leather making process does not start with the hide; instead, it begins with a cow.

Cow hides account for 65% of genuine leather in the United States and, in 2016, the export of raw cattle hides was worth $1.39 billion USD (Leather Goods Market Size and Share, 2019). The market of bovine leather products is driven by inflation and an increase in disposable income; higher levels of income allow people to spend money on higher quality products (Pundir, 2017). While the leather market is worth a lot on its own, it is inherently a byproduct of the meat industry. Leather contributes to 5% of the overall value of a cow, but 75% of the byproduct value. In other words, the cow was primarily raised for meat but the leather is an additional use. As long as there is a demand for meat, the leather industry will never drive the meat industry (Wicker, 2019). However, that does not mean that the current leather making industry does not have other impacts.

2.2 The Current Leather Making Process

The current leather making process consists of three main stages: preparing the raw material, tanning and dyeing, and finishing as seen in Figure 2.2. In preparing the raw material, the skin is removed from the animal through a series of smaller processes such as fleshing, curing, and liming. In fleshing, the flesh is removed from the hide by hand or by using a fleshing

machine. By the end of this process, one side of the hide is a white clean surface (Fowler, 2007). Once the hide is cleaned, it is salted or placed into a salt brine and the curing process begins. Curing is used to help prevent the hide from decomposing and additionally prevents bacterial growth. Next, hair and fat are removed from the hide in liming. Liming uses a chemical solution that often contains calcium oxide. After soaking for about a day or two, the hide will soften, and the hair and fat is removed (Fowler, 2007). Once it has soaked in the chemical bath, the hide is hung since it is full of moisture. In this drying process, the moisture within the hide will cause it to swell and become thicker. Finally, the thicker hide can then be cut into two layers and sent to the tannery (Fowler, 2007).

At the tannery, tanning and dyeing occur. Tanning is a long process that utilizes various machines that aim to convert the raw hide into a stable material. This occurs through a chemical process that can permanently alter the hide's proteins into a stable material that is flexible and durable (Best Leather, 2014). Chrome tanning and vegetable tanning are the two types of tanning typically used in the industry. Chrome tanning is more prevalent and more commonly used and involves matching the reactivity of chromium (III) salt with collagen. This process results in a material that is softer, more durable, and does not discolor. Some of the potential dangers of using chromium in the tanning process includes the waste products because the trimmings and ash contain chromium (IV) which is an environmental danger. Hides are often re-tanned multiple times and treated with agents to ensure its strength, durability, and flexibility (Covington, 2009).

Following the tanning process is dyeing; a lengthy process that adds color to the leather. Dip dyeing is a common process used in tanneries and can either be accomplished using vegetable based or chrome based solutions. The leather product is soaked in a large round drum with water and dye at temperatures ranging from 50 to 60 degrees Celsius. The drum functions

much like washers and dryers, where the rotational movement proves to have more penetration of dye into the product. Both the brush and sponge dyeing processes are used in tanneries for more delicate products like gloves in order to maintain the customer expectations of softness. These processes are costlier for labor since it is very artisanal and requires more time as opposed to dip dyeing where the product is treated in the drum and not by hand. Some tanneries also use spray dyeing machines which consist of a rotary with spray guns. This process shortens industrial time since it is a faster process and includes less labor (Giardini, 2018).

The dyeing process may differ depending on the manufacturer, but a common dye is aniline which is water based without pigments. When the dye is absorbed, the aesthetic aspects of leather such as the wrinkles are exposed. There may be slight variations of color throughout the product depending on the skin's looseness. In addition, semi-aniline dye contains a small amount of pigment which has a better color and may be easier to clean. However, it can be easily damaged with scratches (Moore and Giles, 2020).

After dyeing, finishing occurs in which final touches are added to the leather product. Some attributes that may be added during this time is a patent leather finish, patterns on the leather, or simply putting on the final spray (Best Leather, 2014). After this process, the final leather product is made and undergoes a quality check in order to ensure no mistakes are in the product. While this process produces many high quality products, the amount of waste produced is directly proportional.

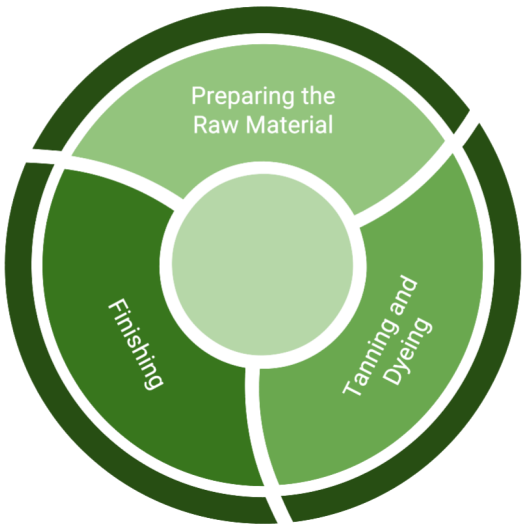


Figure 2.2: Current Leather Making Process.

2.2.1 Waste from the Tanning Process

Over 5.4 billion kilograms (kg) of solid waste is generated from the leather making industry every year. For perspective, processing only 900 kg of leather hides can produce about 200 kg of finished leather, 350 kg of non-tanned solid waste, about 250 kg of tanned solid waste, and about 200 kg of waste lost in wastewater (Li, et al., 2019). The solid waste breaks down to approximately 60% from preparing the raw material through processes outlined in section 2.2 such as fleshing and 40% from tanning and dyeing (BioEnergy Consult, 2020). However, these leather byproducts can be recycled to reduce the overall environmental footprint. More about recycling and rendering of leather byproducts can be found in *Appendix A.1*. The natural keratin from the hair waste can be extracted and used in biomaterials. Similarly, the untanned solid waste and chrome-containing leather solid waste can be treated with the acid hydrolysis method to extract the collagen peptide. This extracted material can be used in biomedical applications

and in cosmetics. Finished leather waste scraps can be used to make smaller items. Tannery sludge can be treated and formed into ceramsite to be used as soil or sand (Li, 2019) as an alternative to wastewater.

Tanning processes cause a great negative impact on the environment due to immense water use and the discharge of many pollutants (Dixit, 2015). An average of 35 cubic meters (m^3) of wastewater is produced per 900 kg of raw hide. It is discharged either directly or indirectly into natural bodies of water through open drains without any treatment. The water that lies in the low-lying areas in developing countries such as India and Bangladesh are polluted to a degree that is unsuitable for public use (Li, 2019). Tannery wastewater and solid wastes can find their way to surface water and the toxins are carried downstream which can contaminate water that is used in bathing, cooking, swimming, and irrigation. These pollutants can include hydrogen sulfide which can enter nearby aquatic systems through wastewater, ammonia being released into the atmosphere, and pesticides used to conserve the hides (Dixit, 2015). Lately, there have been many different initiatives and even some legislation being pushed to find more green policies in this industry. Some methods include treating wastewater to better handle toxic waste, recycling the chemicals used and replacing them with more environmentally friendly one, or even changing the tanning process to allow for different methods (Dixit, 2015). A company called Lanxess is researching and selling their sustainable leather initiative that frames multiple different methods in which the tanning and dyeing processes in the leather industry can become more environmentally friendly (Lanxess, n.d.). Some of the steps in their initiative include a new solution for soaking and dehairing animal hides, using water-based pigments, recycling hide shavings, and using metal and aldehyde free tanning agents (Lanxess, n.d.).

Aside from hydrogen sulfide, another wastewater pollutant is chromium. Chromium waste found in wastewater can seep into the soil which, in turn, contaminates the groundwater systems that provide drinking water and aquatic animals, which are a common food source. Ingesting or inhaling chromium can lead to numerous detrimental effects on the human body, including but not limited to stomach cancer, asthma and respiratory problems, and reproductive problems due to its carcinogenic nature (Shekhawat, 2015). Research also shows that chromium can have a negative impact on the growth and development of plants, which could be detrimental to their ecosystems (Shanker, 2005). A study in Egypt used two systems of wastewater treatment. An electrolytic system resulted in poor removal efficiencies of chemical oxygen demand (COD), total suspended solids (TSS), chromium (III), ammonia, and sulfide. The physico-chemical system involved using calcium hydroxide as a coagulant material for chromium precipitation. Plain sedimentation reduced COD, five-day biochemical oxygen demand (BOD5) and TSS. The results demonstrate a promising future for wastewater treatment with the removal of 98.8% chromium (III), 51.2% TSS, 31% COD, and 25.8% BOD5 (Elsheikh, 2020).

2.3 Making Leather: Alternative Companies Changing the Leather Industry

According to the United Nations Food and Agricultural Organization (FAO), livestock production is a major contributor to land degradation, climate change, biodiversity and water loss. For a frame of reference, 26% of Earth's terrestrial surface is used for grazing and 33% of arable land is being used for feed-crop production (Magic, 2018). Additionally, the livestock sector contributes a large portion of emissions into the atmosphere encompassing 9% of global carbon emissions, 37% of global methane emissions, and approximately 65% of nitrous oxide

emissions (Magic, 2018). Overall, livestock production to produce goods such as meat poses a threat to the environment and needs to be rectified to further relieve stress from the growing population. Therefore, in addition to the creation and production of pleather, a synthetic leather alternative explored in more depth in *Appendix A.2*, many companies have taken the initiative to find alternatives that closely resemble authentic leather to help diminish these problems. Modern Meadow and Bolt Threads are two companies that have made strides in changing the leather industry.

2.3.1 Modern Meadow

Modern Meadow is a startup company that aims to become a top source of leather for the world's makers of accessories, fashion, sporting goods, upholstery, and furniture (Magic, 2018). Modern Meadow is working to transform the industry by finding an alternative to raising animals for slaughter and removing their skin in an intensive process. Their approach involves removing waste from the current leather making process. Their goal is to completely eliminate the use of animals in the leather making process and grow leather in laboratories. Previously, Modern Meadow accomplished this goal using the biofabrication process; however, over the years, they eventually switched to fermentation instead.

Modern Meadow started out utilizing biofabrication to make its leather products. Biofabrication incorporates the use of cow skin cells to yield leather that is "biologically identical" to traditional cow dermis (Magic, 2018). Through this process, Modern Meadow eliminates the waste associated with preparing the raw material in the current leather making process. This bioleather eliminates the possibility of imperfections that may be seen on a cow hide such as scars and disease making the workable material yield a hundred percent as opposed

to sixty percent. No hair or flesh needs to be removed from the hide for they are growing dermis that can be made to a designer's and consumer's specifications including elasticity, flexibility, thickness, and thinness (Magic, 2018). Overall, this bioleather could reduce cost and variability of input material while also decreasing waste and simplifying the supply chain (Magic, 2018). Figure 2.3 below shows the process that has improved performance in the leather industry. The resulting product is then sent to the tannery for additional dyeing and product enhancement.

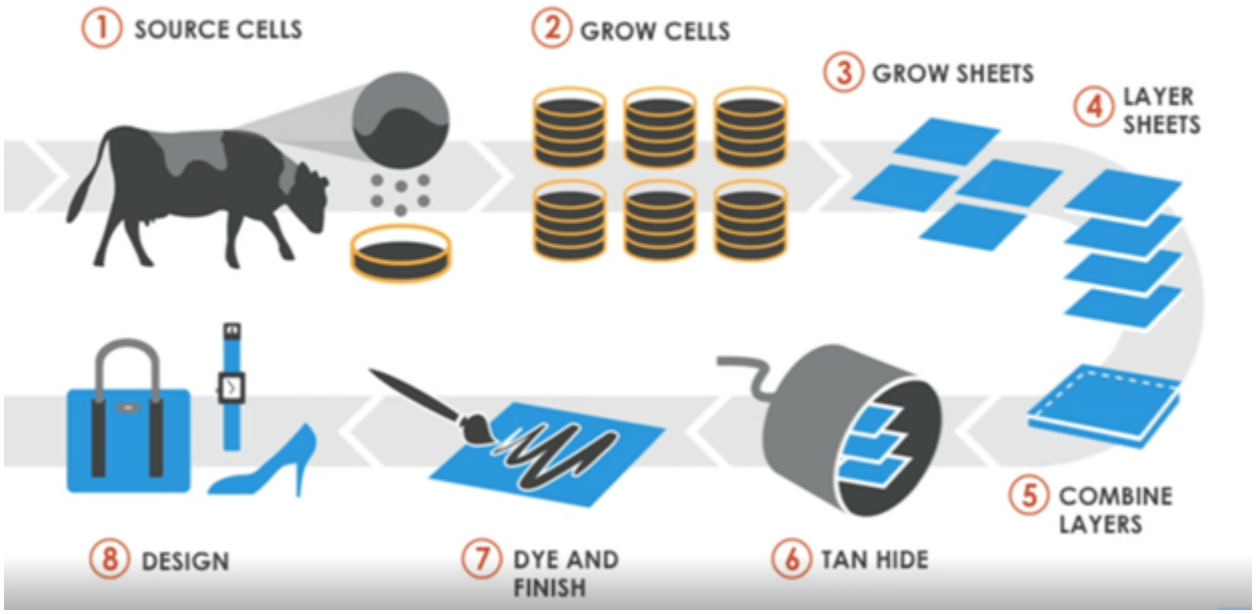


Figure 2.3: Modern Meadow's Biofabrication Lab-Grown Leather Process Diagram (Magic, 2018).

Eventually, Modern Meadow eliminated the use of cow skin cells altogether and started utilizing the fermentation process. For this, the use of a bio-engineered strain of yeast that when fed sugar produces collagen. This collagen is then purified and assembled and tanned to create a material that is nearly identical to leather (Magic, 2018). This process is outlined in Figure 2.4

below. Despite the success that Modern Meadow has seen in eliminating waste, it still faces the challenge of reducing waste from the tanning and dyeing processes. Other challenges faced by the company are making their products affordable. The advantages to the lab-grown leather process will be taken into consideration for our project. However, our team will investigate alternatives to see if we can overcome their shortfall in affordability.

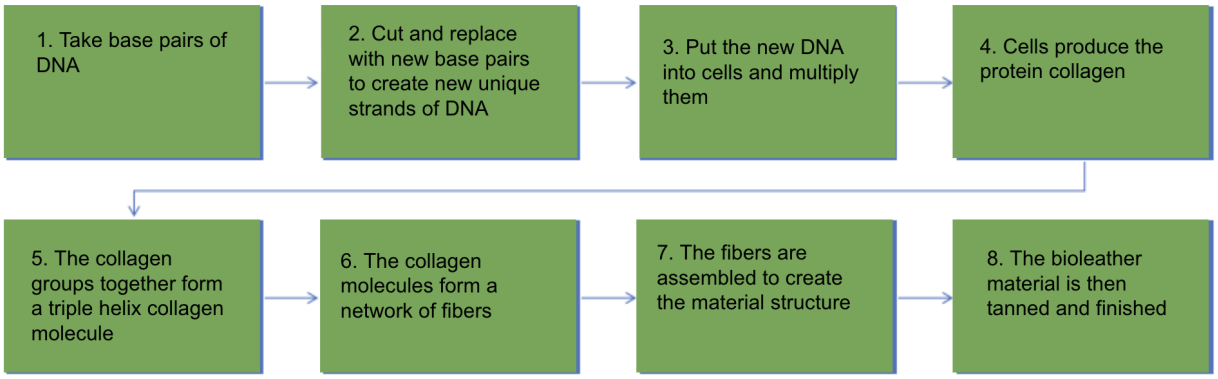


Figure 2.4: Modern Meadow's Fermentation Lab-Grown Leather Process Diagram.

2.3.2 Mylo by Bolt Threads

Bolt Threads, an up and coming company, have similar values and beliefs as Modern Meadow in regards to eliminating animal usage in the leather industry. However, while Modern Meadow utilizes biofabrication, Bolt Threads has a different process to eliminate the use of waste in the current leather making process. Bolt Threads works in collaboration with Material Innovation Initiative (MII), a non profit organization that supports animal free production of materials. Together, they were excited to grow mycelium as animal-free leather called Mylo (Bolt Threads, 2021). Their solution focuses on the use of mycelium, in other words, the use of fungi and mushrooms to make leather. Mushrooms and fungi are abundant and unique materials that can be used as source cells that will grow and evolve into an interconnected

three-dimensional network. This three-dimensional network would then be moved to processing where it would be tanned and dyed. The process to make Mylo can be seen in Figure 2.5 below.

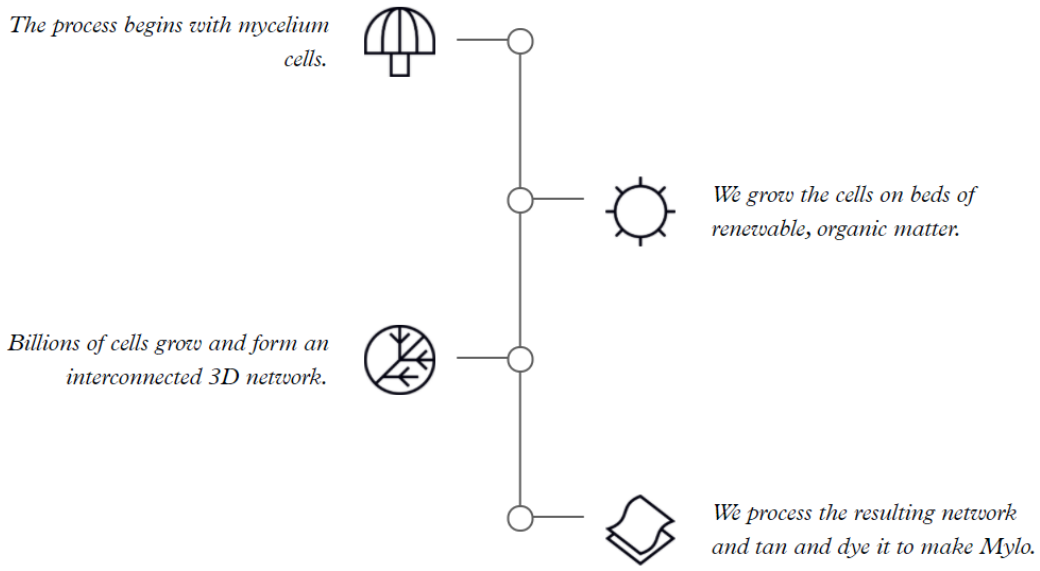


Figure 2.5: Bolt Threads Process for Developing and Producing Mylo (Bolt Threads, 2021).

MII further explains the mycelium leather making process as seen in Figure 2.6 below. MII and Bolt Threads utilize scaffolding to gather agricultural waste. They purify and combine the agricultural waste with other ingredients such as fungal spores. The combined ingredients are placed in an environment that possesses a temperature of 37℃, contains 5% carbon dioxide, and has ample humidity in the airflow (Material Innovation Initiative 2020). With this, a dermal substitute is produced and transforms into a market good through the standard processes of dyeing, cutting, and coating. Bolt Threads additionally eliminates the usage of animals in production of mylo; however, they still face the problem of reducing waste in dyeing and tanning processes.

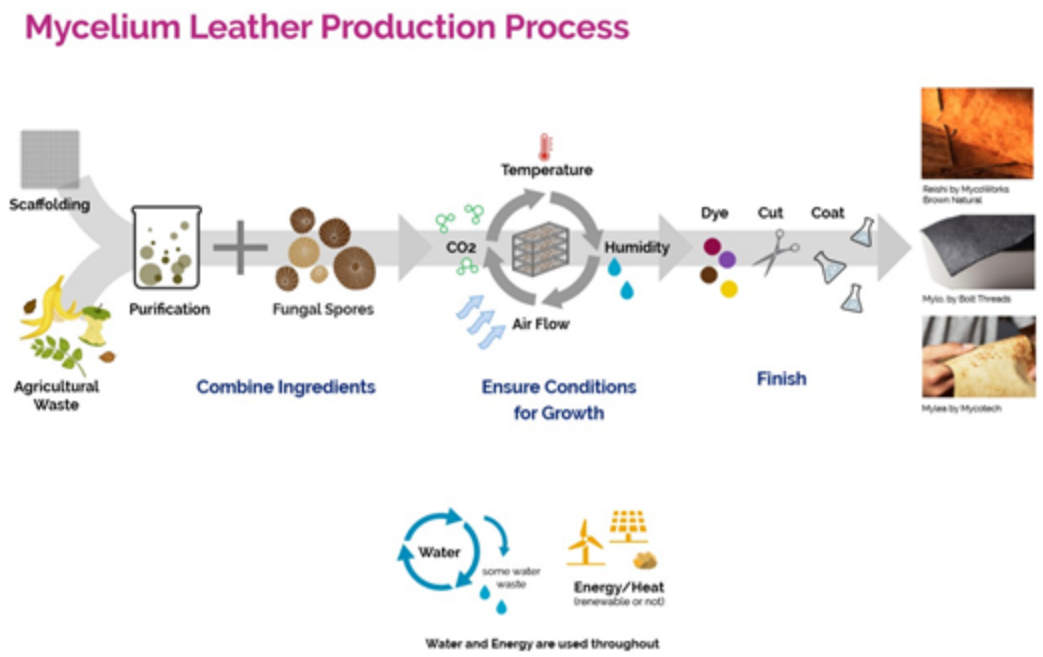


Figure 2.6: MII's description for making lab-grown leather (Material Innovation Initiative 2020).

2.3.3 Comparing Existing Methods

Upon reviewing existing companies and their work in the industry, our team delved into some research on what processes currently exist for making leather including the current process being used. We drew comparisons on what each company accomplished or what they may have lacked in producing leather products. As referenced above, we found two emerging companies within the field which include Modern Meadow who utilizes fermentation to produce leather and Bolt Threads, who have grown mycelium animal free leather. Table 2.1 below compares the current leather making process to these two renowned companies.

Table 2.1: Comparison of Existing Solutions.

Process:	Pros:	Cons:
The Current Leather Making Process	User friendly, reproducible, produces viable leather products	Uses animals, produces waste from tanning and dyeing
Modern Meadow - Fermentation	Eliminates the use of animals, reproducible, produces viable leather products, control over the product's characteristics such as size, shape, color, etc.	Produces waste from tanning and dyeing, not user friendly, costly
Bolt Threads - Mycelium Leather	Eliminates the use of animals, reproducible, produces viable leather products	Produces waste from tanning and dyeing, not user friendly, costly

2.4 Tissue Engineering for Skin Production

Tissue engineering is a branch of biomedical engineering that utilizes the combination of cells and engineering to restore, maintain, improve, or replace different types of biological tissues. For our purposes, we will use cells to grow a dermal layer that can be tanned and dyed to make a leather substitute. We chose this method in order to reduce waste, while maintaining mechanical properties and still using cow "skin."

2.4.1 Stem Cells

Tissue engineering has many real world applications. Currently, stem cells, specifically mesenchymal, are studied as a potential treatment for traumatic skin injuries such as burns (Ude, Miskon, Idrus, Bakar, 2018). When deciding which cell type to use, one must seriously consider what properties will be best for the application. Properties to consider include but are not limited to growth rate, ease of culture, availability, and byproducts produced. Table 2.2 outlines the main

pros, cons, and ethical concerns of three types of stem cells. Adult stem cells can transdifferentiate or change from one mature somatic cell to another without dedifferentiating beforehand, but it is not well studied. There are limitations on the cell culture time and on large scale manufacturing of these stem cells. In addition, there is not extensive research on the differentiating ability. These cells are harvested from adult tissues such as fat or bone marrow, so there are no major ethical concerns raised. Embryonic stem cells can maintain and grow for at least a year in culture and there are established culture maintenance protocols. These cells can differentiate into most cell types. The only limitation is that there is an inefficient process to generate cell lines. In humans, major ethical issues are raised because an embryo must be destroyed in order to acquire the stem cells (University of Nebraska Medical Center, n.d.). One last stem cell type is induced pluripotent. These cells are very abundant, but there are no defined methods of maintenance in the laboratory. The reprogramming of these stem cells can lead to abnormally high proliferation rates, so tumors may result without monitoring (University of Nebraska Medical Center, n.d.).

Table 2.2: Pros and Cons of Cells (University of Nebraska Medical Center, n.d.).

	Adult Stem Cells	Embryonic Stem Cells	Induced Pluripotent Stem Cells (iPSCs)
Pros	Trans differentiate and reprogramming of these cells is possible but is not well studied Success has already been demonstrated in various clinical applications	Can maintain and grow for 1 year or more in culture Established protocols for maintenance in culture ESCs are pluripotent cells that can generate most cell types	Abundant somatic cells of donor can be used
Cons	Limitations on ASC ability to differentiate are still uncertain; currently thought to be multi or unipotent. Cannot be grown for long periods of time in culture Usually, a very small number in each tissue making them difficult to find and purify Currently there is no technology available to generate large quantities of stem cells in culture	Process to generate ESC lines is inefficient	Methods for ensured reproducibility and maintenance, as differentiated tissues are not certain.
Ethical Concerns	No major ethical concerns have been raised	General ethical concerns about growing embryonic stem cells but no major concerns are raised for bovine embryonic stem cells.	Abnormal reprogramming of stem cells can produce tumors

2.4.2 Fibroblasts

While stem cells have their advantages and disadvantages, fibroblasts are an alternate choice for this project. While there is not a lot of research into the use of bovine fibroblasts, normal human dermal fibroblasts (NHDF) however, are often used in cancer research, tissue regeneration, and tissue engineering studies. Since this is the case, there is a possibility of growing bovine skin. The company PromoCell sells NHDFs that are isolated from either juvenile foreskin or adult skin from locations such as the face, breasts, abdomen, and thighs. The company sells these cells in a cryopreserved, proliferating, or cell pellet format.

Bovine fibroblasts are useful for *in vitro* modeling to study wound healing mechanisms and develop therapeutic targets in order to reduce scarring and fibrosis. ScienCell Research Laboratories sells fibroblasts that are isolated from fetal bovine skin. The bovine cells are cryopreserved at passage one and delivered frozen. Each vial contains a minimum of 500,000 cells in 1 milliliter volume. The bovine dermal fibroblasts are characterized by immunofluorescence with antibodies specific to fibronectin and are tested for mycoplasma, bacteria, yeast, and fungi. They are guaranteed to further proliferate for 5 population doublings under ideal conditions (this will be discussed in more depth in Section 4.3.4.2 Cell Count) and cost approximately $427 (Sciencell Research Laboratories, 2020).

2.4.2.1 Dermis

The raw hide before processing is made up of the dermis, epidermis, and a subcutaneous layer. These account for 85%, 1% and 14% of the hide thickness respectively (Leather Resource, 2008). The dermis is primarily made up of fibroblasts that produce collagen, which creates a tough and supportive cell matrix. The dermis consists of two layers. The papillary layer is attached to the epidermis using thin loosely arranged collagen fibers. The reticular layer extends from the papillary layer to the subcutaneous layer. The dermis is largely constructed by the collagen protein which maintains skin toughness and strength. In addition, the thickness of the dermis varies upon location; for example, the thickness is 0.6 millimeters (mm) on the eyelids and 3mm on the back, palms, and soles of the feet (Leather Resource, 2008).

2.5 Cell Culturing

Cell culture involves cells placed in a controlled environment that require an appropriate surface, supply of nutrients, and certain levels of humidity, temperature and gas in the environment in order to thrive. Cells are typically grown in an incubator that is 37 degrees Celsius, and 5 percent carbon dioxide. These concentrations vary depending on cell type and the cell culture conditions. The supply of nutrients in cell culture media needs to be prepared in the lab. The components usually consist of Dulbecco's Modified Eagle Medium (DMEM) basal media, Penicillin Streptomycin, L-Glutamine/Glutamax, and Fetal Bovine Serum (FBS). The cells are usually submerged in the media for more direct perfusion of nutrients.

An important aspect of cell culture is passaging or sub-culturing, which is completed when cells reach confluence; usually around seventy to eighty percent of area coverage on its respective surface. For the scope of our project, the goal is to grow our cells past confluency to grow the sheet of bovine dermis out of fibroblasts. The levels of humidity, temperature and gas concentrations remain consistent and the media composition should still be similar. The media however, requires an important component for growing sheets to self assemble, that being ascorbic acid. Ascorbic acid is derived from vitamin C and promotes collagen synthesis and deposition of glycosaminoglycans important for skin substitutes. It is important for the creation of the sheets of matrix, which allows them to still proliferate over confluency (Saba, I., Jakubowska, W., Chabaud, S., & Bolduc, S. , 2016).

2.6 Scaffolds

Researchers have been looking for ways to support cell growth *ex vivo* for different applications including wound healing and tissue regeneration over the past few decades. For any artificial tissue to grow, a structure to support the cells is required. In some cases, vasculature can transport nutrients to promote cell growth as well. These structures are called scaffolds and can be created with several different biomaterials. Natural polymers can be used as they have extremely high biocompatibility and elicit little to no immunological response, which is necessary for implantable applications (Ninan, 2015). However, these scaffolds usually degrade much too rapidly for most applications. Synthetic polymers have also been used because their properties are much easier to control and apply to the desired application. They can be biocompatible, although there is the risk of eliciting a negative immunological response when implanted in the body (Ninan, 2015). Researchers have even gone as far as to weave strands of lab-grown human fibroblasts to use as a scaffold for different applications (Magnan, 2020).

Plant scaffolding involves using plant leaves with desirable mechanical properties and ability to structurally support and promote cell growth. The vascular network of leaves proves to be beneficial to supply nutrients to cells while they grow. The perfusion of nutrients throughout the leaf provides the scaffold with an opportunity to grow a piece of dermis thick enough to be used for the establishment of a leather product. This vasculature also proves advantageous over other applications. For instance, 3D printers at this stage in development are not capable of using these natural or synthetic polymers to create the beneficial vasculature that the plant scaffolds provide (Gershlak et al, 2017).

Plant scaffolds are also a more ethical option because they don't require animal parts for fabrication. Collagen and gelatin, which are widely used biomaterials, are being used to create natural polymer cell culture scaffolds but the collagen and gelatin usually come from bovine or porcine sources (Song, 2018). Plant scaffolds are also more environmentally friendly because even synthetic polymer scaffolds that are created from renewable sources, such as PGA, still have a negative impact on the environment. The development of PGA still leaks toxic chemicals into the environment and uses large amounts of land and water (Brizga, 2020).

While there are numerous advantages to plant scaffolds, they still have their limitations. For instance, the length of time these scaffolds will support cell culture is unknown given that plant scaffolds are not commonly used in applications today. Meaning, that it is still unknown if plant scaffolds can support long term cell culture.

2.7 Literature Review: Conclusions

The current leather making process consists of three steps: preparing the raw material, tanning and dyeing processes, and finishing. The current way for making leather poses a detriment to society given the vast amount of waste produced during the process. Current solutions exist to assist in diminishing the waste from preparing the raw material. These solutions include making leather through fermentation as used by companies like Modern Meadow, and leather made from mushrooms and fungi as seen in companies like Bolt Threads. Other applications in the tissue engineering field include cell culture experiments on plant scaffolds. The design team intends to utilize these existing solutions to develop a project strategy that will alter the current leather making process for the better.

Chapter 3: Project Strategy

This section will define our initial client statement, evaluate the needs, wants, and constraints for our project, and use this information to formulate our revised client statement.

3.1 Initial Client Statement

When this project began, our general objective was to develop a process for creating leather using lab-grown bovine tissue. We also aimed to use more environmentally friendly methods of tanning and dyeing. With these goals in mind, our original client statement was drafted as follows:

> *"The goal of this project is to develop a process for growing, tanning, and dyeing bovine skin to produce leather such that the methods of the process make significantly less of a negative environmental impact compared to the current leather-producing process."*

3.2 Stakeholders

Once presented with the initial client statement, the design team identified the primary stakeholders for the project. Stakeholders are defined as individuals or organizations that can gain or lose from the success of a system (American Society for Quality, 2021). The designers responsible for establishing a device that will benefit the stakeholders involved are multi-disciplinary in both the biomedical and mechanical engineering fields. The clients support and sponsor the project, while providing the project with context. The users are those who will ultimately utilize the designed product. The primary stakeholders for this project are shown in Figure 3.1 and include designers, clients, and users. While this section focused on the project's

stakeholders, please reference the concept map in *Appendix B.1* which lists the stakeholders for the lab-grown leather industry overall.

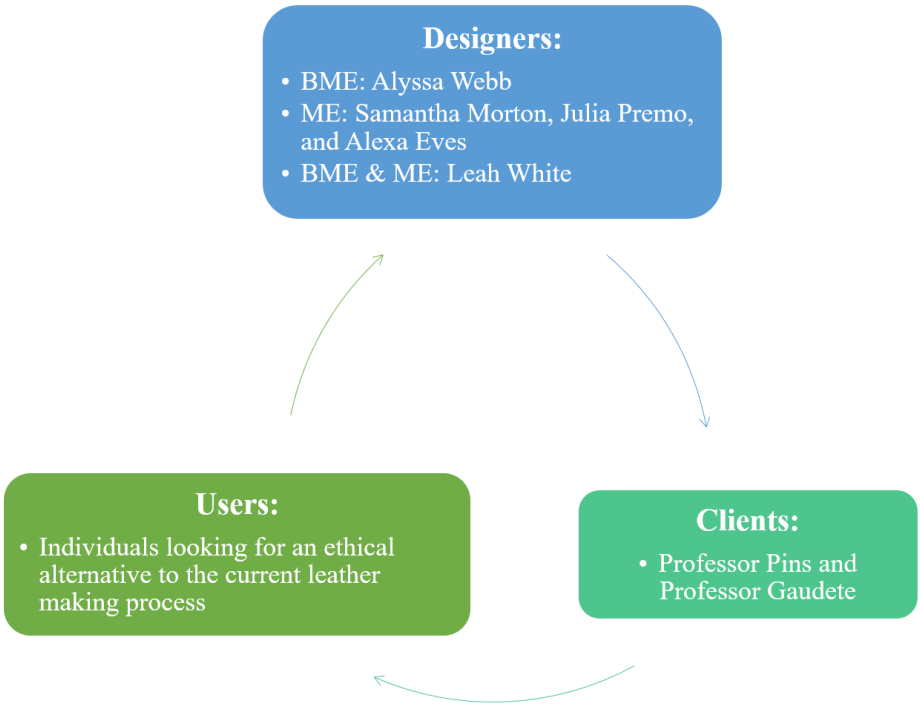


Figure 3.1: The Project's Stakeholders.

3.3 Design Requirements

In order to achieve the goal defined by the initial client statement, the design team needed to consider the objectives, constraints, and needs of the design. The following section will identify these characteristics and help the team make future design decisions.

3.3.1 Initial Objectives

The team began to define the project's initial objectives. For this, we first had to determine the client's objectives, the designer's objectives, and the user's objectives. In order to identify the client's objectives, we analyzed our initial client statement to determine what they were looking for from the design team. From the initial client statement and meetings held with the clients, the team came to the conclusion that the client's wanted the design to accomplish cell viability, sustainability, and to minimize negative impacts on the environment. The design team held similar values and aimed to achieve cell viability, reduction in waste, and also ease of use. Finally, understanding that users will be operating the design, it was determined that the elimination of animal usage, reduction of waste, reproducibility, ease of use, and functionality should be taken into consideration. If the design is user-friendly and reproducible, users can follow various procedures to recreate the lab-grown leather object. This leather object will appeal to others through its elimination of animal use and reduction in waste; however, while it may be an ethical alternative, the leather also needs to be functional. In other words, it should function and possess properties similar to other leather products in the industry. From there, the design team derived a list of objectives that can be further analyzed and addressed.

We also wanted this process to be reproducible so that other researchers could achieve similar results if the same process was followed. This required the team to produce a design that can yield similar amounts of bovine cells every time the process is run. The team, additionally aimed to make this process more user-friendly so that those without significant knowledge in the field can still replicate it. A comprehensive list of all of our initial objectives can be found in Table 3.1 below.

Table 3.1: Describing Objectives

Objective	Description
Scalable	Ability to be used in large scale manufacturing to tailor to the world-wide need
User-Friendly	Want any person not involved in project to reproduce process
Reproducible	Ability for future groups to replicate our processes successfully
Accuracy and Precision	Ability to create the most exact duplicate to regular leather, meet ASTM standards
Ethical	Uses a less invasive and inhumane process
Cost Effective	Saves money or resources required to treat animals/prepare them for butchering
Timely	Can be achieved in a reasonable amount of time

3.3.2 Constraints

Constraints needed to be addressed in advance in order for the design team to successfully work around them. The constraints for this project can be categorized as technical or biological. The specified constraints for these two categories may be found in Table 3.2 below. In terms of technical constraints, our team had to work around time, costs, lab-availability, and size. The time of this project is constrained to a single academic school year. While the COVID-19 pandemic continues to rise, lab accessibility will be limited, constraining the time even further. On the other hand, the budget is constrained to $250 per person, totaling $1,250 for the entire project. Finally, the size of the device needs to be able to fit in an incubator for cell growth to take place. Biological constraints were greatly taken into consideration seeing that cell growth is vital in the success of our project. In order to successfully grow leather, the fibroblasts must grow under optimal conditions. Meaning, that the cells cannot be contaminated. In order to prevent contamination, the design must be able to withstand conditions for sterilization and be made from a biocompatible material.

Table 3.2: Constraints.

Constraint:	Specification:	Description:
Technical	Time	Project must be completed by the end of the 2021 academic school year.
	Costs	The team is allotted only $250 per person ($1,250 total).
	Lab-Accessibility	Given the pandemic, lab-space is limited and only a few members of the team were permitted to be in the lab at a time.
Biological	Sterility	Samples cannot be contaminated.
	Biocompatible	Materials cannot interfere with fibroblast cell functions and growth.

3.4 Final Objectives

Preliminary brainstorming of our project goals took place and the primary discussion points were outlined in the Five Stage Prescriptive Model found in *Appendix B.2*. The resulting objective map as discussed by the designers may be found in Figure 3.2 below. In this figure, primary objectives may be found on the left-hand side and the right-hand side encompasses secondary objectives that stem from the primary objectives.

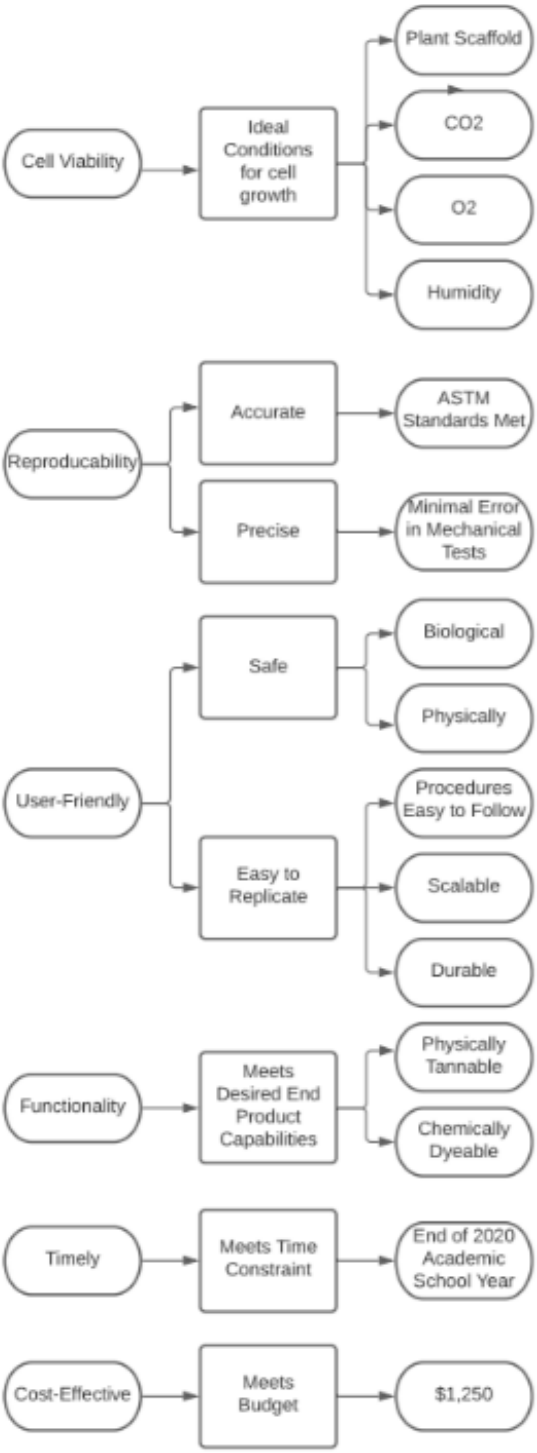


Figure 3.2: The Objective Map.

The primary objectives were ranked in terms of importance using a pairwise comparison chart. Each objective received a score of 0 or 1, when compared to each other. A score of "0" indicated less importance, while a score of "1" meant greater importance. The resulting scores were summed for each objective at the end of each row. A total score of "0" indicated minimal importance in comparison to a score of a "5" which is the objective that possessed the highest importance. In simplest terms, the larger the number, the greater the importance on the objective. The pairwise comparison chart can be seen below in Table 3.3.

Table 3.3: Pairwise Comparison Chart of Design Objectives.

	Scalable	User Friendly	Reproducible	Cell Viability	Cost Effective	Timely	Total
Scalable		1	0	0	1	0	2
User Friendly	0		0	0	1	0	1
Reproducible	1	1		0	1	1	4
Cell Viability	1	1	1		1	1	5
Cost Effective	0	0	0	0		0	0
Timely	1	1	0	0	1		3

The design team worked with the clients at weekly meetings in order to integrate the client's thoughts into the design. Objectives and design functions were deliberated upon at these meetings. In collaboration with our clients, the design team agreed that cell viability was most important. If the device is unable to support the growth and maintenance of fibroblasts, there would be no viable market for the device. Our second most important objective is that our

experiments are reproducible. We want to be able to repeat the experiment and see similar results or make changes to improve results. The third most important objective is that the design process and experiment is timely, given the constraints of an academic school year and the rising threat of the global pandemic.

This process also needs to be scalable. It needs to be able to be performed on larger or smaller surfaces as well as the ability to be mobilized. Our process will be executed on a spinach leaf, however many other leaves with larger surface areas can be utilized to create larger skin grafts for more production. These can include but are not limited to pineapple leaves, iceberg lettuce, Amazonian tree leaves, or any other leaf with an apparent stem and fibers.

To simplify our methodology as much as possible, it is important that the process is user friendly, allowing anyone with no professional experience to be able to execute and understand. To accomplish this, we plan on creating a device that is reproducible through 3D printing and utilizing common tools while cell seeding and performing mechanical tests.

The least important objective is the cost of the lab-grown leather. Our budget is provided by WPI so the amount of money that the design team intends to spend cannot exceed the allocated funds. This can be seen clearly in the budget outlined in *Appendix B.3*.

With our primary objectives in mind, we aimed to develop a new process for the creation of leather for a keychain that will reduce the usage of animal products in the current system. In order to accomplish this, we intend to use tissue engineering to produce bovine dermis. These cells need to produce collagen to build a final product with dimensions that are 2 inches long, 1 inch wide, and 1/16th of an inch thick. Additionally, this process will reduce solid and liquid waste by eliminating hair, scar tissue, and fat waste, water waste, and Chromium waste. In the current process, 70% of untreated leather is discarded (Tarantola, 2014). For Chromium tanning,

tanning one-ton of bovine hide results anywhere between 20 to 80 cubic meters of wastewater from Chromium concentrations around 250 mg/L and Sulfide concentrations around 500 mg/L (Tarantola, 2014). Our goal is to reduce total waste of the tanning process by recommending alternative methods that can be implemented, or put into practice.

3.5 Revised Client Statement

After reviewing our initial client statement and objectives, our team realized that a large portion of this project would be developing a device that would allow us to grow the desired cells on our plant scaffold. This device would need to be able to hold the scaffold properly so that the bovine fibroblasts could be seeded onto the surface of the leaf and media could be perfused or pipetted onto it. Given that one of our primary goals is to reduce the waste created, we aimed to create a system that could collect the media runoff so that any solid waste could be filtered out and the media could then be reused. Ultimately, our initial client statement was revised to read:

> *"The goal of this project is to develop a process that will produce bovine dermal fibroblasts to adhere and proliferate on a plant scaffold that is 5.08 centimeters long, 2.54 centimeterswide, and 0.15875 centimeters thick. The team will design a device which holds a decellularized spinach leaf in place and allows for an automated pumping system to move media through the scaffold. Cells will grow on the device for 4 weeks and media will be perfused at a flow rate of 0.285 milliliters per second through the vascular network of the scaffold. The team will also suggest possible project enhancements as well as alternatives for the tanning and dyeing process to reduce waste."*

At minimum, it is important that we establish a procedure supported by research to tan and dye the leather while having a sheet of dermis that is structurally competent to be used as a sample even if we cannot carry it out in the laboratory.

3.6 Project Approach

Before establishing a proper design process and procedure, our team needed to analyze how to properly execute our objectives and satisfy our client statement. A project flow chart was created and can be seen in *Appendix B.4*.

3.6.1 Management Approach

Our team commenced work in August of 2020. Until October, the team conducted background research and analyzed literature reviews. From October to December, the team created different preliminary designs and completed more research relating to the design process. This research included work on procedures, design functions, specifications, a Five Stage Prescriptive Model, stakeholders, Value Factor Analysis, Pairwise Comparisons, objective maps, and specific components of the design as outlined in previous sections. The team completed the development of the design and performed preliminary mechanical testing at the end of the semester and into the winter break. An important component of the management plan was weekly meetings with our advisors. Each week involved presenting the past weeks' progress, new findings and ideas, and formulating plans for the following week. This ensured the project was continuing to move forward, while additionally helping the team stay on task and remain organized. The team met without our advisors the day before and the day after the weekly

meetings to prepare a discussion and establish and divide individual new tasks for the following week.

However, there were some complications and obstacles in weekly meeting plans given the COVID-19 pandemic. It did not impact A term, August to October, due to the primary nature of the project being more research oriented. In B term, October to December, however, the design portion and preliminary lab work were expected to be completed. These tasks were hindered and time was lost due to the pandemic. In C term, the first two weeks were restricted to fully remote. This meant we could not do any in person lab or project work. The pandemic additionally impacted the team's ability to finalize design concepts. Virtual meetings held over Zoom and various schedules impacted meeting attendance and communication. Information was oftentimes miscommunicated and conflicting information and suggestions were brought to the team's attention. The pandemic also delayed lab access and limited available resources and safety trainings. Additionally, heightened restrictions brought forward in the middle of the term resulted in minimal activity on campus and team members scattered in different places throughout the pandemic.

Chapter 4: Design Process

The design process involved creating many different tools to evaluate and consider various aspects of the project, including constraints and objectives. Pairwise comparisons were done to rank each of the objectives the team came up with. Specific functions needed were outlined in the five stage prescriptive model. Our designs and approach were compared to competitors in the value factor analysis. These tools were used to make sure that the design incorporated all necessary parts and fulfilled the objectives and constraints. The following sections will outline the needs and wants as functions that the final device must consider. Determining these functions allowed the team to adapt a vague idea to a specific model.

4.1 Needs Analysis

After establishing our objectives, the design team further analyzed the primary project needs. We created a table featuring our Needs and Wants, seen below in Table 4.1, describing the purpose of each element and its importance to the final product.

Table 4.1: Defining Design Needs.

Need	**Definition**
Scalable	Ability to be used in large scale manufacturing to tailor to the world-wide need
User-friendly	Anyone can feasibly do this process given the proceedure
Reproducible	Ability for future groups to replicate our processes successfully
Accurate and precise	Ability to create the most exact duplicate to regular leather
More ethical	Uses a less invasive and inhumane process
Cost effective	Saves money or resources required to treat animals/prepare them for butchering
Want	**Definition**
Reduce animal usage in producing leather	Dependent on when lab grown meat market increases and animal farming for meat decreases
Reduce solid waste from process	Dependent on when lab grown meat market increases and animal farming for meat decreases
Reduced liquid waste from process	Dependent on when lab grown meat market increases and animal farming for meat decreases
Timely fashion	for us: done before end of C term

4.1.1 Design Needs

Given that cell viability was the highest ranked primary objective, maintaining proper conditions such as temperature and carbon dioxide (CO_2) will be crucial considerations in our design. Incubators have control over preferred CO_2 levels and temperature. The CO_2 levels will be kept at 5% and the temperature will be maintained at 37° Celsius. Other factors that must be taken into consideration for cell viability are the sterility and biocompatibility of the device itself. With proper sterility and biocompatibility, the cells will not become contaminated throughout the experiment. Therefore, our device needs to be sized to fit in an incubator. In addition, the design must be able to successfully clamp and secure a decelled plant scaffold while media is pumped through the stem of the leaf. The decelled plant cannot rip or tear when media is being pumped through it. If the scaffold breaks and tears, then the cells will no longer be viable for use. Furthermore, in order to actively determine how this project can be scaled, different plant scaffolds will be explored such as spinach and cabbage.

4.1.2 Design Wants

Determined from the functional objectives, design wants are secondary to design needs as mentioned above. For our leather making device, our goal to grow more ethical leather and reduce waste is reflected in our wants. In the future, hopefully tanning and dyeing leather can be done with minimal processes but still meet the aesthetic standards of the consumer. We want a device that grows leather and meets the aesthetic consumer standards. These go hand in hand with the user-friendly design needs as well as the more ethical design needs. By reducing waste, we are reducing the environmental impact and the adverse effects associated with the improper disposal of solid waste such as dumping wastewater into local drinking water sources. Finally, by

eliminating the need for animals, we will eliminate the poor treatment of the animals going forward.

4.1.3 Needs and Wants Design Matrix

A design matrix was created based on the defined design needs and wants. This allowed for a consolidated compilation of our needs and wants which influenced design decisions. Figure 4.1 below shows the complete matrix. This formatted spreadsheet was sourced from the University Massachusetts Lowell and was adapted to include our design. The top portion shows how objectives are correlated and how they can be improved. The customer importance and pre existing solutions are included in the matrix as well. This allows for a clear comparison of our product against current processes. Through ranking the importance of our objectives, the design team was able to determine the needs and wants for our device.

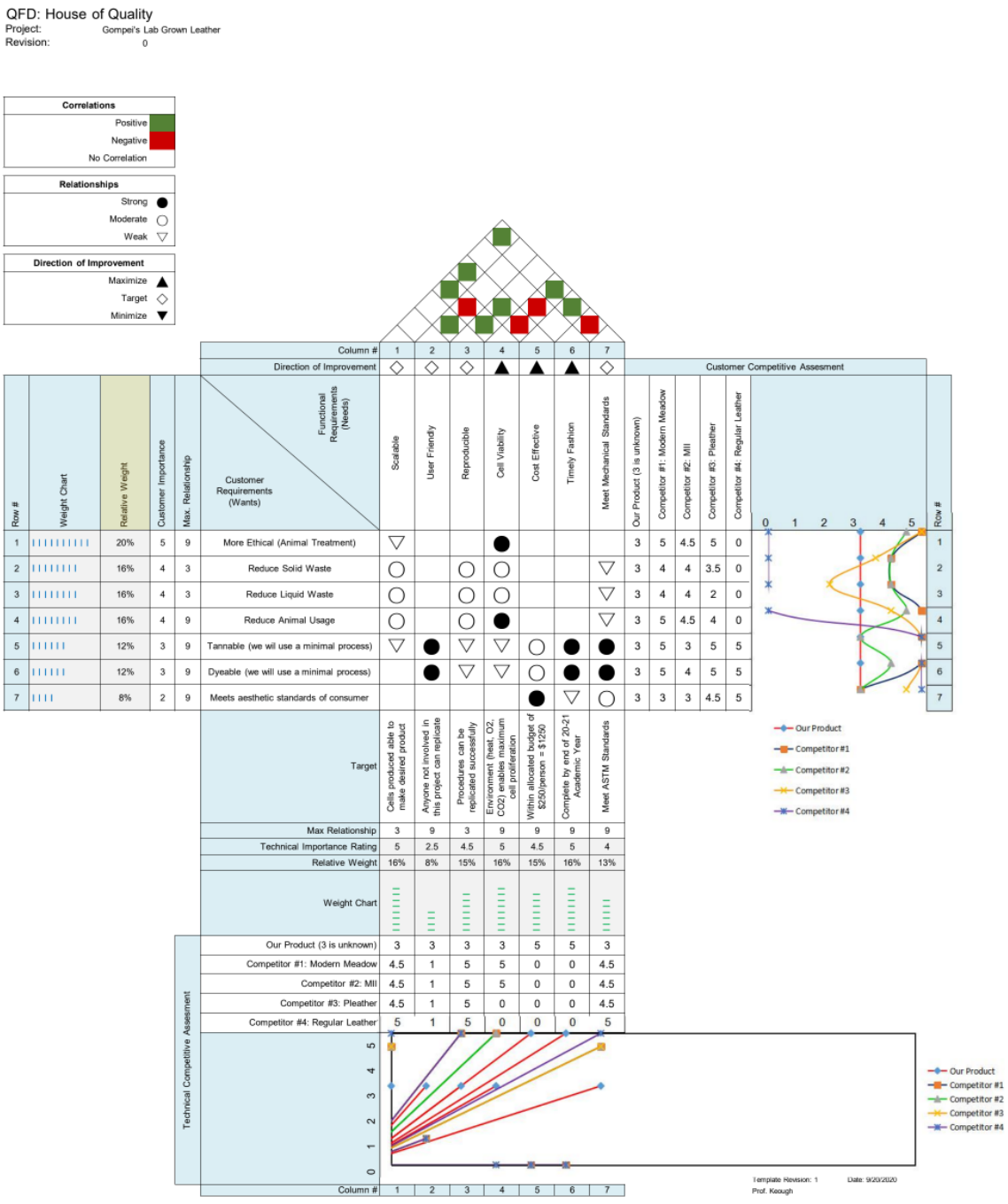


Figure 4.1: Needs and Wants Design Matrix.

4.2 Functions and Specifications

The goal of this project is to make a device that is able to produce lab-grown leather. In order to accomplish this goal, the design team provided several functions and specifications that sought to accomplish the objectives described in earlier sections of the paper. These functions and specifications may be found in Figure 4.2 below.

One of the first functions set out by the design team was to establish a device that was capable of cell viability in order to produce a leather product. Knowing that a cow hide is 90% dermis, the team decided to utilize bovine dermal fibroblasts. We aimed to accomplish cell growth and viability by seeding the bovine dermal fibroblasts onto a decellularized plant scaffold using a 96 cloning well array. Once seeded onto the plant scaffold, the cells needed to remain in a sterile incubation environment that allows for proper cell growth conditions. For this, the team specified an incubation environment of 37 +/- 2 degrees Celsius. Additionally, the environment must possess 5% +/- 2.5% carbon dioxide. Additional conditions to promote cell growth and viability include the perfusion of media throughout the plant scaffold using an automated pumping system. With this system, media flowed through the plant scaffold at a flow rate of 0.285 mL/s. This flow rate will be described more in later sections of this paper. With these conditions, our goal was to grow a dermal sheet that could then be used to create a leather product.

Another objective sought out by the design team was to reduce the negative impact of the current leather making process on the environment. Our main goal in developing this new process was to eliminate waste produced throughout the entire process. Specifically, we wanted to reduce the amount of solid waste that the conventional system generates by 14%. Additionally, we hoped to decrease liquid waste by 6.65% for the pre-tanning and dyeing

processes. These percentages were determined and calculated based on the information presented in *Appendix C.1*.

Other specifications outlined by the design team involved growing the bovine dermal fibroblasts on a spinach leaf that is two inches long, one inch wide, and 0.15875 centimeters thick. The team plans to fulfill this specification by creating the growth area on the leaf of the specified dimension with the clamps. The device should be 6.985 centimeters long, 5.08 centimeters wide, and 3.81 centimeters tall in size and will be capable of fitting on the incubator's shelves.

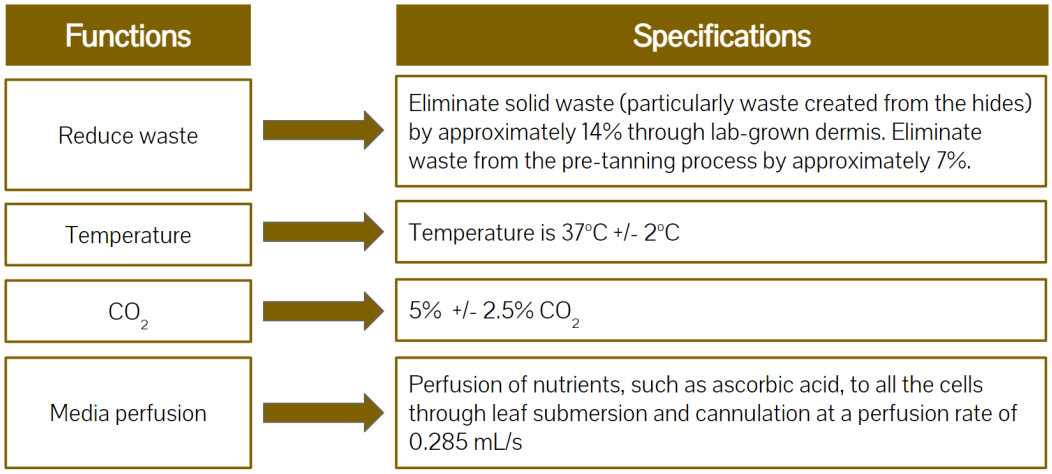


Figure 4.2: Functions and Specifications.

4.3 Conceptual Design Phase

The design team worked individually to produce two to three designs based on the functions and specifications detailed previously. These designs may be found in *Appendix C.2*, along with the pros and cons for each. Once the team created individual preliminary designs, the

design team discussed the advantages and disadvantages of each. The pros of each individual device were taken into consideration to determine our first conceptual design.

4.3.1 Conceptual Design Elements

After determining the various functions and specifications of the design, the methodology utilized to establish each design component was developed. These components include a pump to automate media, a clamping system, and techniques for cell seeding. For each component, the pros and cons were discussed by the design team. By combining advantages and discussing alternatives across different design elements, the final design concept was established.

4.3.2 Brainstorm Design Elements

Preliminary testing and brainstorming for a clamp design involved the use of chip clips holding a regular store-bought spinach leaf under running sink water. This allowed us to get a better idea of what method would be best to clamp the leaf in the device. This can be seen in Figure 4.3. Having three clips on the sides of the spinach proved to provide better support to the leaf when there is added moisture and liquid is run over. There would be some differences using tubing and a pump as compared to running sink water along the top with the spray function. The pressure the team decides to use will most likely be less than our preliminary tests to allow the cells to continue to adhere. In addition, the clamps should have more coverage to ensure that the leaf will not tear the way it did in this improvised trial.

Figure 4.3: Preliminary Clamp Testing for Design Brainstorm.

The design team was cognizant that optimal cell growth required incubation. Therefore, the design team planned to create and develop a device that can be sized to fit within an incubator. This size could range from a standard sized petri dish which can span from having a diameter of 30 to 200 millimeters, as seen in Figure 4.4. The design could additionally take the shape and size of a 75 to 100 squared centimeter cell culture flask, as seen in the figure below.

Figure 4.4: Petri Dish (Grainger) and Cell Culture Flask (Bellco, 2020).

4.3.3 Pros & Cons of Individual Components

Clamping System:

The clamping system consists of two pieces made from Polyethylene terephthalate, PET. The first piece is connected to the pump's tubing and will hold the cannulation system in place. The second piece can connect to the first piece when media is being pumped through the system. However, it may also be removed for incubation once the media has completely pumped through. The second piece will hold the decelled spinach leaf on three sides. The fourth side of the decelled spinach leaf, the stem, will be connected to a cannula that is locked into place with a locking mechanism. The pros and cons of this clamping system can be seen in Table. 4.2. This system is designed to be stackable in order for the project to produce larger portions of dermis sheets. Beneath the clamping system is a media collection container. This will be set at an incline so that media that passes through the plant scaffold will be collected with the help of gravity. This media will then be filtered and cycle through the pump again.

Table 4.2: Pros and Cons of Clamp.

Pros:	Cons:
Detachable for incubation which assists in cell growth.	Horizontal orientation makes cannulation difficult.
A rubber, biocompatible seal will be placed between components.	Clamping system to hold the pump tubing and cannulation device will require work.
PET is biocompatible and has a glass transition temperature that can withstand heats involved in UV sterilization.	

Cell Seeding:

The cannulation process is very intensive and requires a lot of extra thought into planning for the associated design parameters and limitations. However, this allows the nutrients to perfuse through the cells properly since they will be thicker than the required 200 μm of diffusion. Proper testing is required to find the pressure when seeding cells and pumping media through. The vertical design orientation would allow for easier cannulation and media perfusion due to gravity. However, we cannot cell seed on the vertical surface so the horizontal arrangement is better for our applications. The advantages and disadvantages of seeding horizontally are outlined in Table 4.3. Instead, we shall cell seed on top of the leaf and allow media to run over the leaf's surface as well as through the leaf by assembling a pump mechanism outlined in the next section. We intend to use media made up of the components as outlined in Table 4.4 below.

Table 4.3: Pros and Cons of Cell Seeding Procedure.

Pros	Cons
Easy cell seeding on top	Pump mechanism still needed for media perfusion
Cells have less of a chance of falling off	Cannulation on decelled leaves can be difficult

Table 4.4: Cell Subculturing Media Components.

Component	Stock Solution	Volume	Final Conc.	Range
Dulbecco's Modified Eagle Medium (DMEM) basal media		88.0 mL		
Penicillin Streptomycin	100 X	1.0 mL	1 X	1 X
L-Glutamine/Glutamax	200 mM	1.0 mL	2 mM	2-4 mM
Fetal Bovine Serum (FBS)		10.0 mL	10%	2-20%
Total		**100 mL**		

Peristaltic Pump:

In order to reduce liquid waste and be more cost effective during the cell growth process, our team investigated a pumping system that could filter excess media back through to be reused. We needed a pump that would easily attach to our device and could be automated to deliver media without intervention of experimenters. In our research, we found a couple of different pumps often used during cell culture, including syringe pumps, vacuum pumps, and peristaltic pumps. Showcased below in Table 4.5, the pros and cons of each pump type we considered based on the needs of the project is featured (Byun, 2013).

Table 4.5: Pros and Cons of Pump Options.

Pump Type	**Pros**	**Cons**
Syringe Pump	- Wide variety of vendors - Uses a pressurized system to push liquids out of a syringe - Constant flow rate	- Can only hold as much liquid as fits in the syringe - Requires instrumentation that may make it harder to use - Fairly high in cost
Vacuum Pump	- Wide variety of vendors - Uses a pressurized system to pull liquids through conduit - Constant flow rate - Can hold a large amount of liquid to be pumped through	- Requires instrumentation that may make it harder to use - Fairly high in cost
Peristaltic Pump	- Can be low in cost - Can be operated with as much liquid as wanted or on a closed system to recycle liquid - Can pump liquid bidirectionally	- Pinching can cause cellular damage - Mechanical components can wear out over time - Short lifetime (2-4 weeks)

For our project specifically, we needed a simple pump that would be able to perform constantly without physical assistance, as we could not always be in the lab. Commonly used in scaled down bioreactors due to its size, ease of use and varying price ranges, peristaltic pumps seemed most fitting for our project. These pumps can be found for sale for as low as ten dollars and as high as a few hundred dollars, depending on its size and how technically advanced it is. We also wanted a cheaper pump due to our low budget. This type of pump is compatible with an Arduino or a Raspberry Pi to be coded to perform continuously at a rate we desire and will be easy to set up and control.

4.3.4 Preliminary Design Sketches

Once the primary and secondary objectives were defined, the design team was able to develop a block diagram of the final device, as seen in Figure 4.5. The design team understands that the device must be able to viably produce cells. The procedure followed must additionally be reproducible and timely, while additionally accounting for scalability to increase or decrease the size of the product being made. With this in mind, the diagram below provides a simplified view of key elements that would be incorporated into the final device.

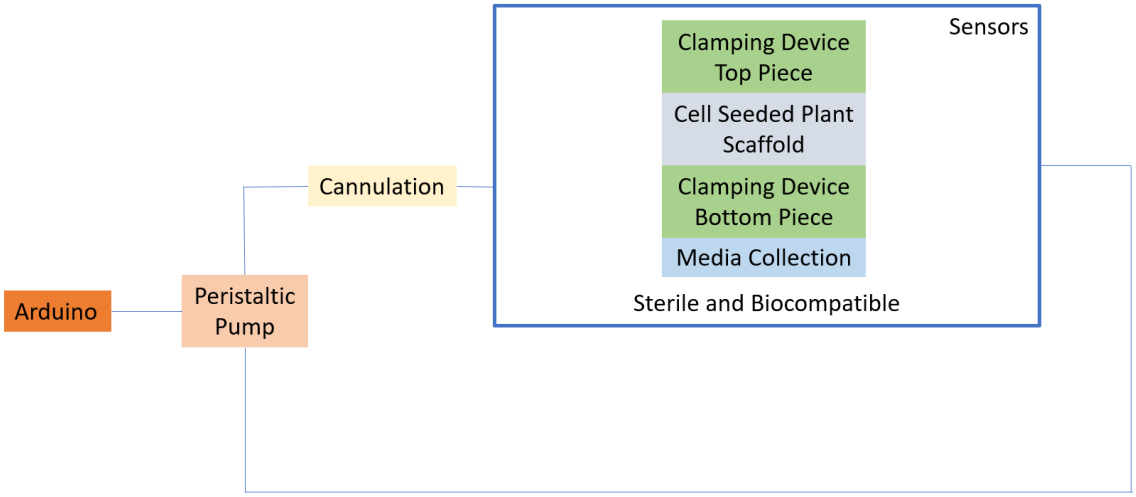


Figure 4.5: Conceptual Design Drawing.

Each designer came up with preliminary designs and then the team met as a whole to rank the individual designs, discuss the advantages and disadvantages of each, and created a design that featured the advantages that were seen in the individual designs. After reviewing the individual designs (found in *Appendix C.2*) two preliminary drawings were created. We then got client feedback and narrowed it down to one final design which factors in the advantages and disadvantages of the other designs. Using the objectives outlined previously in the paper, we

gave a rating out of 5 to each aspect. This rating will rank each design aspect and how well each design fulfills this requirement. Our designs completely fulfill the requirements for cell viability, media perfusion, and ease of use.

The first design allowed for perfusion of media through cannulation. The leaf would be attached using four clamps to provide support at all sides. The leaf would be placed at an angle to aid in this perfusion but to still allow for cell seeding on the surface of the leaf without the cells falling off. This device is essentially a well plate with three layers. The top portion would house the leaf and a removable plastic layer. This functions to seal off the leaf and limit the amount of media needed to submerge the leaf to limit waste. There is also a mesh layer below the plastic layer so when it is time for media replacement, the plastic layer is removed and waste would be collected in the mesh layer which is also removable. The media will fall into the lower compartment and then enter an escape tube to allow for filtration and could potentially be pumped back into the cannula.

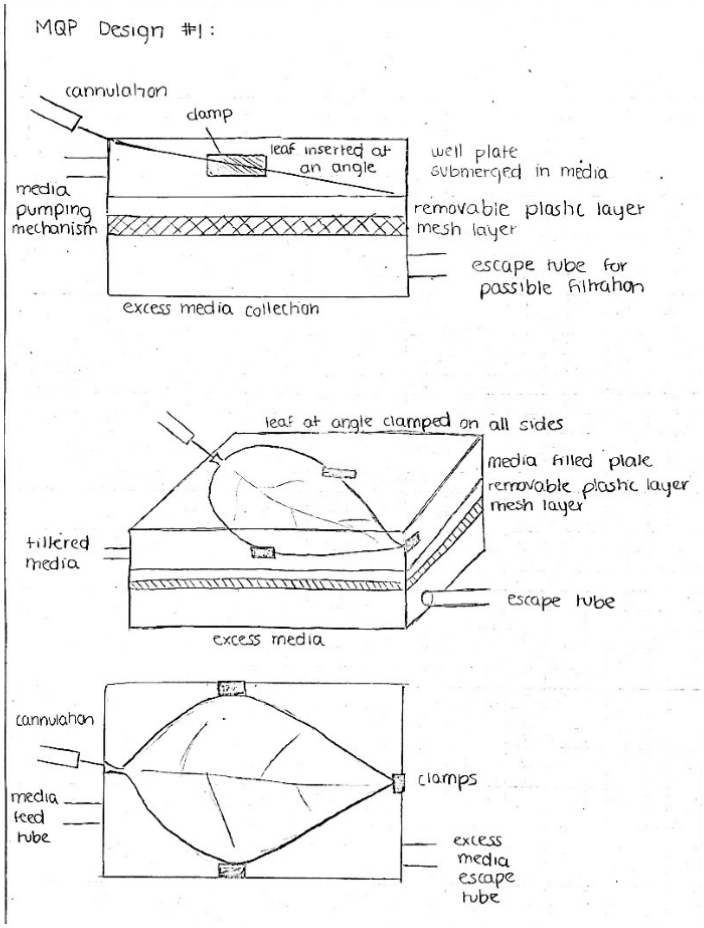


Figure 4.6: Preliminary Design 1.

The device shown in Figure 4.6 above can be used with cannulation if that option is desirable but can also be used without. The design draws inspiration from the Pins-Gaudette device. The Pins-Gaudette device is a circular, concealed clamping system that holds a decelled plant scaffold. The system is capable of incubation and provides an opening for cell seeding. In comparison to the Pins-Gaudette device, the leaf is placed in the upper level, and cells can be seeded on top through the opening. This device is composed of three pieces. The top piece includes screws to connect the second piece. On the top piece there are several air holes to allow proper incubation and CO_2 levels. The opening in the uppermost piece is utilized for cell seeding onto the leaf. The second layer would be a (detachable) biocompatible mesh that will collect

media and other wastes allowing the media to "fall" through the mesh to the bottom layer. For this, ideally, the middle component would have a smaller screw connecting the mesh to the plate. This can be removed to replace the mesh and likewise the third piece will have indentations to account for the extruded screw heads. The bottom layer is a waste collection for the media that is not absorbed by the plant scaffold. We aim to reuse the waste produced from this system and feed it back into the media pump if feasible.

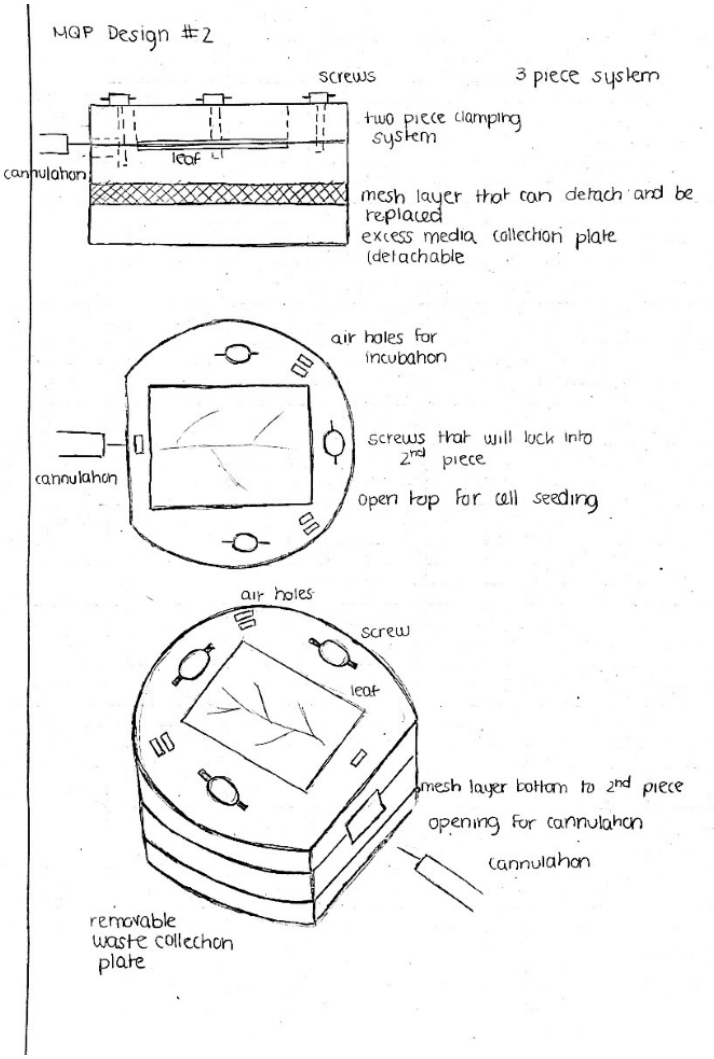


Figure 4.7: Preliminary Design 2.

The second design was preferred by the clients and is shown in Figure 4.7 above. This design showed advantages such as a specified area for cell growth, a stackable system, and it allows for the potential use of pumping pressurized media rather than cannulation as well as a recirculating pump. One disadvantage to note was that the opening for cannulation shown in the diagram may not be as successful as other designs given minimal space and view of the scaffold's stem. While trying to put the cannula in the stem in this design, the team may damage the scaffold and media may not properly be delivered.

Taking all of these factors into account, we drafted a final preliminary design incorporating the desired traits of the previous designs, such as the mesh layer to catch solid waste, the stackable shape, and the clamping system to keep that scaffold in place. In addition to this, we added a detachable piece to hold the cannula in place to minimize any possible damage that could be done and input a pump to recycle the media from the bottom well plate to be reused again. This final preliminary design can be viewed in Figure 4.8 below.

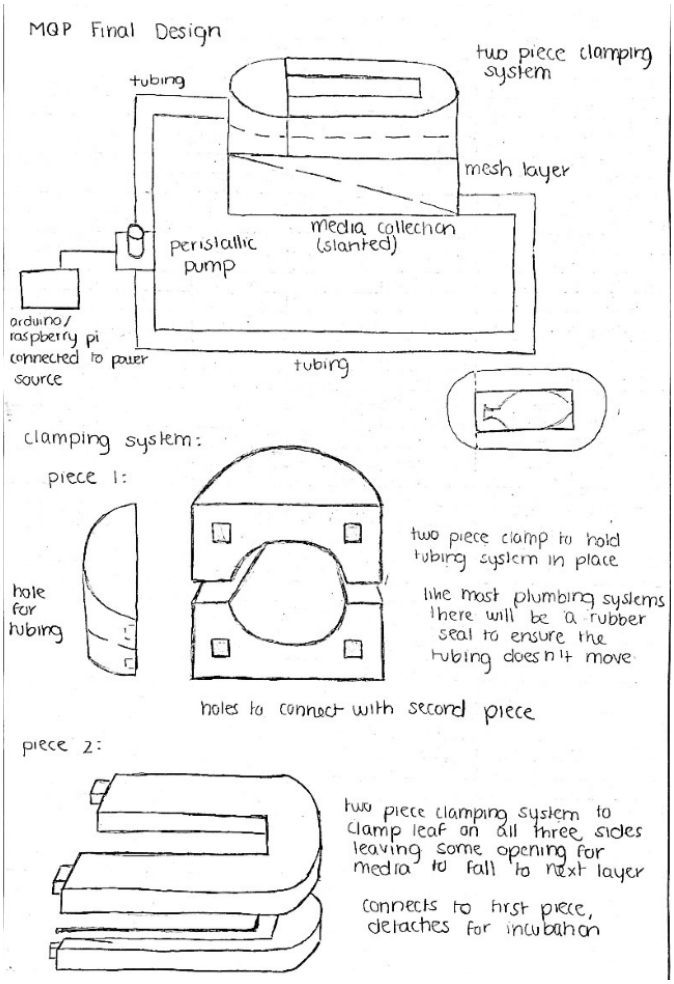


Figure 4.8: Final Preliminary Design.

4.3.5 Analysis of Design Elements

In order to ensure that design elements were achieving objectives, the design team further explored various components of the overall design. The next section will provide understanding and address key components to this project's success including decellularization of leaves, mechanical testing, cell seeding, cell count and perfusion.

4.3.5.1 Decellularization of Plant Leaves

The decellularization process involves the removal of non-structural components of a spinach leaf. These non-structural components of the spinach leaves include the chlorophyll. Once completed, the spinach leaf will be void of color and only consist of vascular structure. The plant's vascular network will be a delivery mechanism for the nutrients in cell media to the cells that will be seeded on top of the leaves. The procedure used to decellularize the spinach leaves may be found in *Appendix C.3.1*.

Plant scaffolds were also one of the most sustainable scaffolding options for growing these cells. They can be cannulated from the stem of the leaf and perfuse media through once the plant cells are cleared out. Cannulation describes the process by which venous access to a leaf is provided using a cannula. Depending on the type of leaf, the location of cannulation may be different. First, the plant should be treated with hexanes and 1x phosphate buffered saline (PBS) to remove the plant cuticles. A 10% sodium dodecyl sulfate (SDS) in deionized water should then be delivered by cannula through the stem, or other location depending on leaf type, for five days. In addition, a 0.1% Triton-X-100 in a 10% sodium chlorite bleach in deionized water solution should be perfused for 48 hours followed by an additional 48 hours of sterilized deionized water (Gershlak, Hernandez, Fontana, Perreault, Hansen, Larson, Binder, Dolivo,Yang, Dominko, Rolle, Weathers, Medina-Bolivar, Cramer, Murphy, and Gaudette, 2017). To accomplish our purposes while working around laboratory restrictions, the leaves were soaked in DI water and placed on a shake table for 10 minutes to rinse. The decellularization solution we used included: 5 mL of SDS, 50 mL of bleach, and 445 mL of DI water. The leaves were then placed on a shake table for 10 minutes and let to soak for 72 hours. The solution was replaced every 48 hours and repeated until the leaves appeared visually clear and decelled. These

decellularized leaves were further analyzed for their mechanical properties which is outlined in the following section.

4.3.5.2 Preliminary Mechanical Testing

Bovine dermis has a specific modulus of elasticity that will need to be replicated in order to meet consumer expectations when producing leather in the laboratory. Additionally, decellularized plant scaffolds need to be strong enough to support cells during cell seeding experiments and dermal growth. The most important part of bovine dermis that contributes to leather's overall tensile strength is the grain layer, which is discussed in *Appendix C.3.2*. The grain layer gives dermis its strength, which when produced into leather prevents cracks and breaks in the surface to make it more aesthetically pleasing. Before the plant scaffolds were used, we carried out mechanical tests with rice paper as a proof of concept since the modulus of elasticity of rice paper and decellularized spinach leaves are around the same value (Gershlak et al, 2017). During these tests, the target tangent modulus was equal to the value for decellularized spinach leaves at approximately 0.3 MPa. We completed four different types of mechanical testing; two at-home tests and two tests with the Instron 5544 provided in Goddard Labs. The at-home tests consisted of one testing rice paper and one testing decellularized spinach leaves while the Instron tests involved one with rice paper and one with decellularized cabbage. The procedures for these tests can be found in *Appendix C.3.3*. We later performed tests with leather and decellularized leaves on the Instron, which are discussed in section 5.1.2.

The at-home mechanical tests were performed on three decellularized spinach leaves. These leaves were decellularized using the at home decellularization process mentioned in Appendix *C.3.3.1*. This decellularization process is different from the final process used on the leaves tested in section 5.1.3, which is why they were used for preliminary experimental testing.

The at home mechanical test required the use of decellularized spinach leaves, two binder clips, a plastic zip lock bag, water, and a scale to measure the amount of water being used. Images outlining the at home mechanical tests can be found in Figure 4.9 and Figure 4.10 below.

Figure 4.9: At Home Mechanical Testing of Decellularized Spinach Leaves. The image on the left is the decellularized spinach leaf prior to the mechanical test. The middle image shows the mechanical test being performed. The image on the right shows the torn leaf from mechanical testing.

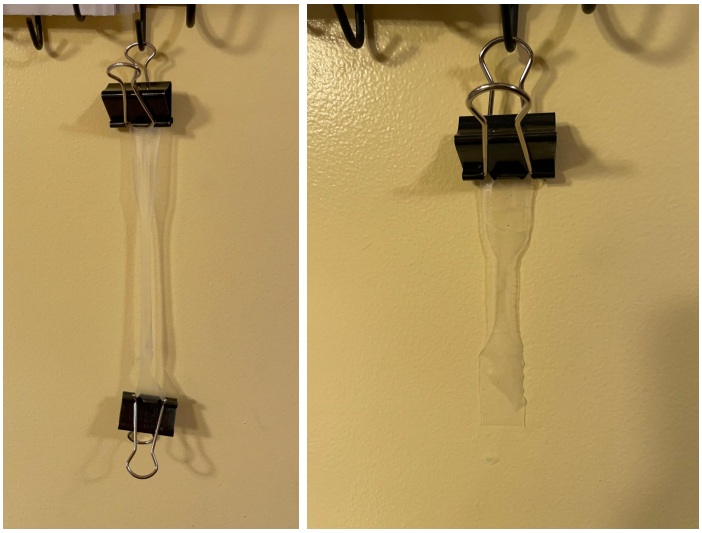


Figure 4.10: At Home Mechanical Testing of Rice Paper.

Both at-home tests were executed to prove that mechanical testing was possible if the laboratory was inaccessible for the foreseeable future due to COVID-19 restrictions. These tests served as examples that our team was capable of properly executing accurate mechanical tests with limited equipment. Wet rice paper was used for the first test as it has very similar mechanical properties to decellularized spinach leaves. For this test, there were three samples used. The rice paper samples were cut into dog-bone shapes with scissors while the paper was still dry. Each sample reached failure when attached to a 9 gram binder clip. Multiplying this value by the force of gravity (9.81 m/s^2), the breaking force was determined to be .08919 N. Data from this test can be seen below in Table 4.6.

Table 4.6: Data Collected from At-Home Mechanical Test With Rice Paper.

Test	**Weight of Binder Clip (kg)**	**Total BreakingPoint Load (kg):**	**Total BreakingPoint Load (N):**
1	.009	.009	.08919
2	.009	.009	.08919
3	.009	.009	.08919

Spinach leaves were decellularized at home and used in the second at-home test. Three samples were used for this test as well and the results were very similar to those from the rice paper, as seen in Table 4.7. Testing decellularized spinach leaves with the Instron is discussed further in depth in section 5.1.2.

Table 4.7: Data Collected from At-Home Mechanical Test with Decellularized Spinach Leaves.

Test	Weight of Binder Clip (kg)	Amount of Water at Breaking Point (ml)	Amount of Water at Breaking Point (kg)	Total BreakingPoint Load (kg):	Total Breaking Point Load (N):
1	.003	5	.005	.008	.07928
2	.003	5	.005	.008	.07928
3	.003	6	.006	.009	.08919

The two laboratory mechanical tests were performed on rice paper and decellularized cabbage leaves. Similar to the at home tests, the Instron tests with the rice paper proved that the verification of mechanical properties was viable. For this test, nine samples were used. Rather than cutting the pieces into dog-bones, we decided to test them in a rectangular shape, as it was very difficult to trace a dog-bone shape with wet rice paper and a scalpel without tearing the paper. A sample can be seen in Figure 4.11 below. Each sample had a gauge length of 75 mm and a strain rate of 0.4167 mm/sec or 25 mm/min. Cross sectional areas for each sample varied due to the thickness of the rice paper. Exact measurements can be seen below in Table 4.8. Compared to the at-home tests, the force values to reach failure were much higher. The data and comparison chart can be seen in Table 4.9 and Figure 4.12 below.

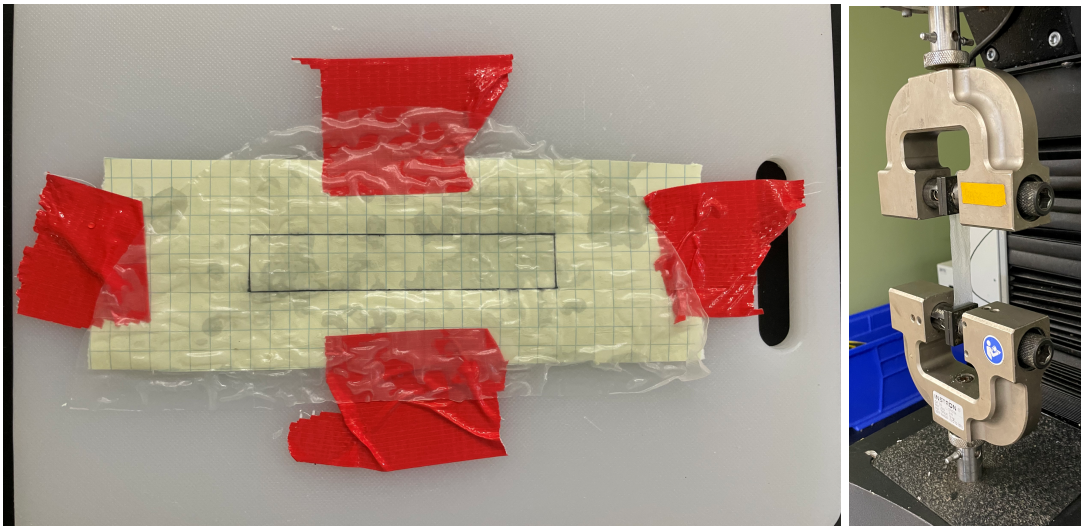


Figure 4.11: Instron Mechanical Testing of Rice Paper.

Table 4.8: Measurements of Rice Paper Samples.

Sample	Gauge Length	Width	Thickness
1	75 mm	19 mm	1.45 mm
2	75 mm	19 mm	1.4 mm
3	75 mm	19 mm	1.5 mm
4	75 mm	19 mm	1.3 mm
5	75 mm	19 mm	1.8 mm
6	75 mm	19 mm	1.6 mm
7	75 mm	19 mm	1.1 mm
8	75 mm	19 mm	1.0 mm
9	75 mm	19 mm	1.8 mm

Table 4.9: Data from Instron Testing on Rice Paper.

Sample	Failure Load (N)	Modulus (MPa)
1	0.234	0.0150
2	0.332	0.0150
3	0.188	0.0164
4	0.212	0.0144
5	0.363	0.0137
6	0.297	0.0138
7	0.344	0.0142
8	0.229	0.0118
9	0.194	0.0142
MEAN	**0.266**	**0.0143**
STD	**0.068**	**0.0012**

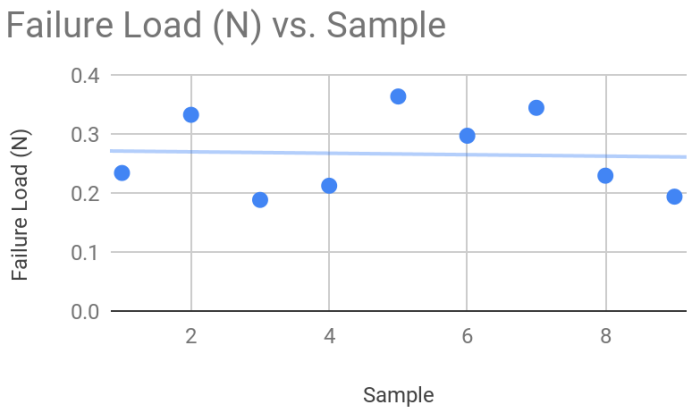


Figure 4.12: Failure Load vs. Test Comparison Graph.

The data from the Instron tests were consistent throughout the process with an average failure load at 0.266 N. The variation between the at-home and laboratory results can most likely be attributed to lack of proper resources to accurately measure weight and force. As it was not possible to measure the extension of the rice paper or spinach leaves at home, the Instron was

able to account for this value to then calculate the elastic modulus. The stress-strain curves from the Instron tests of each sample of rice paper can be found in *Appendix C.3.3.2.* In Figure 4.13 below, the stress-strain curve from test 1 can be seen. The comparison chart of the calculated elastic modulus values is shown below in Figure 4.14.

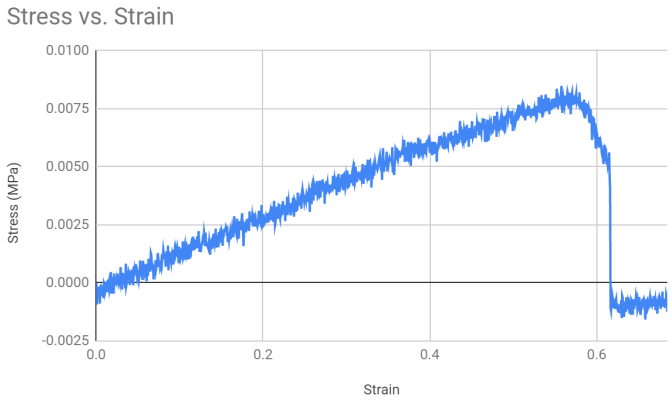


Figure 4.13: Stress-Strain Curve for Rice Paper Test 1.

In order to calculate the elastic modulus, the line of best fit of the cumulative data is to be calculated, ranging from the point where stress is equal to 0 to the point where the stress reading is highest. For test 1, this value was 0.0150 MPa.

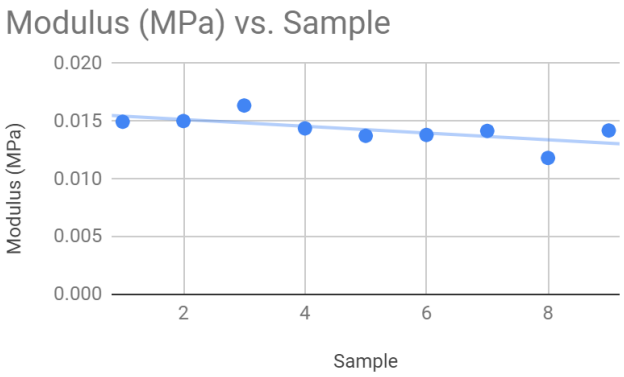


Figure 4.14: Elastic Modulus Values Calculated from Each Test.

The values calculated are consistent, with an average modulus of 0.0143 MPa with a standard deviation of +/- .0012. However, this is drastically different from the original values of the decellularized leaves. While the yield load was relatively similar, these tests showed that rice paper has a tangent modulus approximately twenty-times smaller than spinach leaves based on the reference value of 0.3 MPa.

Though we decided not to pursue cell seeding with cabbage leaves, it is still a widely available and good alternative to spinach due to the vascular nature. We decided to verify if the strength of decellularized cabbage is comparable enough to spinach to where it would be both a convenient yet functional substitute. Unfortunately, while decellularizing the first round of cabbage leaves, the wrong solution was used which caused the cabbage leaves to dissolve and rip in the flask. There were 2 scaffolds that were able to be salvaged for mechanical testing, however the data was very inaccurate and was removed from consideration.

4.3.5.3 Bovine Fibroblast Isolation

Having a viable plant scaffold to house the cells allowed the team to begin extracting fibroblast cells for use. Rather than purchasing bovine fibroblasts from a supplier, the team decided that the most cost-effective decision would be to procure a skin sample from a cow and isolate the cells ourselves in the laboratory. Our team had never done this process before, and we had to complete some research on isolating fibroblast cells from mice and adapt the procedure to essentially create our own for cows outlined in *Appendix C.3.4*. The isolation was performed but we did not know if the process was successful for two weeks. However, because we used diligent aseptic technique, there was no contamination and we were able to observe the fibroblasts through a microscope as shown in Figure 4.15.

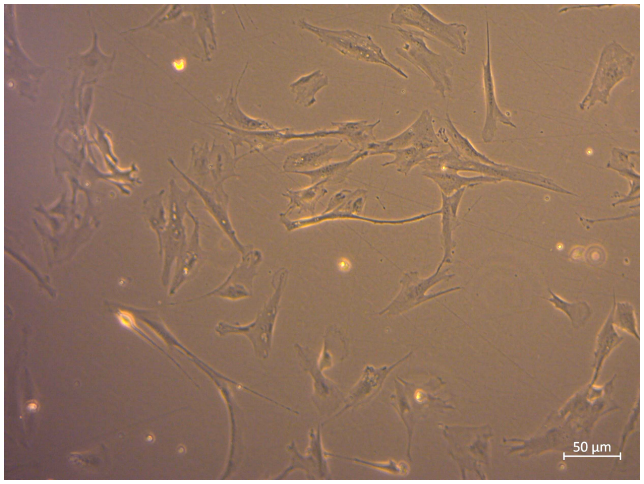


Figure 4.15: Bovine Dermal Fibroblasts.

4.3.5.4 Bovine Fibroblast Freezing and Passaging

After isolating the cells, we had to ensure there was no contamination with the procedure we followed, meaning there is no bacteria, yeast, or other cultures other than the fibroblasts that we isolated. From there, the cells were allowed to grow to close to 100% confluency and we determined that we should freeze some for transportation purposes. This was necessary since the isolation was completed in the Gateway Lab and most of our project work is located in the specific MQP lab in Goddard. At this point of confluency, some cells were frozen and some were allowed to continue growing after being passaged for the second time. The procedure for freezing and passaging these cells can be found in *Appendix C.3.5*.

4.3.5.5 Cell Seeding onto Plant Scaffolds

After freezing and passaging, cell seeding experiments can be performed. The cells can be seeded after being rinsed with phosphate buffered saline (PBS). Typically cells are seeded onto plant scaffolds with cloning rings. Cells are then seeded directly into these rings and are

allowed to adhere to the scaffold. This allows for easy identification and localization of the cells (Gershlak et al, 2017). From here, a hyper confluent cell layer should form, which will become a dermal sheet. In future sections, cloning rings in regards to our device will be discussed.

4.3.5.6 Cell Count

While cloning wells may help cells adhere to the plant scaffold, the design team then needed to account for how many cells are needed in order to grow our sheet of dermal tissue. We needed to do some preliminary calculations in order to find the necessary amount of cells. Since there are 300 million cells for 0.00347 cubic meters of skin in an average adult, there would be 8.652×10^{10} billion per cubic meter (Premier Dermatology, 2017). From here, we can convert this value to cells per cubic inch or 8.652×10^{16} million cells/ cm^3. We aim to create a $2.048m^3$ sheet; this would be $1/8in^3$ for total volume which we can then multiply by the number of cells per cubic inch identified previously to find the total number of cells, being 159,439 cells. This number won't be entirely accurate in terms of actual cell attachment, since plating efficiency is only 90%, but it will give us an estimate that we should be seeding even more cells than our approximation (Lacombe, J., et al, 2020). In order to achieve this amount of cells, perfusion will be used.

4.3.5.7 Perfusion Model

The following sections describe perfusion through a decellularized plant scaffold. For more analysis on the below model. Please reference *Appendix C.3.6*.

4.3.5.7.1 Physical Perfusion Model

Spinach leaves, like any other plant scaffold, have various veins that make up its intravascular network. This network of veins is what makes spinach leaves an ideal option for perfusing media for cell growth given its porous properties on the surface as well as its mechanical properties. An image of how fluid flows through the vascular network of a spinach leaf can be seen in Figure 4.16.

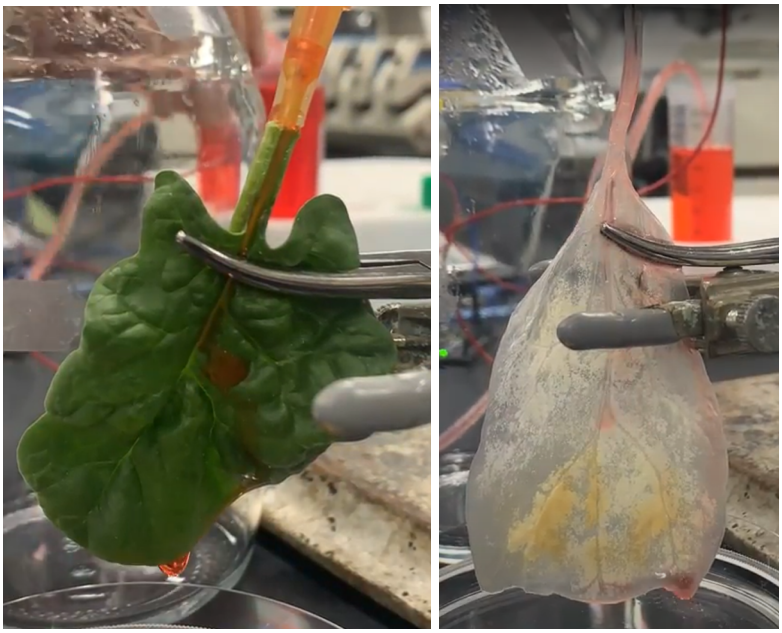


Figure 4.16: Perfusion Through a Spinach Leaf.

Perfusion is the delivery of solutes such as oxygen, media, and ascorbic acid through diffusion. For this project in particular, perfusion of media and ascorbic acid will be analyzed as a microfluidic application. This means that the fluid possesses an extremely small Reynold's number and has a laminar flow as seen in *Appendix C.3.6.1*. For a microfluidic problem of this scale, the governing equation for this perfusion analysis is that the change in pressure is equivalent to the flow through the area of this vasculature. This equation is represented below

where ΔP is the change in pressure of the fluid, A is the cross-sectional area of interest, and Q is the flow of the fluid.

$$\Delta P = AQ$$

In this application, assume that the pressure will be linear and the flow is constant. After passing through the inlet, or main stem of the spinach leaf, the fluid will divide and disperse among the many veins of the leaf. The flow through these different veins may be treated as resistors in series and parallel. However, in addition to resistance dispersed among the veins, there is also resistance throughout the mesh of the leaf itself. Meaning, that diffusion additionally occurs through the walls of the veins and into the rest of the leaf. The flow and resistance of this process can produce a pressure and flow curve. Whereas, diffusion can be modeled through mass flow rate. Mass flow rate is equal to the area multiplied by viscous coefficient of the fluid. That is then multiplied by the concentration divided by the thickness. This can be seen in the equation below.

$$\dot{m} = \mu A^*(\Delta C/\Delta x)$$

In the above equation, $\dot{m}$ is the mass flow rate of the fluid, μ is the viscous coefficient, A is the area of interest, ΔC is the change in concentration, and Δx is the thickness.

4.3.5.7.2 Perfusion Rates

This experiment will address the physical movement of fluid throughout a spinach leaf, and will additionally compute a flow rate for perfusion. A perfusion rate describes the delivery of a certain amount of solutes through a specified volume and time. Perfusion rates are proportional to pressure (P). It is known that more flow is delivered with greater pressures, and less flow with

smaller pressures. Therefore, in this application, the design team will investigate the perfusion versus the need for media and ascorbic acid in the scaffold. This analysis will be determined through the investigation of how gravity and pressure changes affect the perfusion model through the leaf. With gravity and pressure in mind, more perfusion occurs at the bottom portion of the leaf. It is important to mention that the spinach veins are distensible, meaning that the veins will expand and not remain rigid when fluid passes through them. In order to account for distension, we look at the change in diameter of the veins. A change in size indicates and results in a change in flow, resistance, and pressure. However, given the complex structure of the spinach leaf, distension will not be modeled.

In order to devise and understand perfusion rates for our application, the design team ran food coloring dye through a decellularized spinach leaf. Images of this process may be seen in Figure # below. From this experiment, our device utilizes a flow rate of 0.285 milliliters per seconds. These calculated values can be further explained in *Appendix C.3.6.1.* The perfusion rate and characteristics will be modeled through an application called COMSOL Multiphysics. This analysis may be seen in the following section.

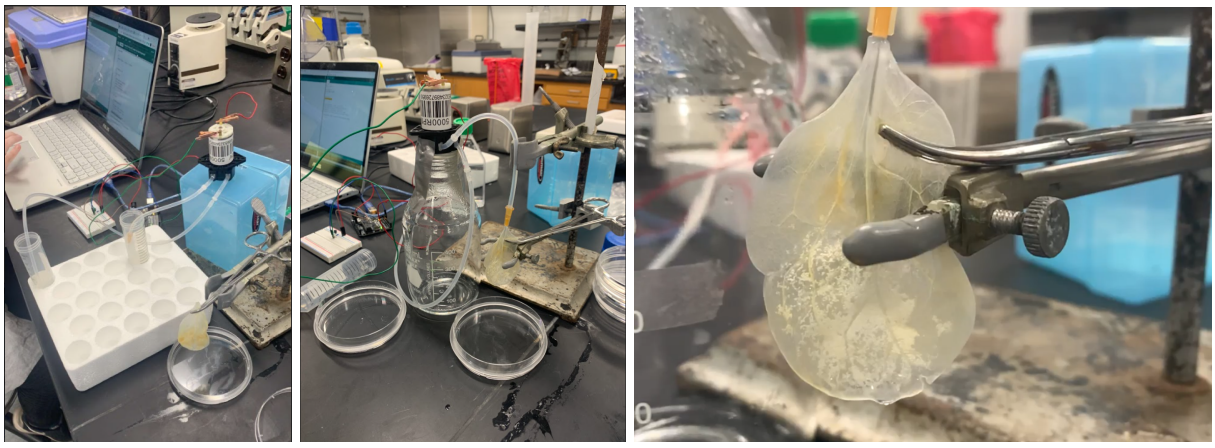


Figure 4.17: Perfusion Experimental Set Up

4.3.5.7.3 COMSOL Multiphysics Representation

The spinach leaf's perfusion of media through the main stem will be modeled using COMSOL Multiphysics graphs and simulations. Some background research and initial conditions seen in the COMSOL model can be found in *Appendix C.3.6.1* The pre-simulation model can be seen in Figure 4.18, and explains the various attributes contributing to perfusion.

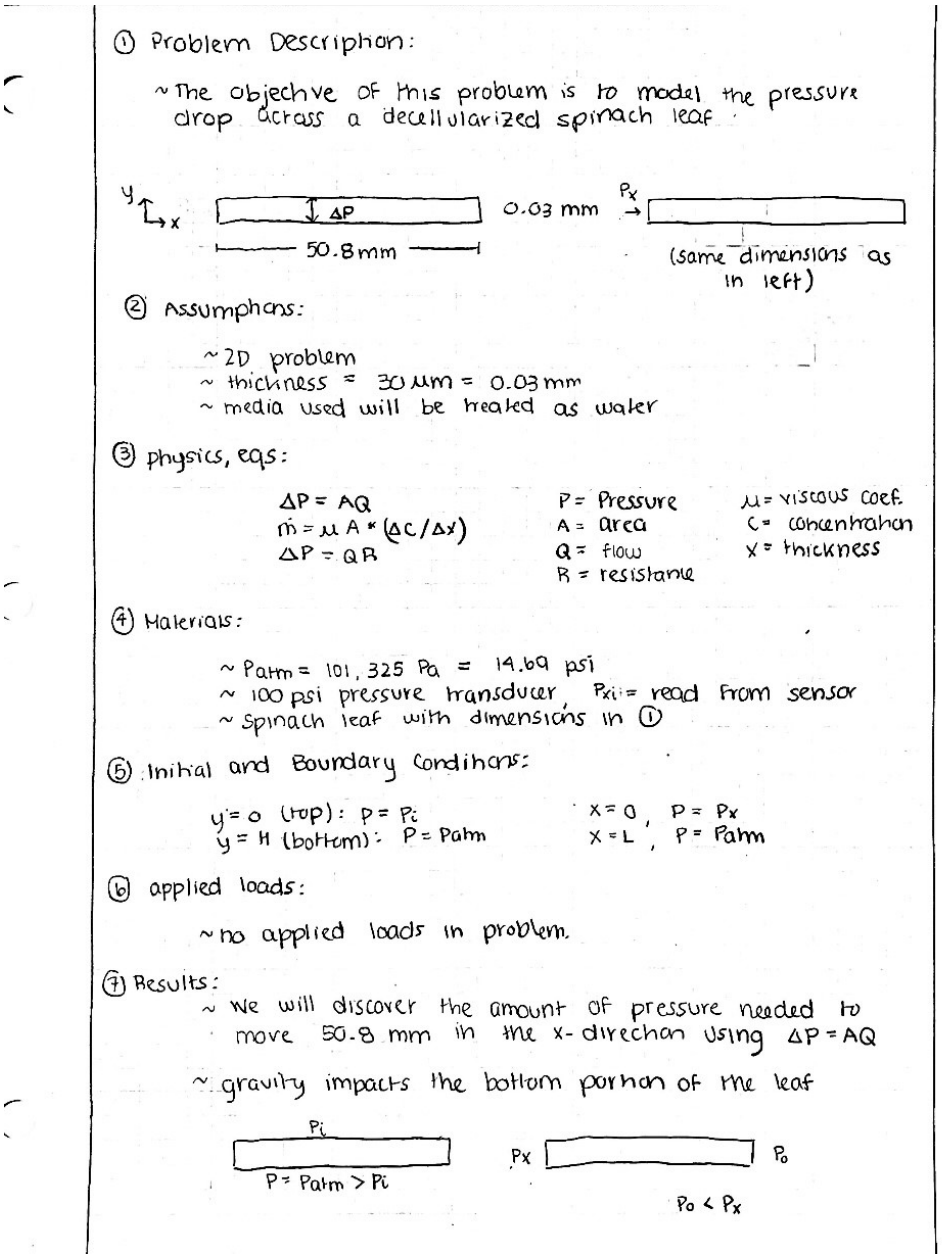


Figure 4.18: COMSOL Multiphysics Pre-Simulation.

Using the pre-simulation model, the following graphs shown in Figure 4.19 were produced. These graphs show the relationship between flow, structure, velocity, and pressure. Part a in Figure 4.19 shows the mesh structure of the spinach leaf stem. Meanwhile, Part b in Figure 4.19 shows the velocity profile of the spinach stem. The stem abides the no-slip condition and possesses a parabolic velocity profile. The walls possess zero velocity while the center of the stem possesses the maximum velocity value of 0.55 meters per second. Part c in Figure 4.19 shows the pressure drop across the main stem of the spinach leaf. The left side of the stem, or the inlet for media flow, holds the greatest pressure of 0.2 psi. The pressure decreases the further media progresses through the stem. At the end of the stem, the pressure is equivalent to atmospheric pressure, or approximately 0 psi.

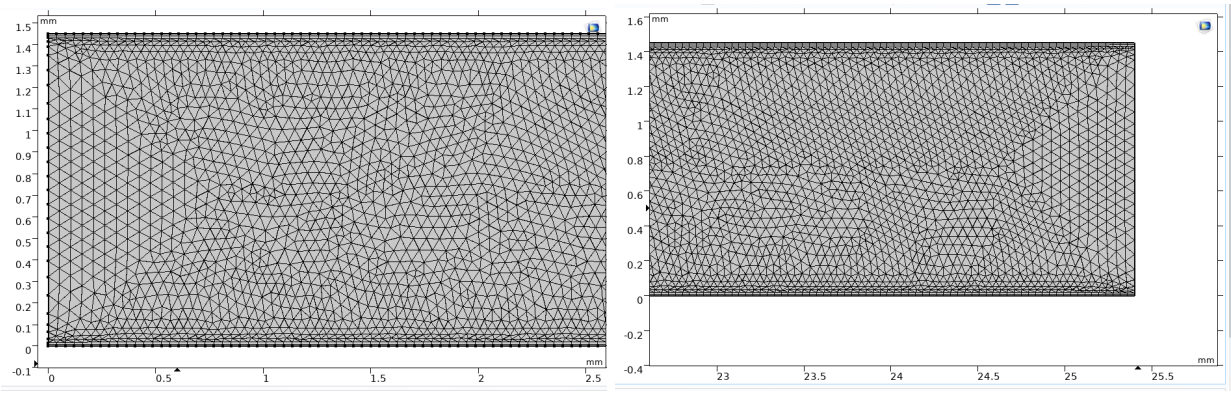


Figure 4.19a: Mesh of Spinach Leaf Fluid Flow.

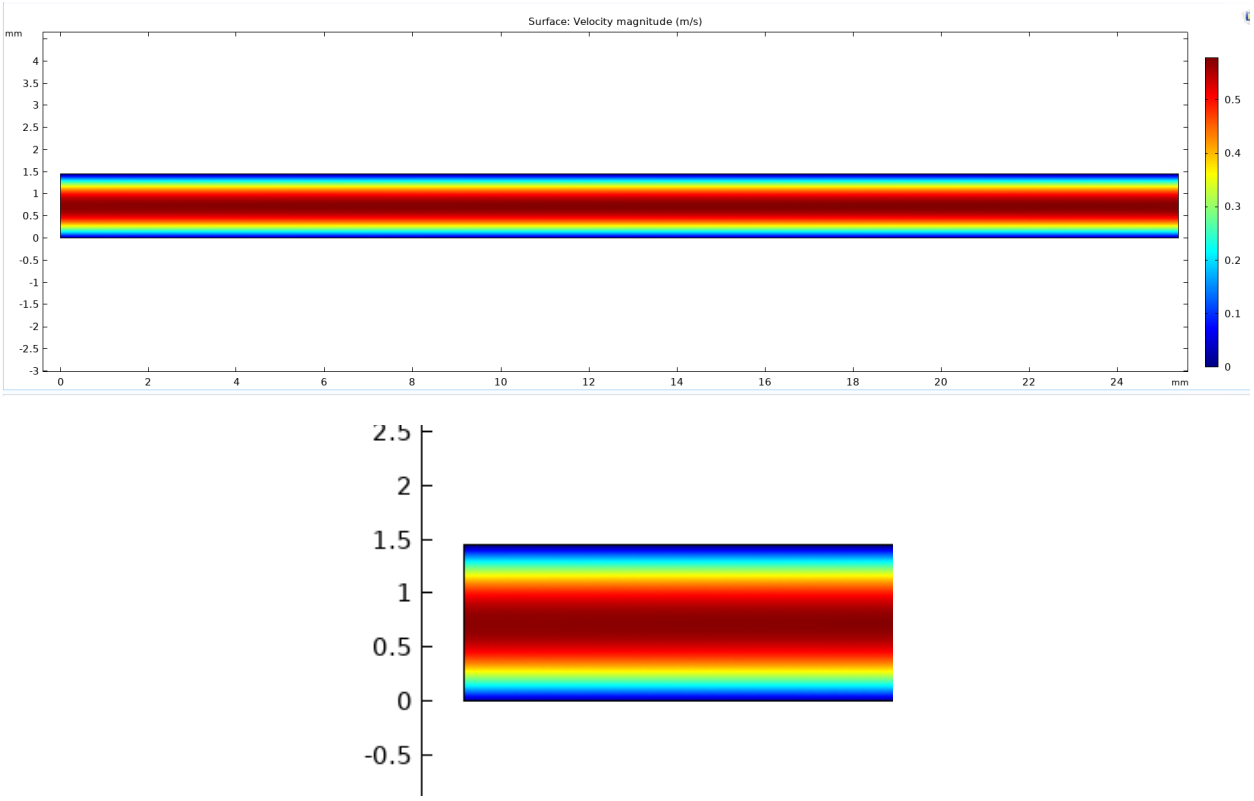


Figure 4.19b: Velocity Profile of Spinach Leaf Fluid Flow.

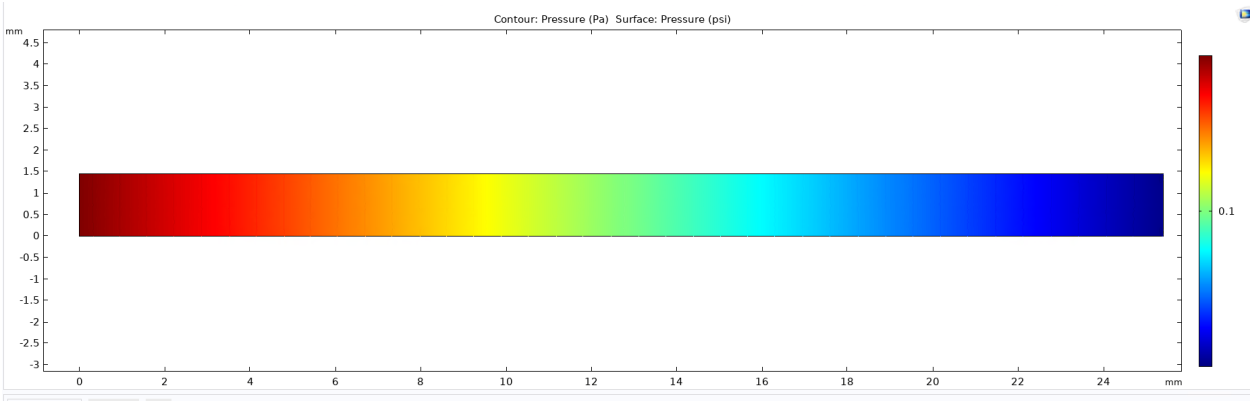


Figure 4.19c: Pressure Drop of Spinach Leaf Fluid Flow.

From these graphs, we understand that the maximum velocity in the parabolic profile is approximately 0.55 m/s. The increase from the initial velocity of 0.1714 m/s makes sense given the relationship between velocity and pressure as outlined in Bernoulli's equation. Calculations

for this scenario may be seen in *Appendix C.3.6.1*. Through further analysis and the use of a dataset cut line, we find the average velocity to have a magnitude of 0.38597 m/s. Therefore, from the graph, we can conclude that velocity relies and has a direct relationship to pressure.

The max pressure in this scenario is shown to be approximately 0.2 psi. However, it is important to analyze these results. As mentioned in earlier sections, it is hard to fully represent the flow through the spinach leaf given its complex vasculature structure which includes distensibility, multiple branches, and diffusion. Therefore, in the analysis made in COMSOL Multiphysics, there are currently too many constraints to fully represent the proper fluid flow through the leaf. In a typical biological scenario, we would look at an initial pressure of approximately 2 psi, not 0.12 psi which was recorded by the team's pressure transducer. If our application ran with an initial pressure of 2 psi, then there needs to be some mechanism that provides friction to increase energy. Without this mechanism, as we have shown it in our COMSOL Multiphysics analysis, it is physically impossible to go from 2 psi to 0.1 psi. Therefore, it is recommended to further analyze this set up under turbulent flow as opposed to laminar flow. This is because laminar flow consumes a lot of energy. With a further examination of the spinach leaf's vasculature and flow, a better understanding of perfusion can be drawn.

Chapter 5. Final Design Verification

Several procedures and experiments were developed in preparation for experimentation of our final design. The goal of these smaller experiments was to ensure the success of the large experiment to validate our final product and have a set of controls to compare our final results to so we could evaluate any problems that arose.

5.1 Standard Laboratory Testing Procedures

Several procedures were required in order to move forward with experimental analysis of individual components, as well as the design verification process. Those procedures are outlined below.

5.1.1 Decellularization of Spinach Leaves

For this project, a batch of approximately 7 spinach leaves are treated and decellularized over the course of seven days. This process occurred in three parts. The first batch of spinach leaves were performed in an at-home environment due to the restrictions placed on laboratory access during the pandemic. The next two batches of spinach leaves were decellularized in the laboratory once restrictions were lifted. Images for both of these processes may be seen in Figure 5.1 below. Again, the procedure to decellularize a spinach leaf for both the at home and laboratory set up may be found in *Appendix C.3.1*.

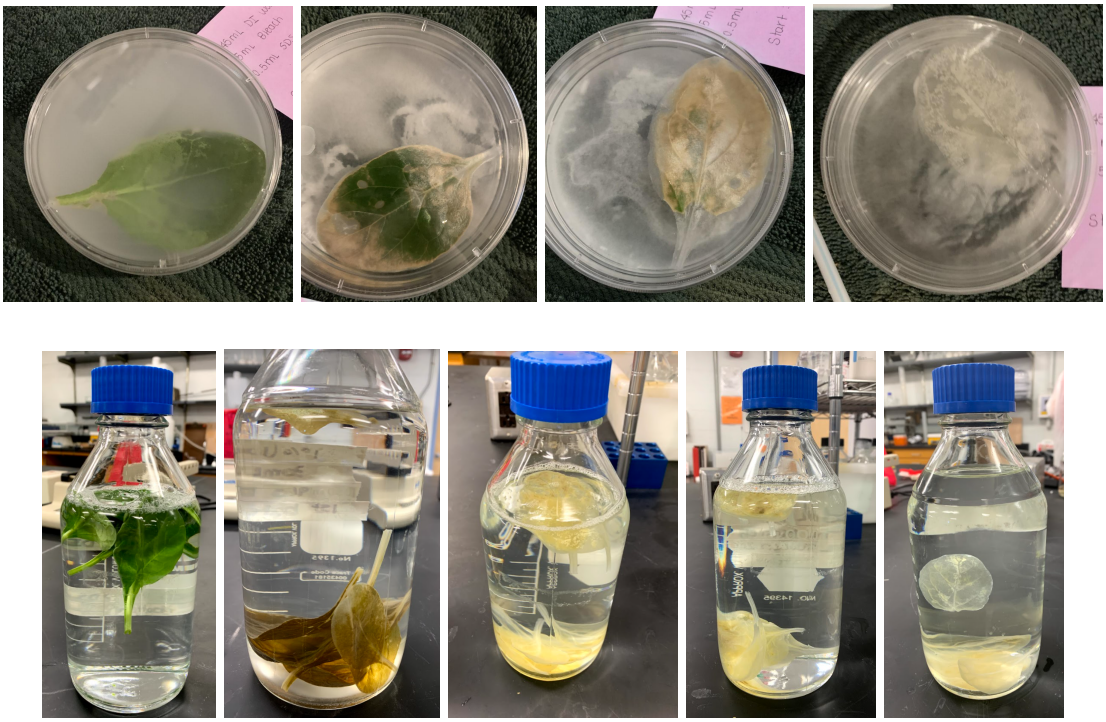


Figure 5.1 Decellularized Spinach Leaf Progression (At Home - top, Lab - bottom).

5.1.2 Mechanical Testing of Decellularized Spinach Leaves and Leather

Once the final spinach leaves were decellularized, we were able to complete mechanical testing in the lab with the Instron. The procedure performed was the same procedure used for the rice paper tests with the same strain rate of 25 mm/min. The written procedure can be found in *Appendix D.1.1.* There were nine samples used for this test. Since each leaf was a different size, they all had different measurements which can be seen below in Table 5.1. An image of two of the leaves is shown below in Figure 5.2. There were five spinach leaves that were not completely clear, but followed the same decellularization procedure as the others. While the leaves may not have appeared fully clear, their modulus values aligned with the fully clear leaves.

Table 5.1: Measurements of Each Spinach Leaf Sample.

Test	Width	Thickness	Gauge Length
1	16.72 mm	.70 mm	15.50 mm
2	26.05 mm	1.57 mm	31.70 mm
3	25.99 mm	.57 mm	23.70 mm
4	30.09 mm	.60 mm	26.00 mm
5	17.90 mm	.65 mm	13.27 mm
6	28.01 mm	.57 mm	20.88 mm
7	30.01 mm	.53 mm	27.78 mm
8	23.23 mm	.38 mm	22.68 mm
9	19.24 mm	.74 mm	13.08 mm

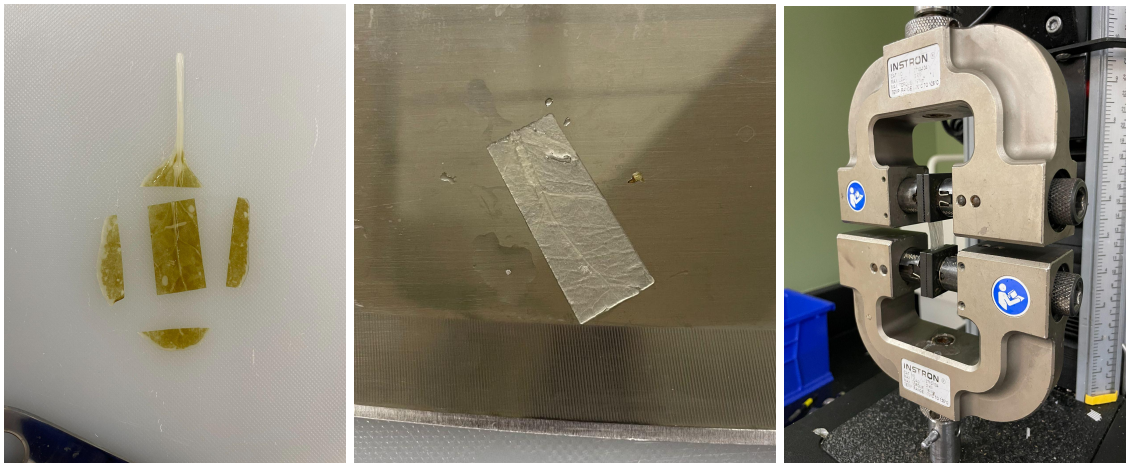


Figure 5.2: Sample of Decellularized Leaf Used for Testing.

The results of these tests were different from the reference modulus of 0.3 MPa. While some of the values were around 0.3 MPa, many were not. This can be concluded as a result of biological variance. All stress-strain curves for the samples can be found in *Appendix D.1.1* and the stress-strain curve for the first test can be found below in Figure 5.3. The calculated moduli and a comparison chart can be seen below in Table 5.2 and Figure 5.4. The average modulus for

the spinach samples was calculated to be 0.504 MPa and a standard deviation of +/- 0.3499. Like the rice paper, this value is the line of best fit for the data -- calculating the slope of the range from the value when the stress is at 0 to the point where the stress reading is highest.

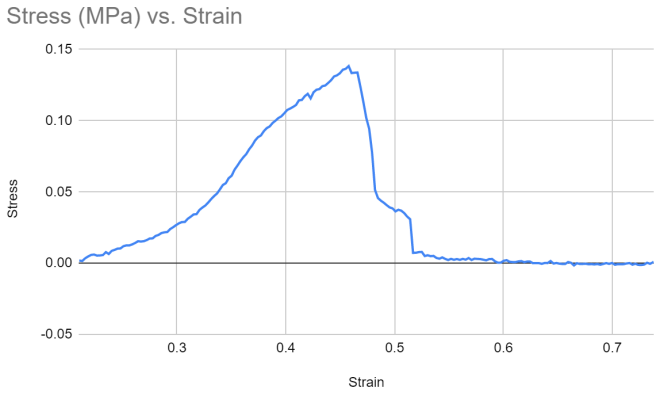


Figure 5.3: Stress-Strain Curve of Test 1 of Decellularized Spinach Leaves.

Table 5.2: Calculated Modulus Values for Each Spinach Leaf Test.

Test	Modulus (MPa)
1	0.6130
2	0.2400
3	0.0710
4	0.0701
5	0.6267
6	0.9109
7	0.6678
8	1.029
9	0.3074
MEAN	**0.5040**
STD	**0.3499**

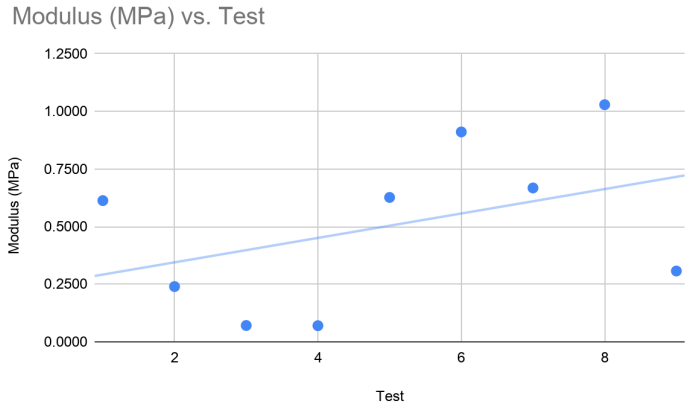


Figure 5.4: Comparison Chart of Spinach Leaf Moduli.

We also performed the same mechanical tests on samples of leather. Since leather is less likely to rip, samples were cut into dog bone shapes with a scalpel. The same procedure and strain rate used for rice paper and decelled spinach leaves was used. Samples can be seen below in Figure 5.5.

Figure 5.5: Samples of Leather Used for Mechanical Testing.

The written procedure and all stress-strain curves can be found in *Appendix D.1.2.* The stress-strain curve for the first test can be found below in Figure 5.6. There were nine samples for testing leather. Measurements for each sample and results from the tests can be found in Tables 5.3 and 5.4 below. Additionally, a comparison chart of the moduli may be found in Figure 5.7. The average modulus for the leather samples was calculated as 38.692 MPa with a standard deviation of +/- 6.232. Leather made with different thicknesses or from different animal hides can exhibit various elastic moduli. This test specifically was to function as a preliminary test for our group to reference if we were to produce a piece of leather of a similar size. Like the rice paper and the spinach leaves, this value is the slope of the data set ranging from the value when the stress is at 0 to the point where the stress reading is highest.

Table 5.3: Measurements of Each Leather Sample.

Test	Gauge Length	Gauge Width	Thickness
1	49.00 mm	8.49 mm	1.45 mm
2	55.07 mm	7.70 mm	1.45 mm
3	51.13 mm	11.14 mm	1.39 mm
4	52.92 mm	8.93 mm	1.39 mm
5	49.46 mm	9.11 mm	1.38 mm
6	53.13 mm	9.80 mm	1.43 mm
7	51.82 mm	9.72 mm	1.23 mm
8	52.25 mm	10.98 mm	1.28 mm
9	48.00 mm	11.10 mm	1.22 mm

Table 5.4: Calculated Moduli for Each Leather Sample.

Test	Modulus
1	48.550
2	40.514
3	44.633
4	43.565
5	39.726
6	35.118
7	33.498
8	32.143
9	30.480
MEAN	**38.692**
STD	**6.232**

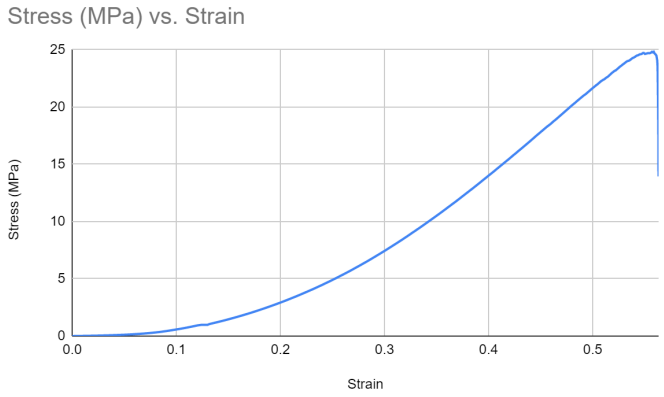


Figure 5.6: Stress-Strain Curve of Leather Sample 1.

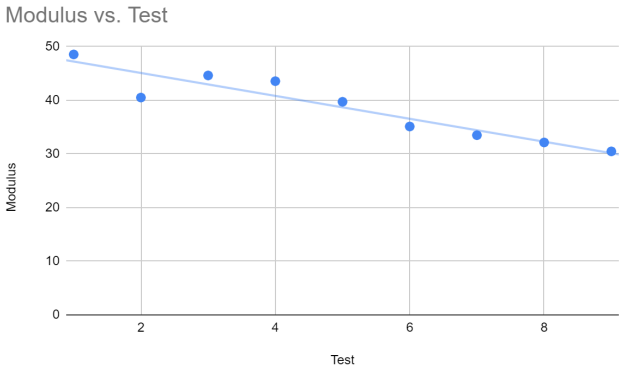


Figure 5.7: Comparison Chart of Leather Sample Moduli.

5.1.3 Cell Seeding onto Spinach Leaf in our Device with Cloning Wells

The standard laboratory practice for seeding onto plant scaffolds is to use cloning wells to seed the specified cell type, this case being bovine fibroblasts. A 96 cloning well array was a component of our design and slid into our device as seen in future sections of this paper. The wells are sterile plastic cylinders on a rectangular shaped plate that will slide over the leaf and allow the cells to attach and grow on the spinach leaf. The procedure followed is in *Appendix D.2*, and is additionally used for the device procedure found in Section 6.1.4.

5.1.4 Petri Dish Control

The purpose of seeding cells in a petri dish alone, will prove that the cells were properly isolated and can proliferate. This allowed us to compare the scaffold control and our design accordingly, knowing that the cells were viable. The results of this control can be seen in Figure 5.8.

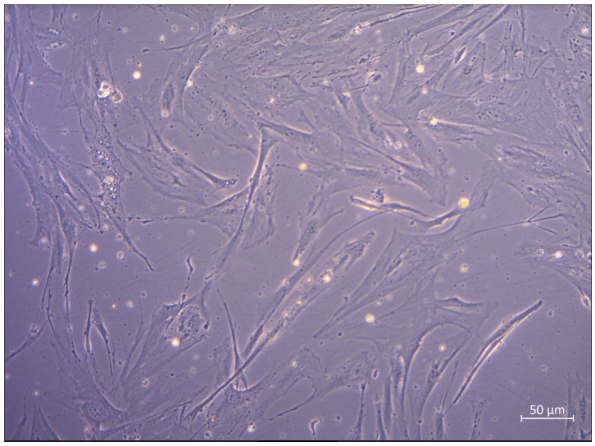


Figure 5.8: Results of Petri Dish Control.

5.1.5 Plant Scaffold in Petri Dish Control

This control experiment allowed us to show the success of spinach scaffolding for cell growth. We seeded the cells onto the spinach scaffold with cloning wells in a petri dish. We were able to compare this control to the petri dish control in order to prove that cells could adhere and proliferate on a plant scaffold in a similar manner to cells proliferating in a petri dish without scaffolding as shown in Figure 5.9. The final result of this scaffold control can be shown in Figure 5.10 which demonstrates contamination.

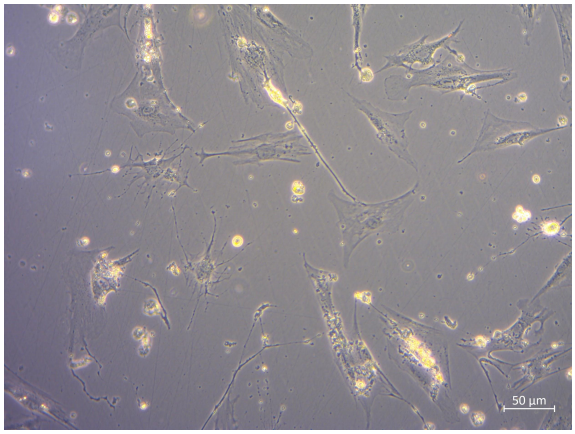


Figure 5.9: Plant Scaffold Control.

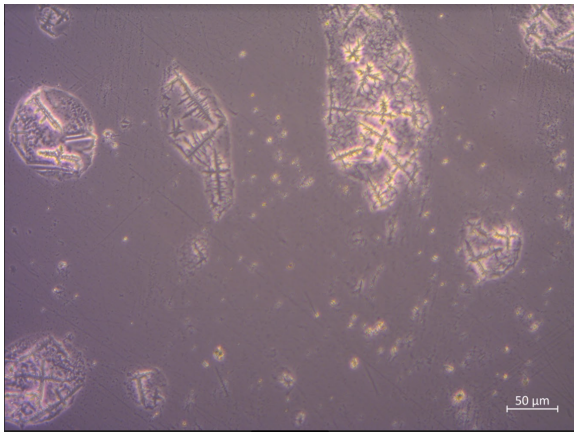


Figure 5.10: Results of Plant Scaffold in Petri Dish Control.

5.1.6 Selected Design

We were successfully able to grow cells in a tissue culture environment, but had consistent contamination in our experiments with our device. Future work should eliminate contamination sources, which is discussed in the recommendations section of this paper.

Chapter 6: Final Design Validation

This chapter reviews the individual components of the final design and evaluates how well our final product achieves the objectives that were outlined in Section 3.4: Final Objectives.

6.1 Final Design

The final device design aimed to fulfill our final objectives; making the design reproducible, scalable, and user-friendly while also maintaining cell viability.

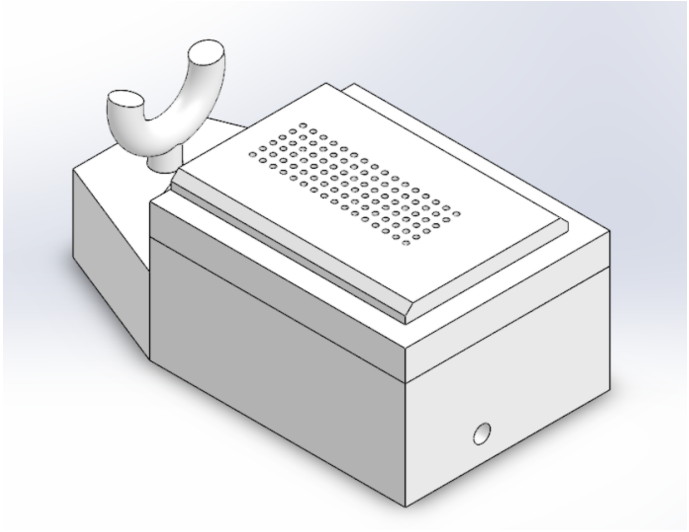


Figure 6.1: Final Design CAD Drawing and Prototype.

Our final design incorporates a 3D printed assembly as seen in the CAD drawing in Figure 6.1 This assembly incorporated the implementation of a series of Arduino sensors, including temperature, pressure, and pH sensors, in order to detect the viability of our recycled media. Figure 6.2 below shows the Arduino set-up used for the pumping system and the pH sensor.

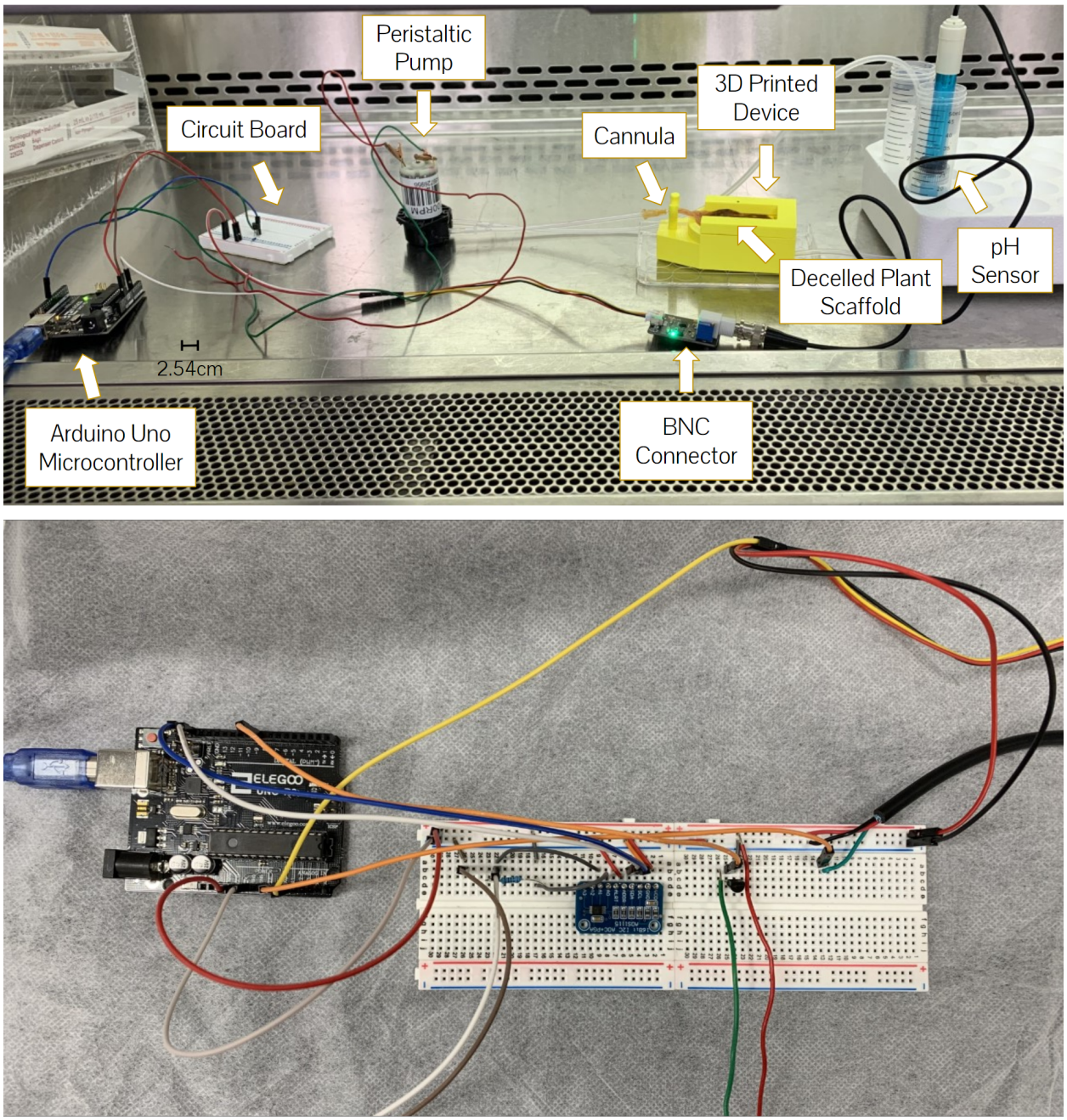


Figure 6.2: Arduino Circuit Diagram and Image of Pump and Sensors.

6.1.1 Hardware

The final design consists of four physical components that are 3D printed. These components include: a 96 cloning well piece, the top portion of the clamp, a media collection

piece that additionally acts as the bottom portion of the clamp, and a cannula holder. All of these parts can be seen in Figure 6.3. The first component is the 96 cloning well piece, labeled 1 in Figure 6.3, which assists in the seeding of fibroblast cells. The next two components, labeled 2 and 4 in Figure 6.3, are crucial in securing the spinach leaf into place. This clamp keeps the decellularized plant scaffold in place for both cell seeding experiments and for the perfusion of media through the system. Part 4 labelled in Figure 6.3 additionally acts as the media collection component which is responsible for collecting excess media that perfuses through the plant scaffold. Finally, the last printed component, labeled 3 in Figure 6.3, is a part capable of holding a cannula in place which is applicable for the delivery of media to the plant scaffold.

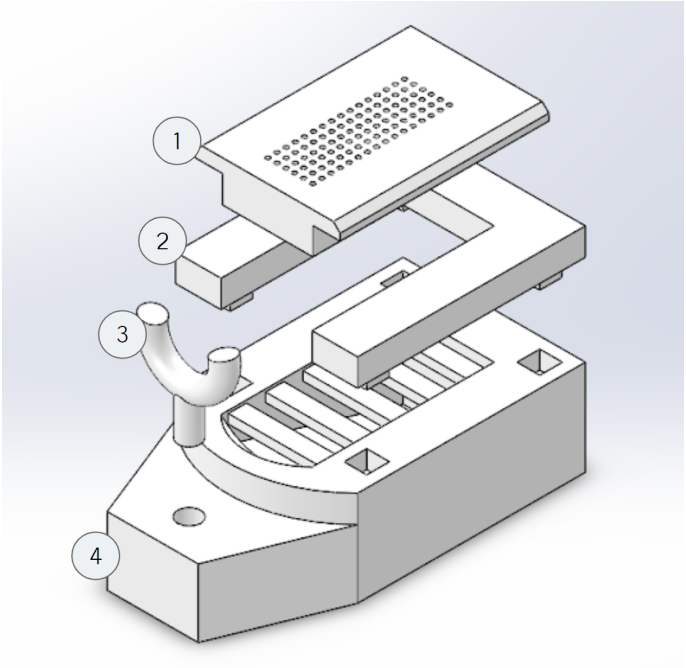


Figure 6.3: Solidworks Model of the Six 3D Printed Components.

These four physical components were 3D printed and may be seen individually in Figure 6.4 below. Meanwhile, Figure 6.5 shows an assembly of the device, with the exception of the 96

cloning well piece. The image illustrates the function of the clamping system holding the decellularized plant scaffold. Finally, Figure 6.6 shows the final assembly of the device.

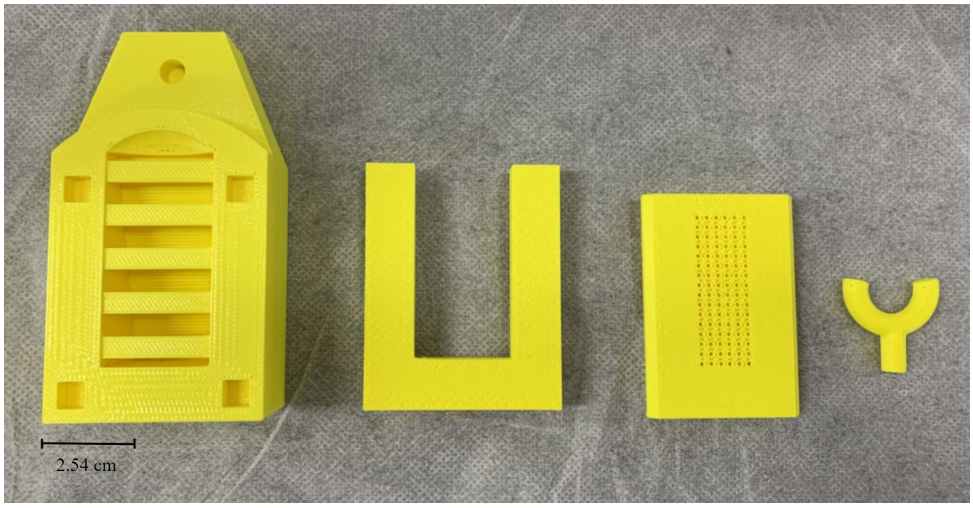


Figure 6.4: 3D Printed Hardware of the Design.

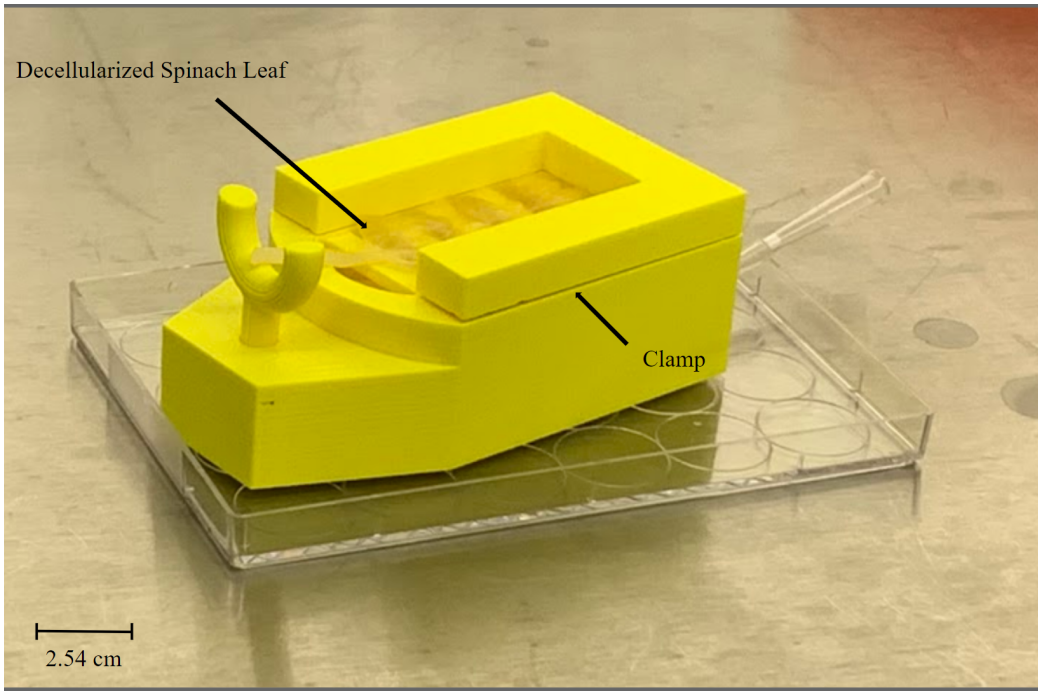


Figure 6.5: Assembled Device Highlighting Scaffold Clamp.

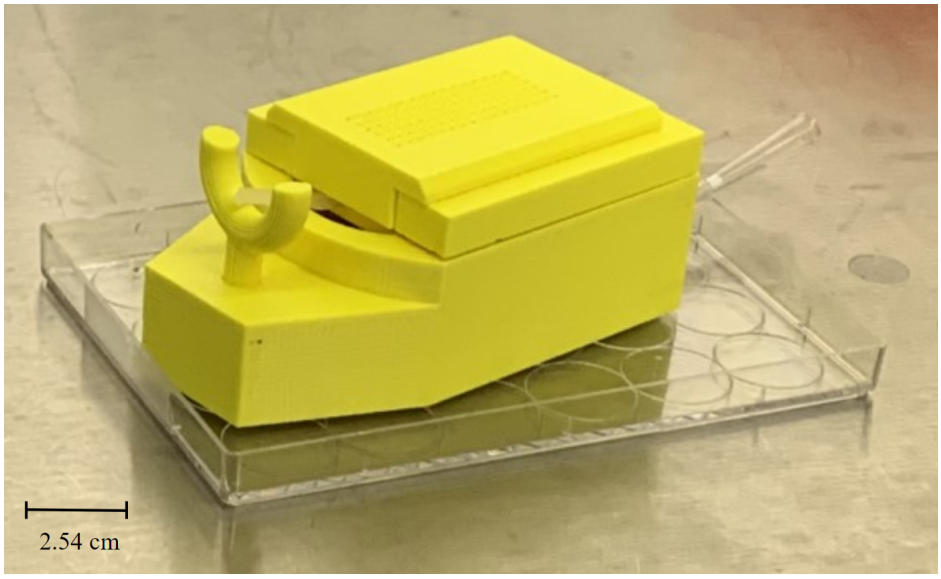


Figure 6.6: Final Device Assembly of Hardware.

Aside from the six 3D printed components mentioned, there are an additional three sensors and a pump to assist in growing fibroblast cells on a plant scaffold. These components include a peristaltic pump to move the media through the system, a pH sensor to help determine the viable life of the media being used, a NTC thermistor, and a pressure sensor to assist in the fluids and heat analysis associated with our design.

6.1.2 Electronics and Instrumentation

The goal of implementing electronic components into our prototype was to assist in the delivery of media to the plant scaffold. Additional contributions of these electronic components was to provide figures for calculations in the fluids and heat transfer analysis of this design. Our final design consisted of four electronic components to achieve these goals. The sensors were powered by a computer as well as a 9 Volt battery.

The first is a Gikfun 12 Volt DC Peristaltic Pump with silicone tubing. The pump is responsible for driving a microfluidic laminar flow to the plant scaffold as well as collecting excess media from perfusion and recycling it through the system.

Next, is the GAOHOU pH 0-14 Value Detect Sensor with +pH Electrode Probe and BNC. This device was utilized to gather and send sensor information regarding the pH value of the media being moved through the system. The sensor reads the pH of the media while also notifying the design team via email when the media is not at a pH value of 7.4. This pH value signified that the media was no longer viable for cell growth and needed to be replaced by the team.

An NTC 10K Thermistor and a 100 PSI Pressure Transducer was used to enhance and provide finite values for a heat transfer and fluids analysis. The NTC thermistor helped provide a heat exchanger analysis that could further enhance the design. Meanwhile the pressure reading assisted in completing an in depth perfusion analysis regarding pressure drop.

All of these components were a part of the final design to ensure proper growing conditions for the cells in their entire proliferation phase. The assembled final design may be seen in Figure 6.7. These sensors and monitoring systems are described below and contribute to the quality control of the system. With these applications, the system operator can continuously monitor and ensure that the culture environment is ideal for cell growth, producing the highest quality of dermis at the end.

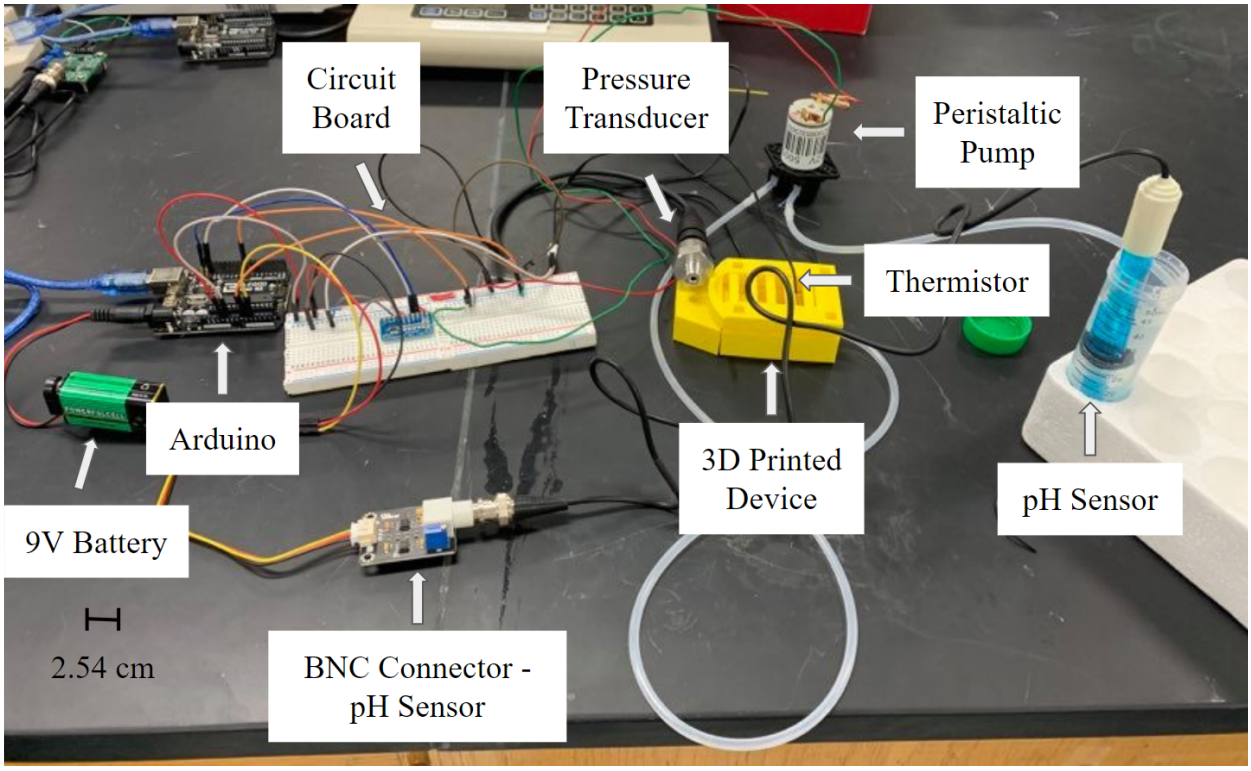


Figure 6.7: Experimental Design Assembly with Arduino Sensors.

6.1.3 Software and Coding

In order to properly use our peristaltic pump and sensors, we needed to code using an Arduino and the corresponding coding language. In order to create a fully functioning system, we worked with each individual part and ran the code to assure that each part worked. In Table 6.1 below, each part with it's described functions are shown. The corresponding source codes and circuit wiring can be found in Appendix E.

Table 6.1: Arduino Sensor Functions.

Arduino Part	Function
Peristaltic Pump	Pumps the used media from the well in the bottom of the device back through the cannula to recycle it.
Thermistor	Senses the temperature of the media in the well at the bottom of the device to confirm it is within the temperature range for viable media, which is between 35 and 40 degrees Celsius.
Pressure Sensor	Senses the pressure of the flow of media coming out of the cannula to ensure safe pressure values for the scaffold.
pH Sensor	Senses the pH value of the media to ensure it is still within the range for viable media, which is between 6.5 and 8.5.

We were able to calibrate the NTC 10K thermistor by graphing thermometer readings against the thermistor's readings. Using a regression model that mimicked a fifth degree polynomial function, coefficients were drawn and used in the source code. Below, Figure 6.8 shows the calibration graph and respective equation for the thermistor.

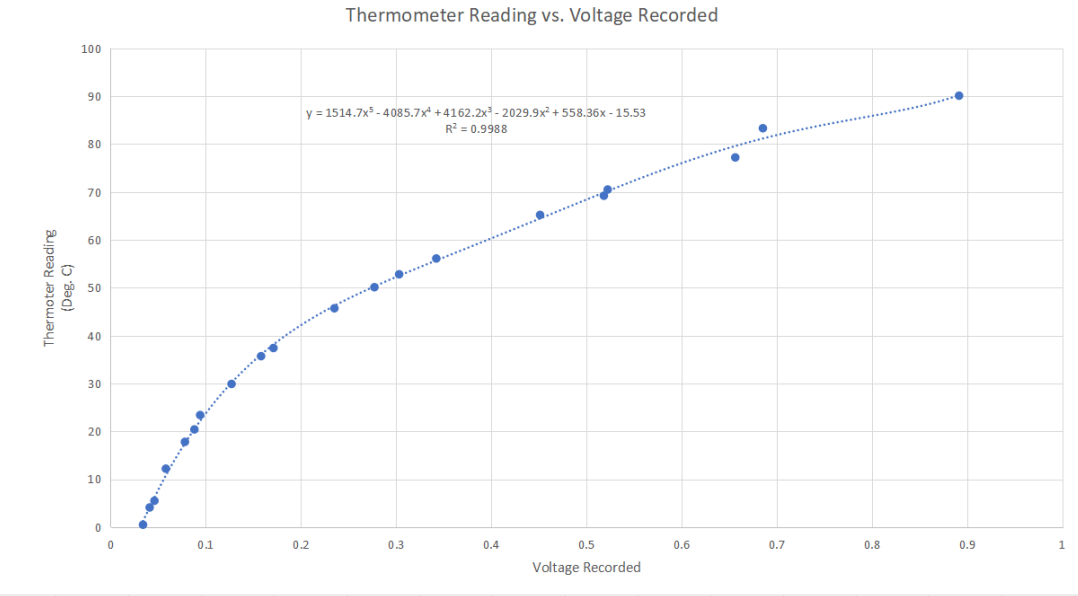


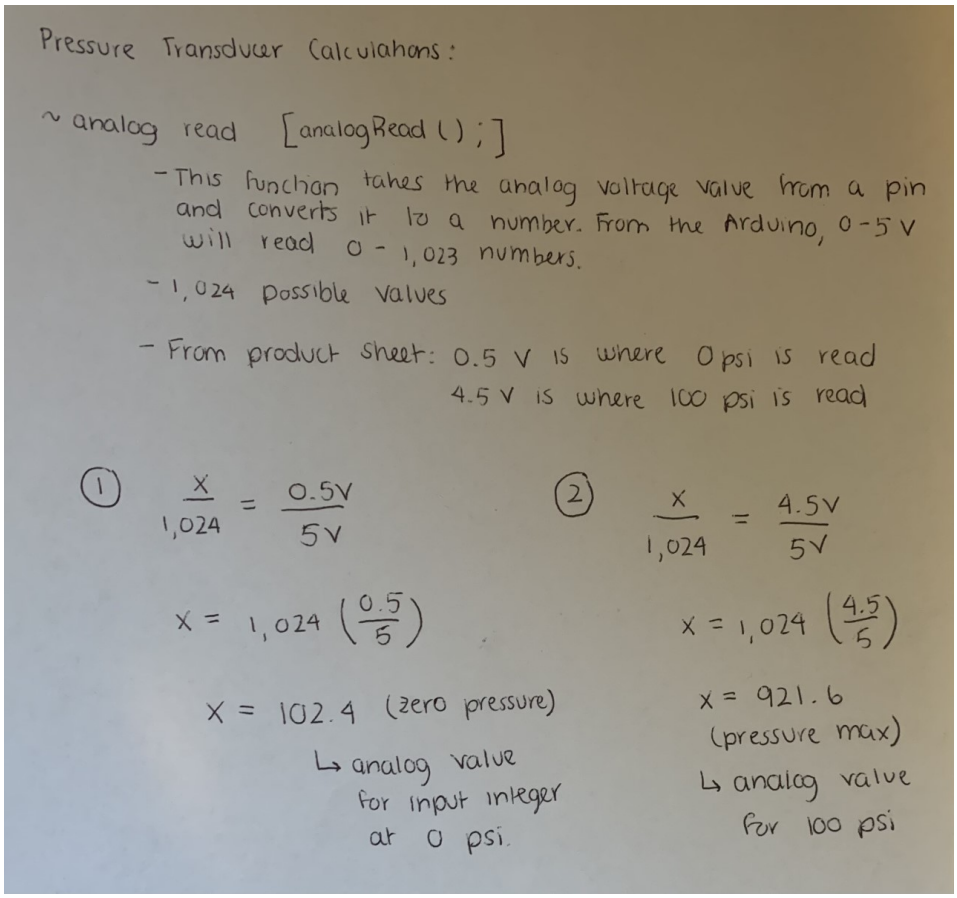
Figure 6.8: Thermistor Calibration Graph.

For the pH sensor, we adjusted the trimmer on the BNC board. While running a calibration code, various solutions with known pH values were tested. For these solutions, the trimmer was adjusted until the code read the correct value on the Serial Monitor. The accuracy of the pH sensor's calibration was determined with the known pH values of solutions tested. The results of this test can be seen in Table 6.2 below.

Table 6.2: pH Sensor Accuracy Testing Values.

Substance:	**Sensor pH Value:**	**Actual pH Value:**
Lemon Juice	2.26	2
DI Water	7.40	7
Bleach	13.39	13

For the pressure sensor, we used existing equations in the code to calibrate the sensor. In Figure 6.9 below, the equations and calculations used to calibrate the sensor can be viewed.



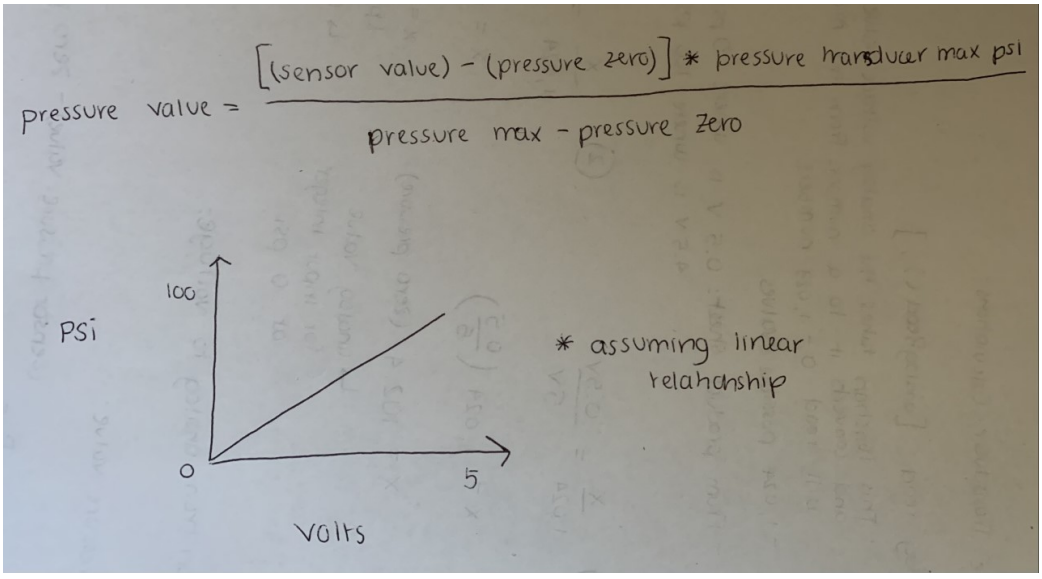


Figure 6.9: Pressure Sensor Calibration Equations and Calculations.

The sensors were wired to display their respective variable values on a computer screen for the user to monitor. The system also functions to send an email to the team whenever a sensor

reads values that are not within the viable range. The final code corresponds to the image of the complete circuit set up including all of the separate sensor codes wired together and is shown in Figure 6.10 below. Once calibrated, the sensor was tested by blowing on it. This action should cause a slight increase in pressure and the serial monitor would show a result. The pressure slightly increases from 0.1 psi for the room to about 2 or 3 psi.

```
# include <OWire.h>
#include <Adafruit_ADS1015.h>
Adafruit_ADS1115 ads(0x48);
int16_t rawADCvalue;
float volts = 0.0;
float voltMax = 4.096;
float a0 = -15.53, a1= 558.36, a2 = -2029.9;
float a3 = 4162.2, a4 = -4085.7, a5 = 1514.7;
float TempDegC=0;
unsigned long StartTime = 0;
const int pressureInput = A0; //select the analog input pin for the pressure transducer
const int pressureZero = 102.4; //analog reading of pressure transducer at 0psi
const int pressureMax = 921.6; //analog reading of pressure transducer at 100psi
const int pressuretransducermaxPSI = 100; //psi value of transducer being used
float pressureValue = 0;
const int pump = 8;
#define SensorPin 1          // the pH meter Analog output is connected with the Arduino's Analog
unsigned long int avgValue;  //Store the average value of the sensor feedback
float b;
int buf[10],temp;

void setup(void)
{
Serial.begin(9600);
ads. setGain(GAIN_ONE);
ads. begin();
StartTime = millis();
pinMode(pump, OUTPUT);
pinMode(pressureInput, INPUT);
pinMode(13,OUTPUT);
Serial.begin(9600);
Serial.println("Ready");    //Test the serial monitor
}

void loop(void)
{
rawADCvalue = ads.readADC_Differential_0_1();
volts = (rawADCvalue/32767.0) * voltMax;
TempDegC = a0 + a1*volts + a2*pow(volts,2) + a3*pow(volts,3) + a4*pow(volts,4) + a5*pow(volts,5);
unsigned long CurrentTime = millis();
float ElapsedTime = (CurrentTime-StartTime)/1000.0;
Serial.print("Time (sec)"); Serial.print(ElapsedTime,3);
Serial.print(", Volts Measured = "); Serial.print(volts,2);
Serial.print(", Temp. (deg C) = "); Serial.print(TempDegC,2);
Serial.print(", (deg F) = "); Serial.println(TempDegC*1.8+32.2);
pressureValue = analogRead(pressureInput); //reads value from input pin and assigns to variable
pressureValue = ((pressureValue-pressureZero)*pressuretransducermaxPSI)/(pressureMax-pressureZero); //conversion equation to convert analog reading to psi
Serial.print(pressureValue, 1); //prints value from previous line to serial
Serial.println("psi"); //prints label to serial
  for(int i=0;i<10;i++)       //Get 10 sample value from the sensor for smooth the value
  {
    buf[i]=analogRead(SensorPin);
    delay(10);
  }
  for(int i=0;i<9;i++)        //sort the analog from small to large
  {
    for(int j=i+1;j<10;j++)
    {
      if(buf[i]>buf[j])
      {
        temp=buf[i];
        buf[i]=buf[j];
        buf[j]=temp;
      }
    }
  }
  avgValue=0;
  for(int i=2;i<8;i++)                      //take the average value of 6 center sample
    avgValue+=buf[i];
  float phValue=(float)avgValue*5.0/1024/6; //convert the analog into millivolt
  phValue=3.5*phValue;                      //convert the millivolt into pH value
  Serial.print("    pH:");
  Serial.print(phValue,2);
  Serial.println(" ");
  digitalWrite(13, HIGH);
  delay(500);
  digitalWrite(13, LOW);
 digitalWrite(pump, HIGH);
 delay(500);
 digitalWrite(pump, LOW);
 delay (500);
```

Figure 6.10: Final Sensor Code.

6.1.4 Device Procedure

This device provides the user with a procedure to produce lab grown leather. The layout of the design has been discussed (see Figure 6.3). These components in combination with various sensors (see Figure 6.7) make a system to grow lab grown leather. Any producer that is utilizing this device at an independent location would be able to replicate the process outlined in the steps below.

1. First, ensure that all components of the device are printed, sterilized, and filed to ensure a proper assembly. Additionally, ensure that media is made and pump connections are secure.
2. Take a decellularized spinach leaf and the piece containing the 96 cloning well array for seeding. After the leaf is sterilized, place it into a petri dish and begin seeding bovine fibroblasts cells onto the scaffold through the use of the 96 cloning well array. Incubate with the petri dish with the leaf submerged in media and with the cloning well array on top of the scaffold.
3. Ensure the adhesion of fibroblast cells to the plant scaffold through fluorescent dyeing experiments.
4. Once fibroblast cells adhere to the plant scaffold, place the spinach leaf in the device. Ensure that all components of the device are connected, and secure the stem of the leaf with a cannula.
5. Meanwhile, place one segment of the pump's silicon tubing into a fresh batch of media and connect the other end to the cannula. Pump media through the plant

scaffold. Once complete, let the cells incubate for two days. Repeat this process, while replacing media when necessary.

This is a rough outline of the process; however, it shows how simple and user friendly that this design is to operate in order to produce lab grown leather products. An image of our device being operated within a sterile environment may be seen below in Figure 6.11.

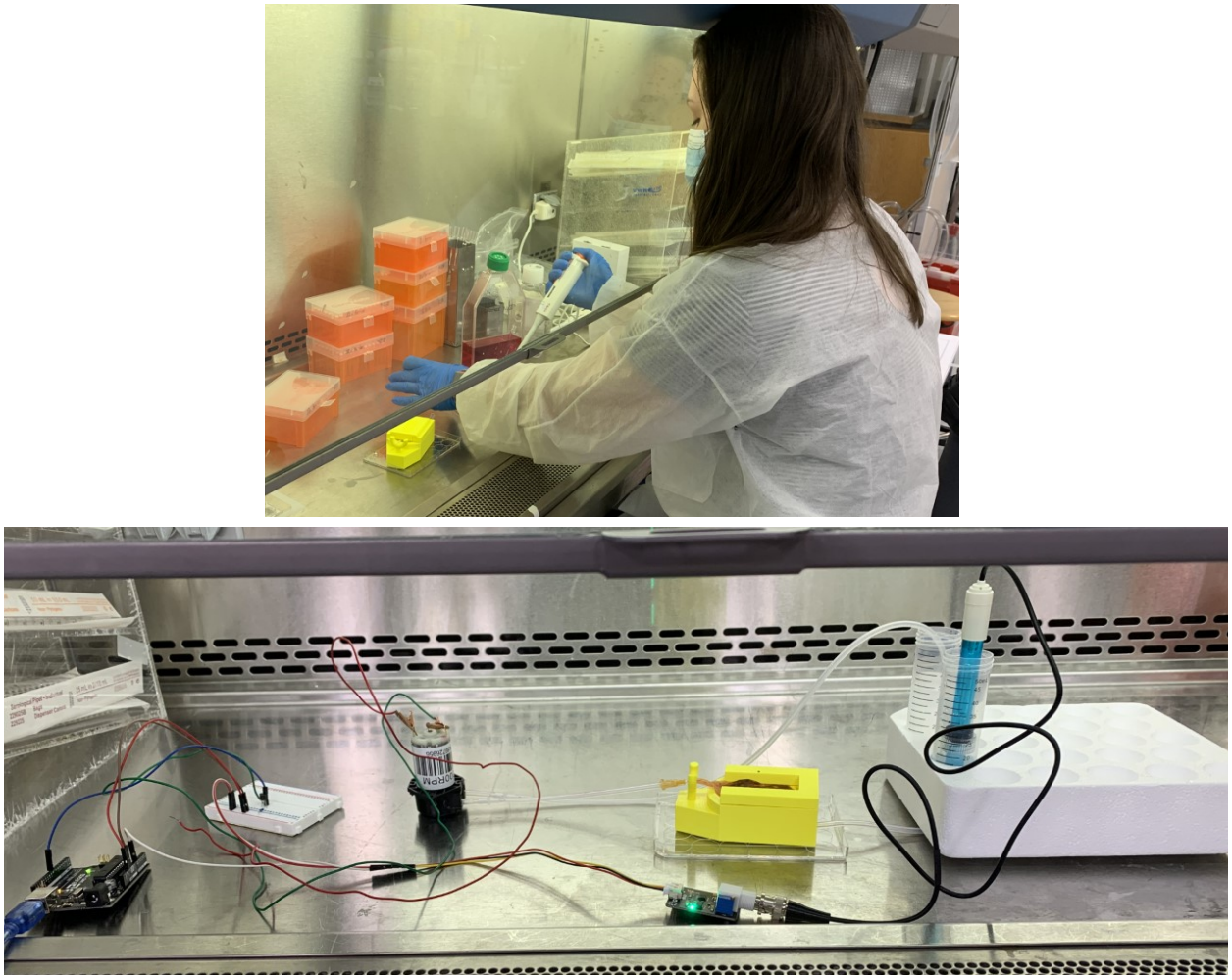


Figure 6.11. Operating Device in a Sterile Environment.

6.2 Evaluation of Objectives

In Chapter 3, the constraints of our project and the main objectives we used to frame our final design were discussed. In the sections below, we will evaluate how the constraints affected our final product versus how we addressed the main objectives.

6.2.1 Evaluation of Constraints

In section 3.3.2: Constraints, we identify our primary constraints as time, cost, lab accessibility, and sterility and biocompatibility of the materials used in the lab. Over the course of this project, our team was able to readjust our plans according to our limitations.

Lab accessibility was the largest challenge our team had to face. As our lab work was intended to begin in January 2021, our plans were pushed back until mid-February of 2021 due to COVID-19 restrictions. The team adjusted our plans and began decellularizing spinach and cabbage leaves at home.

6.2.2 Evaluation for Design Criteria

Using the design criteria mentioned previously, our team ensured that our device successfully achieved all of these objectives. These included cell viability, scaffold adequacy, cost effective, and user friendly.

6.2.2.1 Ensure Cell Viability

One of the primary objectives of the design is that the cells in culture remain healthy and viable, ensuring the product would meet ASTM standards. The leaf is both submerged in media

and cannulated through the stem so that the nutrients in the media can easily be accessed by the cells. By designing the device to fit within an existing incubator space, the carbon dioxide and temperature requirements for adequate cell growth are maintained and the design requirement for pH maintenance and waste reduction is ensured by efficient and mechanically powered media changes within the incubation space.

6.2.2.2 Scaffold Adequacy

After careful consideration of different scaffold options, the group ultimately decided to use decellularized spinach leaves. This was mainly due to the fact that spinach is the most commonly used plant scaffold with its porous properties on the surface and mechanical properties. Additionally, the intravascular network made the scaffold suitable for perfusion, making it easier to transport media during cell seeding.

6.2.2.3 Cost Effective

Our team had $250 per student dedicated to the project budget. In total, $1,250 was designated to us by Worcester Polytechnic Institute in order to complete the project. The limited budget allowed us to make use of the materials already available in the lab, and a portion of the budget was designated to cover these specific lab fees. Seen below in Table 6.3 is our team's proposed and actual use of the given budget.

Table 6.3: Proposed versus Actual Budget.

Cell Seeding and Decellularization Materials				
Item	Cost	Quantity	Total Cost	Comments
Spinach Leaves	$2.99	1	$3	1 lb - Purchased from Price Chopper
Cabbage Head	$2.34	1	$2	Purchased from Price Chopper
Fibroblasts		1	$0	
Petri Dishes	$10.99	1	$11	Pack of 30, 90x15mm
Cell Culture Flasks	$60.00	1	$60	For a pack of 100
96 Well Plate	$55.75	1	$56	For a pack of 50
Fine Scissors	$4.99	1	$5	Set of 5
Fine Forceps	$9.99	1	$10	
SDS	$248.39	1	$248	For 1 kg
DI Water	$40.00	1	$40	For a pack of 4 one-gallon jugs
Bleach	$15.00	1	$15	For a 1.21 ounce jug
Liquid Nitrogen	$159.00	1	$159	
Centrifuge Tubes	$22.49	1	$22	50 pieces 20 mL
Micropipettes	$42.99	1	$43	Pipettor with 100 pipette tips
Pipettes	$15.99	1	$16	For a pack of 100
Centrifuge Tube Rack	$15.99	1	$16	
Daigger Vortex Genie 2	$390.95	1	$391	
Cyquant Cell Proliferation Assay Kit (Thermofisher)	$438.00	1	$438	10 microplates
Fibroblast Medium	$124.00	1	$124	
Fetal Bovine Serum	$479.00	1	$479	500 mL
GlutaMAX	$39.20	1	$39	
Penicillin-Streptomycin	$20.58	1	$21	
Trypsin/EDTA	$21.00	1	$21	
Trypsin Neutralization	$21.00	1	$21	
Liberase Blendzyme 3	$352.00	1	$352	10 mg
70% Ethanol	$23.00	1	$23	32 ounces
DPBS	$16.00	1	$16	
PLL	$16.00	1	$16	
Ascorbic Acid	$66.40	1	$66	A4403-100MG
Centrifuge	$61.99	1	$62	
Incubator	$176.99	1	$177	
Shaker Table	$97.99	1	$98	
		1	$0	
		1	$0	
Total Cost:			$3,051	

Mechanical Testing Materials				
Item	Cost	Quantity	Total Cost	Comments
Rice Paper	$5.95	1	$6	12 ounce pack of 22 cm round
Flat Tip Forceps	$8.99	1	$9	
Calipers	$10.49	1	$10	
Ruler	$4.73	1	$5	
Duct Tape	$5.97	1	$6	
Cutting Board	$6.62	1	$7	
Meat Cleaver	$9.14	1	$9	
Ziploc bags	$8.89	1	$9	280 pack
Instron	$14,900.00	1	$14,900	
Binder Clips	$10.00	1	$10	For a combo pack of 100
Chip Bag Clips	$5.99	1	$6	For a set of 7
Total Cost:			$14,977	

Device Materials (Including Arduino)				
Item	Cost	Quantity	Total Cost	Comments
3D Printing Materials	$50.00	1	$50	
Arduino Uno	$15.00	1	$15	
Pressure Sensor	$16.00	1	$16	
Thermistor	$10.00	1	$10	
pH Sensor	$20.00	1	$20	
Peristaltic Pump	$16.50	1	$17	
Jumper Wires	$7.99	1	$8	560 pieces, variable lengths
ADC Converter	$11.99	1	$12	3 pack, 16 bit
Soldering Iron	$52.99	1	$53	
9V Battery	$11.99	1	$12	
9V Battery Connector	$5.99	1	$6	
Total Cost:			$218	

Overall Budget	
Cell Seeding and Decellularization Materials	$3,051
Mechanical Testing Materials	$14,977
Device Fabrication Materials	$218
Total Cost	$18,246

Cell Seeding and Decellularization Materials				
Item	Cost	Quantity	Total Cost	Comments
Spinach Leaves	$2.99	1	$3	1 lb - Purchased from Price Chopper
Cabbage Head	$2.34	1	$2	Purchased from Price Chopper
Fibroblasts		1	$0	
Ascorbic Acid	$66.40	1	$66	A4403-100MG
Lab Space Surcharge	$50.00	5	$250	Charged per student
Total Cost:			$322	

Mechanical Testing Materials				
Item	Cost	Quantity	Total Cost	Comments
Rice Paper	$5.95	1	$6	12 ounce pack of 22 cm round
Total Cost:			$6	

Device Materials (Including Arduino)				
Item	Cost	Quantity	Total Cost	Comments
3D Printing Materials	$30.00	1	$30	This is probably an overestimate.
Pressure Sensor	$16.00	1	$16	
pH Sensor	$20.00	1	$20	
Peristaltic Pump	$16.50	1	$17	
9V Battery	$11.99	1	$12	
9V Battery Connector	$5.99	1	$6	
Total Cost:			$100	

Overall Budget	
Cell Seeding and Decellularization Materials	$322
Mechanical Testing Materials	$6
Device Fabrication Materials	$120
Total Cost	$448

Though we had lab resources and tools that were provided for us by the university, the relative budget of our project remained on the more affordable spectrum. The costs involved were mainly fees and printing costs, as well as buying materials to perform cell culture. The amount our team had spent was a total of $448, which is significantly lower than our allotted budget. It is reasonable to conclude that a process that costs less than $1,000 with access to necessary machinery is considered affordable. If there was no access to an Instron (being the most expensive component) the budget would have totaled $3,346. This total is quite low considering the complexity of the process and materials used. Once again, it can be concluded that this process is relatively affordable.

6.2.2.4 User Friendly

The design was created using SolidWorks and 3D printing in order for the device to be easily fabricated. We kept the device small so it could be used in a university lab-grade incubator and kept simple geometry for the parts so it was easy to replicate. We also wanted to include our own piece with cloning wells instead of using multiple individual cloning wells so it was easier to successfully seed the cells onto the scaffold. In addition to this, we used 96 wells on our scaffold because, if we were to scale up our design, the technology within the biomanufacturing community uses a standard of 96 wells.

6.3 Additional Considerations

While creating our design, we wanted to keep in mind how our project would be influenced by other outside factors. This section aims to evaluate our project within the

following domains: economics, environmental impact, societal influence, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability.

6.3.1 Economics

The leather industry continues to increase its value every year, as mentioned in section 2.1: The Leather Industry. The demand for leather will still exist even if replaced with a lab-grown solution. In order for a completely lab-grown leather market to be introduced, lab-grown meat would have to take precedence first because leather is a byproduct of the meat industry. This could jeopardize the businesses of slaughter houses, butcher shops, and local farmers. The cattle industry would be affected as well, and farmers may not be able to collect enough funds to properly care for their animals relying on livestock crops alone. This process could also jeopardize international societies such as India, where leather production contributes heavily to their economy.

On the positive end, with our scalable and reproducible process, there is the potential for mass-producing lab-grown leather in years to come. By maintaining a relatively low budget, recreating this process in different locations and doing it continuously can prepare loads of dermis for leather production for a fraction of the cost.

6.3.2 Environmental Impact

This leather production industry has a large negative impact on the environment, as outlined in sections 2.1: The Leather Industry and 2.2: The Current Leather Making Process. Our project is capable of eliminating fleshing and curing waste from the current leather-making process from the leather-making equation. With our design and procedures, there will be little to

no solid waste since we are only growing the cells that are necessary for creating leather. We also have outlined recommendations in later sections for tanning and dyeing that utilizes vegetable tanning methods and water pigments. This protocol aims to eliminate use of the harmful chemicals that pollute the environment when they are improperly disposed of such as chromium.

Overall, with the implementation of our outlined procedures for the growth of dermal cells and environmentally friendly tanning and dyeing processes, the need for raw hide from slaughterhouses should be reduced. This will not stop the innumerable environmental problems that stem from the meat and leather production industry in tandem, but it will minimize the need for the leather-making byproduct of the meat industry. If plant-based or lab-grown meat substitutes become more prevalent in the coming years, these processes can contribute to an overall decrease in the negative environmental impact of the meat and leather production industries as a whole.

6.3.3 Societal Influence

Over the last few years, there has been an increase in criticism for the consumption of animal products whether it be through food or material. Since leather currently remains a byproduct of the meat industry, it causes minimal additional animal cruelty on top of what the meat industry does on its own. Other leather alternatives such as pleather, as mentioned in *Appendix A.2*, have their own criticisms. These are mainly due to the fact that it is creating a surplus of non-recyclable plastic. As mentioned previously, the creation of lab-grown leather can significantly reduce greenhouse gases and carbon emissions which, from an environmentalist standpoint, would be a desirable solution. People with a vegan or vegetarian lifestyle may be indifferent to this solution as it still involves animal products. However it could be a convincing

solution when partnered with the fact that an animal didn't have to die in order for the leather to be produced. It can also generate more action for reducing animal cruelty overall.

6.3.4 Political Ramifications

Referencing section 6.3.1: Economics, the lab-grown leather industry could have a negative impact on the agricultural economy and local farmers whose welfare depends on selling their animal products for revenue. This could also negatively affect international trade relations between countries because hide imports and exports contribute to their overall economy. However, the positive effect on the environment could reduce climate change on the global scale. As acts and movements for climate change have been increasingly discussed in recent years, this proposition is favorable to those who advocate for eco-friendly solutions.

6.3.5 Ethical Concerns

Lab-grown leather allows people to purchase leather products without the guilt of knowledge that it is made from an animal product. As mentioned in 6.3.3: Societal Influence, people following a vegan or vegetarian lifestyle for ethical reasons may feel inclined to own leather products as it was produced without killing an animal, and they wouldn't be polluting the Earth by purchasing synthetic leather. Additionally, with the reduction of chemical contamination from tanning and dyeing with a vegetable dyeing proposition, which is discussed further in the recommendations section, consumers can purchase more ethically produced leather in terms of work conditions for employees and the environmental impact that improperly treated waste has on the surrounding communities.

6.3.6 Health and Safety Issues

By altering the leather tanning and dyeing processes, there is a significant reduction in chemical exposure to the people working directly with the tanning chemicals. Referencing section 2.2.1: Waste from the Tanning Process, there is currently heavy contamination from chromium, ammonia, and hydrogen sulfide that pollutes large waterways and drinking water sources in countries with tannery sites, which affects all inhabitants of that area. With laboratory-based processes, all leather production can potentially be done in multiple regulated locations; reducing fume exposure and overall environmental hazards.

6.3.7 Manufacturability

Some of the main design objectives for this project were to create a scalable and user-friendly design as well as be able to save time and money. Tremendous consideration was placed not only on the ease of use of this system, but its efficiency to minimize user interaction time. For instance, a 3D print is far more cost effective and timely than some traditional manufacturing techniques. As such, 3D printing is affordable and allows for less waste production in customizing parts and making prototypes. In terms of timeliness, 3D printing will reduce lead time and time to market. For scalability, our 3D printed design is capable of being stacked as multiple units. This ability to stack units in the design allows users to maximize the potential for replication and mass production.

6.3.8 Sustainability

While our device iterations were 3D printed using inexpensive materials, this is not sustainable for the final product. Given our budget and the objectives we were trying to meet, cost effective options such as 3D printing materials were preferred but are not a viable option for long term and large scale use. As popularity of these devices increases, the team has taken the potential environmental impact into consideration and would recommend that stainless steel be used. This material is easily sterilizable and can improve large-scale manufacturability. The devices would maintain their biocompatibility as well, and are much more durable than a 3D printed part. The dimensions could be altered to accommodate larger leaves to produce larger dermis sheets. Overall, alternative materials should be considered for future use.

Chapter 7: Discussion

The following chapter will discuss the key features of our completed work with this project presently, explain our results from the seeding and growing process, potential for scalability, and ease of use. The team has provided how we met these objectives to allow for growth in the future.

7.1 Seeding and Growing Process

The processes as described in Chapter 5, allowed us to test and compare our design to the simpler control methods. Our device was able to successfully adhere and proliferate dermal fibroblast cells. This means that in future applications, it possesses the possibilities to grow a sheet of dermal fibroblasts that can be transformed into a piece of leather. While our design may not be able to seed and proliferate cells at a faster rate, it possesses several key attributes that can help progress the leather industry. Our device is manufacturable, user-friendly, and has the possibility to be replicated by future researchers.

7.2 Potential for Scalability

Throughout the design process, the design team has kept in mind the scalability of the final device. With this application, there is endless potential for scalability and increased efficiency of the prototype. The first opportunity to scale the design relates to the SolidWorks model. The device is currently scaled to fit the size of a spinach leaf which has surface dimensions of approximately 2.54 centimeters by 5.08 centimeters, and is currently 5.21 centimeters tall. The size of this system can easily be increased to scale the amount of leather

being produced, or accommodate larger plant scaffolds. This size of the device can be increased to have dimensions up to 67.31 centimeters by 36.83 centimeters by 55.88 centimeters if still utilizing 3D printing (Sculpteo, n.d.).

The second opportunity to increase the scale of the design is to stack multiple units in the system. In other words, one could print multiple clamping systems to hold several spinach leaves. This stacked system provides the device with the potential to grow several sheets of cells simultaneously. These sheets could then be combined to produce a larger or thicker portion of lab-grown leather.

The third opportunity to increase the scale of the leather being produced is to alter the scaffold being used. The design team explored various options to use as a scaffold, but decided to go with a spinach leaf for this project. The team particularly explored cabbage as an alternative scaffold. The decellularization process is the same for spinach, but the cabbage has a slightly larger area to grow cells. Cabbage could widen the area of cell growth from approximately 2 square inches to 6 square inches. While the design team focused on spinach and cabbage, further research and analysis shows that there are more possibilities. For instance, a ficus hispida leaf has been decellularized in previous experiments and could be used in this application. This would increase the potential area for cell growth to 40 square inches (Valke, D., 2005). These applications were not fully explored in the scope of this project, but have the potential to enhance the scalability of our design.

7.3 Additional Features for Ease of Use

In order to make our design more user friendly, we used relatively basic Arduino circuitry and coding because anyone with no professional experience or training can quickly learn. Our

decision to use an Arduino microcontroller was due to its accessibility and ease of use, as was the pump on our device. A peristaltic pump was chosen for its low cost and because it can be easily wired and controlled with any small scale microcontroller. An Arduino Uno can be found on Amazon for as little as $12 USD and the peristaltic pump was $17 USD. The Arduino IDE coding software is available for free download on the Arduino website, as well as through a web-platform which requires no download. All decellularization processes and mechanical tests are able to be performed at home using common household materials, and our final device was 3D printed using inexpensive materials. All tests were to the ASTM standards to allow for completion by following a clear protocol.

Chapter 8: Recommendations and Conclusions

The following chapter will discuss future recommendations provided by the team such as possible options to continue the project and make continuous improvements. The conclusions of the work that has been completed with this project presently will explain our results and interpretations of data.

8.1 Recommendations

The following sections are different areas of recommendations for continuing future work to expand and improve upon the current state of the project.

8.1.1 Tanning Recommendations

Methods are currently being developed to use natural tannins in an effort to reduce the negative impacts that the use of chromium creates (Kanth, 2009). These processes fall under the umbrella of vegetable tanning. Vegetable tanning relies on using a process called pickling first, which aims to dehydrate the raw hide in preparation for tanning, which transforms the hide into a non putrescible material; the material can no longer be decomposed by microorganisms. (Kanth, 2009). In historic times, vegetables were used for tanning. The animal hide was laid in a puddle containing various plant materials. The polyphenols in plants leached from the vegetables into the hide and reacted with collagen while the tannins in the plant material also fixated to collagen (Covington, 2009). Vegetable tannins, which are made from natural ingredients like tree bark, leaves, and roots, are then loaded into large drums with the hide and run for specified amounts of time that are dependent upon each company's base procedure (Kanth, 2009). Figure 8.1 below

shows the process used in Kanth's experiment for a control vegetable tanning process. The first step is pickling and the rest are tanning, with Wattle being the vegetable tannin used (Kanth, 2009).

add	Water	100%
	NaCl	10%; run the drum for 10 min
	H_2SO_4	0.75%; 3 feeds at 15 min interval + 30 min; pH of the cut section has been 4.5–4.7; Then 50% float (solution) has been drained.
add	Basyntan P[a]	2%; run the drum for 1 hr
	Wattle	10%; run the drum for 1 hr
add	Wattle	10%; run the drum for 3 hr, then complete penetration of vegetable tannin has been ascertained.
add	Formic acid	0.1%; run for 45 min, final pH has been found to be 3.5–3.7.

Figure 8.1: Example Vegetable Tanning Procedure (Kanth, 2009).

Overall, while vegetable tanning can take longer than the alternative of chromium tanning, leather production companies have found that, in addition to improvement of environmental impact, vegetable tanned leather has a better aesthetic appeal and wears much nicer over time (Galen Leather, 2019). This specific method was not feasible for the scope of our project, but could be on a larger scale.

Currently, there are companies looking to produce other more sustainable methods of tanning leather so that we can minimize the negative environmental impact of the current processes. The company Lanxess has been looking to develop a more sustainable process for the creation of leather. One of their initiatives includes the development of X-Tan®, a metal and aldehyde free tanning process that aims to keep toxic materials out of waste water and reduce the need for the materials used (Lanxess, n.d.). Throughout this process, the chromium tanning

solution has been exchanged with a polycarbonyl sulfate (PCMS) solution, which binds more efficiently with the raw hide and leaves significantly less material in waste water and improves the susceptibility to dye, which minimizes dye waste. While this method may not be ideal for smaller scale leather making processes, when on a large-scale manufacturing level, it would be simple to implement and reduce the overall negative impact of the current tanning process.

8.1.2 Dyeing Recommendations

One of the more sustainable methods for dyeing includes vegetable or plant dyeing. A study was conducted using flower petals to extract more eco-friendly dye. The process involves sonicator dyeing to extract the yellow and brown color from the *Coreopsis tinctoria* flower petals. Overall, their findings showed that there was an improvement in the leather appearance using their determined optimal conditions. Dyeing with these flower petals also did not impact mechanical characteristics (Velmurugan, Palanivel., Shim, Jaehong., Seo, Sang Ki., Oh, Byung-Taek., 2016).

Fungi has also been used to dye leather in an effort to improve sustainable practices. In this study, the dyeing was uniform and had an intense bright shade. Visual tests were also performed to assess the dye qualitatively. The mechanical properties were also not significantly impacted using this dyeing method (Palanivel Velmurugan, Seralathan Kamala-Kannan, Vellingiri Balachandar, Perumalsamy Lakshmanaperumalsamy, Jong-Chan Chae, Byung-Taek Oh, 2010).

8.1.3 Sterilization

Due to consistent issues with contamination, our team would recommend finding a proper sterilization method for our device. Since 3D printed materials are porous, it is difficult to ensure isopropanol is completely removed from the device, which can be toxic to cells. 3D printed designs cannot be autoclaved as well, which is a challenge for using such devices for cell culture. For a more sustainable option, stainless steel is recommended and can help improve the sterilization process. Stainless steel has greater potential for proper sterilization, since it can be autoclaved and is not porous. We further suggest that once a proper sterilization method is found, that future researchers ensure cell viability, with a Live Dead Assay, and adhesion to the scaffold. Additionally, this staining method could verify that just fibroblasts are present on the plant scaffold. Some of these methods can be found in *Appendix F.1*.

8.1.4 Perfusion - A Further Analysis

Given the complex structure and microscopic size of a spinach leaf, a more in-depth analysis of fluid flow through the spinach leaf's vasculature and perfusion can be drawn. This analysis was outside the scope of this project, but is recommended for future research. In future work, teams can delve more into the spinach leaf's complex branched structure, treating each path as an electronic resistor. It is additionally encouraged that future researchers consider distensibility and diffusion through the stem's walls in this analysis.

8.1.5 Heat Exchanger

The team additionally suggests that the device be enhanced using a counterflow heat exchanger. The purpose of this heat exchanger would be to heat the media before entering the plant scaffold. This way, the team no longer has to heat the media before use. Appendix F.2 shows the calculations and description of how this heat exchanger would be applied.

8.2 Conclusions

The current leather making process poses as a detriment to society. Therefore, our team's solution was to develop a device capable of growing a sheet of bovine dermal fibroblasts. This sheet could be further used and be transformed into a leather product.

In order to accomplish this feat, the team successfully decellularized spinach leaves to use as plant scaffolds. Plant scaffolds have specific mechanical properties that needed to be considered as we attempted to grow cells. We completed various uniaxial tensile tests with rice paper, decellularized spinach leaves, and leather as proof of execution. We performed two at-home tests and three tests using an Instron 5544. During these tests, the target tangent modulus was equal to the value for decellularized spinach leaves at approximately 0.3 MPa (Gershlak et al., 2017). The at home test results showed similar tear force with the spinach leaves and the rice paper, both being at around 0.08 N, but the Instron tests showed rice paper to have a modulus about 20 times smaller than spinach scaffolds - being around 0.015 MPa instead of 0.3 MPa. The tests on the decellularized spinach with the Instron varied as well, with an average modulus of 0.446 MPa rather than 0.3 MPa. Varying values may be a result of biological variability. The last Instron test performed was on deerskin leather. This was purchased from a

local craft store and a 23.8125 cm x 8.89 cm sheet was cut into 9 relatively equal samples. The average calculated modulus was 38.692 MPa.

In order to gain a better understanding of the dispersal of media through the spinach leaf, a perfusion model was established. The design team modeled fluid flow through the main vein of the spinach leaf as a thin walled pipe. It was assumed that the fluid through the spinach leaf was water. Additional considerations were to neglect distensibility and diffusion. Using COMSOL Multiphysics and values measured from our device, important characteristics of perfusion were determined. The model abides by the no-slip condition and possesses an inlet velocity and maximum allowable pressure of 0.1741 meters per second and 7,116 Pascals respectively. These values were further modeled as graphs in COMSOL Multiphysics as flow through a thin walled pipe. The maximum velocity was approximately 0.55m/s given velocity's relationship to pressure through Bernoulli's equation. The pressure drop was also represented to decrease from approximately 0.2 psi to 0 psi across the spinach leaf. It is important to note that with the complex structure and small size of the spinach leaf, a more in-depth analysis of fluid flow can be drawn.

Our design needs to be further explored and used in cell culture experiments before the team can make conclusions regarding cell adhesion and proliferation. The main advantages of our device was the achievement of some of our initial objectives. The design was easy to use and allows for the possibility of scale-up manufacturing. However, before we could carry out assays to determine cell growth and proliferation (i.e. a live-dead or cyquant assay), our cells were contaminated. Further research into sterilizing our device and cellular experimentation are needed to further enhance and progress the leather making industry.

Our results suggest that our device maintains the necessary conditions for adhesion and proliferation of bovine dermal fibroblast cells. In the future, our device can be tested for longer time periods using bovine dermal fibroblasts on a decellularized scaffold, and can theoretically be used to construct a piece of dermis on a decellularized leaf. Ultimately, we envision use of this system to reduce the cost and time associated with making leather. If our design were to be scaled up, the associated costs would increase as well due to the potential use of bioreactors. While our team aimed to reduce cost, we would need a full cost analysis on a scaled up model to confirm that we addressed this objective. Future testing is needed to verify that we met our objectives.

With the proper materials and training, lab-grown leather production becomes much more attainable. With these processes, we can significantly reduce waste from the current processes. The leather industry could be revolutionized but must go hand-in-hand with the meat industry. Leather production could now begin in the lab and may end in your local stores. Are you and your wallet ready to meet the future?

References

Ambady, S. “Cell Culture Procedure and Protocols.” A Manual for Biomedical Engineering MQP Module, Nov. 2010.

American Society for Quality. (2021). What Are Stakeholders? Retrieved from https://asq.org/quality-resources/stakeholders#Analysis

Best Leather. (2014). Leather Tanning: The Tanning Process Explained. Retrieved October 16, 2020, from http://www.all-about-leather.co.uk/what-is-leather/how-is-leather-made.htm

Bellco. (2020). Tissue Culture Flasks. Retrieved December, 2020.

BioEnergy Consult. (2020, June 13). Wastes Generation in Tanneries. Retrieved from https://www.bioenergyconsult.com/waste-from-tanneries/

Bolt Threads. (2021). Mylo. Retrieved from https://boltthreads.com/technology/mylo/

Brizga, J. et al. (2020). The Unintended Side Effects of Bioplastics: Carbon, Land, and Water Footprints. *One Earth* **3** 45-53. doi: 10.1016/j.oneear.2020.06.016.

Byun, C.K., Abi-Samra, K. Cho, Y.K., & Takayama, S. (2013). Pumps for microfluidic cell culture. Electrophoresis 35(2-3) 245-257. doi: 10.1002/elps.201300205

Covington Anthony, D. Tanning chemistry: The science of leather. Royal Society of Chemistry. 2009.

Dixit, S. et al, (2015). Toxic hazards of the leather industry and technologies to combat threat: A review. *Journal of Cleaner Production* **87** 39-49. doi: 10.1016/j.jclepro.2014.10.017.

Elsheikh M. A. (2009). Tannery wastewater pre-treatment. Water science and technology : a journal of the International Association on Water Pollution Research, 60(2), 433–440. https://doi.org/10.2166/wst.2009.351

Galen Leather (2019, March 31). *What is Vegetable Tanned Leather and is it Superior in Quality?* Galen Leather Company. https://www.galenleather.com/blogs/news/vegetable-tanned-leather

Giardini, E. (2018, September 14). Leather Dyeing Techniques: Tanneries Vs. Leather Craftsmen. Retrieved from https://blog.leatheredgepaint.com/leather-dyeing-techniques-tanneries-vs.-leather-craftsmen

Global Leather Goods Market: Growth: Trends: Forecast (2020 - 2025). Home. https://www.mordorintelligence.com/industry-reports/leather-goods-market.

Grainger. (n.d.). Polystyrene Sterile Petri Dish; PK500. Retrieved December, 2020.

J.R. Gershlak, S. Hernandez, G. Fontana, L. R. Perreault, K. J. Hansen, S. A. Larson, B. Y. Binder, D. M. Dolivo, T. Yang, T. Dominko, M. W. Rolle, P. J. Weathers, F. Medina-Bolivar, C. L. Cramer, W. L. Murphy, and G. R. Gaudette, "Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds," Biomaterials, vol. 125, pp. 13–22, 2017. https://www.sciencedirect.com/science/article/pii/S0142961217300856

Jones, M. et. al. (2021) Leather-like material biofabrication using fungi. Nature Sustainability 4 9-16. doi:10.1038/s41893-020-00606-1

Kanth, S. V. et. al. (2009) Cleaner tanning practices for tannery pollution abatement: Role of enzymes in eco-friendly vegetable tanning. *Journal of Cleaner Production* **17**(5): 507-515. doi: 10.1016/j.jclepro.2008.08.021

Lacombe, J., Harris, A. F., Zenhausern, R., Karsunsky, S., & Zenhausern, F. (2020). Plant-Based Scaffolds Modify Cellular Response to Drug and Radiation Exposure Compared to Standard Cell Culture Models. Frontiers in Bioengineering and Biotechnology, 8. doi:10.3389/fbioe.2020.00932

Lanxess (n.d.). Sustainability & innovations. Retrieved February 25, 2021, from https://leather.lanxess.com/sustainability-innovations/

Leather Goods Market Size & Share: Global Industry Report, 2019-2025. Leather Goods Market Size & Share | Global Industry Report, 2019-2025. (2019, February). https://www.grandviewresearch.com/industry-analysis/leather-goods-market.

Leather Resource, LLC. (2008). What is Leather? Retrieved from http://www.leatherresource.com/whatisleather.html

Li, Y., Guo, R., Lu, W., Zhu, D. Research progress on resource utilization of leather solid waste. J Leather Sci Eng 1, 6 (2019). https://doi.org/10.1186/s42825-019-0008-6

Liu, Cheng-Kung & Latona, Nicholas & Taylor, & Eble, & Ramos, M. (2015). Characterization of mechanical properties of leather with airborne ultrasonics. J. Am. Leather Chemists Assoc. 110(3):88-93. 2015. Liu, C.-K., Latona, N. P., and Taylor, M., Eble, C., and Ramos, M. L. Journal- American Leather Chemists Association. 110. 88-93.

Magic, D. (2018). Modern Meadow: Using Additive Manufacturing to Reimagine Fashion and

Food. Retrieved September 07, 2020, from https://digital.hbs.edu/platform-rctom/submission/modern-meadow-using-additive-manufacturing-to-reimagine-fashion-and-food/

Magnan, L. et al, (2020). Human textiles: A cell-synthesized yarn as a truly “bio” material for tissue engineering applications. Acta Biomaterialia 105 111-120. DOI: 10.1016/j.actbio.2020.01.037.

Material Innovation Initiative. (2020, September). Reports. Retrieved from https://www.materialinnovation.org/reports

Moore & Giles. (2020). How Leather is Made. Retrieved from https://www.mooreandgiles.com/leather/resources/how-leather-is-made/

Ninan, N. et al, (2015). Natural Polymer/Inorganic Material Based Hybrids Scaffolds for Skin Wound Healing. Polymer Reviews 55(3) 453-490.

Palanivel Velmurugan, Seralathan Kamala-Kannan, Vellingiri Balachandar, Perumalsamy Lakshmanaperumalsamy, Jong-Chan Chae, Byung-Taek Oh, (2010), Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather, Carbohydrate Polymers, Volume 79, Issue 2, Pages 262-268, ISSN 0144-8617, https://doi.org/10.1016/j.carbpol.2009.07.058. (https://www.sciencedirect.com/science/article/pii/S0144861709004202)

Premier Dermatology. (2017, September 09). Skin Fun Facts. Retrieved from https://pdskin.com/blogs/skin-fun-facts/#:~:text=The average person has about,up to 300 sweat glands

Pundir, Ankit Singh. (2017, May). U.S. Bovine Leather Goods Market by Type - 2023: AMR. Allied Market Research. https://www.alliedmarketresearch.com/us-bovine-leather-goods-market.

Saba, I., Jakubowska, W., Chabaud, S., & Bolduc, S. (2016). New and Improved Tissue Engineering Techniques: Production of Exogenous Material-Free Stroma by the Self-Assembly Technique. Composition and Function of the Extracellular Matrix in the Human Body. doi:10.5772/62588

Sciencell Research Laboratories. (2020). Bovine Dermal Fibroblasts. Retrieved from https://www.sciencellonline.com/bovine-dermal-fibroblasts.html

Sculpteo. (n.d.). Understand Units and Sizes for your 3D printing Dimensions. Retrieved March 17, 2021 from https://www.sculpteo.com/en/3d-learning-hub/design-guidelines/3d-printing-dimensions/

Shanker, A. et al. (2005). Chromium toxicity in plants. Environmental International 31(5) 739-753. doi:10.1016/j.envint.2005.02.003

Shekhawat, K., Chattergee, S., & Joshi, B. (2015). Chromium Toxicity and its Health Hazards. International Journal of Advances Research 3(7) 167-172.

Song, R. et al. (2018). Current development of bioegradable polymeric materials for biomedical applications. *Drug Design, Development and Therapy* **12** 3117-3145. doi: 10.2147/DDDT.S165440

Tarantola, A. (2014, June 3). *How Leather Is Slowly Killing the People and Places That Make It*.

Gizmodo. https://gizmodo.com/how-leather-is-slowly-killing-the-people-and-places-tha-1572678618.

Ude, C.C., Miskon, A., Idrus, R.B.H., Bakar, M.B.A. Application of stem cells in tissue engineering for defense medicine. Military Med Res 5, 7 (2018). https://doi.org/10.1186/s40779-018-0154-9

University of Nebraska Medical Center. (n.d.). What are Stem Cells?: Stem Cells: University of Nebraska Medical Center. Retrieved 2021, from https://www.unmc.edu/stemcells/stemcells/

Valke, D. (2005). Fiscus Hispida. Retrieved from http://www.flowersofindia.net/catalog/slides/Hairy Fig.html

Velmurugan, Palanivel., Shim, Jaehong., Seo, Sang Ki., Oh, Byung-Taek. (2016), Extraction of natural dye from Coreopsis tinctoria flower petals for leather dyeing − An eco-friendly approach. Fibers Polym 17, 1875–1883 (2016). https://doi.org/10.1007/s12221-016-6226-0

W-Fowler. (2007). How is leather made? Retrieved October 16, 2020, from http://www.all-about-leather.co.uk/what-is-leather/how-is-leather-made.htm

Wells, H.C., Holmes, G. and Haverkamp, R.G. (2016), Looseness in bovine leather: microstructural characterization. J. Sci. Food Agric., 96: 2731-2736. https://doi.org/10.1002/jsfa.7392

Wicker, A. (2019, January 31). Is Leather Truly a Byproduct of the Meat Industry? Retrieved from https://ecocult.com/is-leather-truly-a-byproduct-of-the-meat-industry/

Appendices

Appendix A: Additional Background Information

Appendix A.1 Recycling and Rendering of Leather Making Byproducts

Recycling and rendering can also be extremely useful in reducing leather waste. 27.5 million hides were used in domestic and global leather production and 5.5 million got discarded into landfills. These hides could be used to make 99 million pairs of shoes, 110 million footballs, and 2 million sofas (Real Leather is Greener than Imitations, 2020). Rendering involves repurposing raw animal materials into something useful. 56 billion pounds of raw materials such as bone and fat from the US and Canada can be used in pet food, animal feed, cosmetics as well as reusing retinol, gelatin, and collagen found in animal products. Rendering's contribution to carbon emission reduction in the US and Canada is equal to removing 12 million cars annually from the road. Rendering converts 99% of meat and meat production by-products safely into ingredients for animal feed, pet food, biofuel, fertilizer, industrial and consumer products. All U.S. landfills would be full within four years without rendering, posing serious public health and environmental problems. Without rendering, approximately 50% of the animal parts considered inedible by Americans would be wasted. Renderers in the U.S. and Canada invest more than $500,000 annually in research by the Fats and Proteins Research Foundation to seek solutions to challenges such as odor control. Rendered fats and oils account for 30% of the feedstock used in biodiesel and renewable diesel production in the U.S. The rendering industry recycles more than 2.3 billion pounds of meat and poultry from retail food waste which is used for animal food

ingredients and 2.4 billion pounds of used cooking oils from foodservice operations, much of which is used for biodiesel production (Tribe, 2020). These processes will greatly reduce net waste production as well as the environmental impact. In addition, shifting to synthetics will reduce the waste dramatically.

Appendix A.2 Pleather Background

Pleather is a commonly used leather substitute. Leather created in a lab uses plastic. Most pleather is made using mostly polyvinyl chloride (PVC) or polyurethane (PU) (Christian, 2016). While the idea of pleather sounds appealing, as it would minimize harm to animals in using synthetic polymers over animal skin, it still has a largely negative impact on the environment. Specifically, in PVC, production of pleather releases dioxins, hazardous chemicals, that can get into water supply through air deposition, chemical factory waste, and air emissions near lakes and reservoirs (Christian, 2016). They can cause people cancer, developmental problems, damage the immune system, and more. Dioxins also break down extremely slowly, meaning they can last in the water supply for a long time (EPA, 2020). On the other hand, while PU doesn't produce this problem, it can get much more expensive due to the extensive manufacturing processes needed to prepare it (Christian, 2016). However, it is much higher quality than PVC pleather. Overall, though, synthetic leather doesn't usually last as long as real leather and as it wears, it doesn't look as aesthetically pleasing. Also, synthetic leather can't really be recycled without more extensive processing, so it just ends up creating waste.

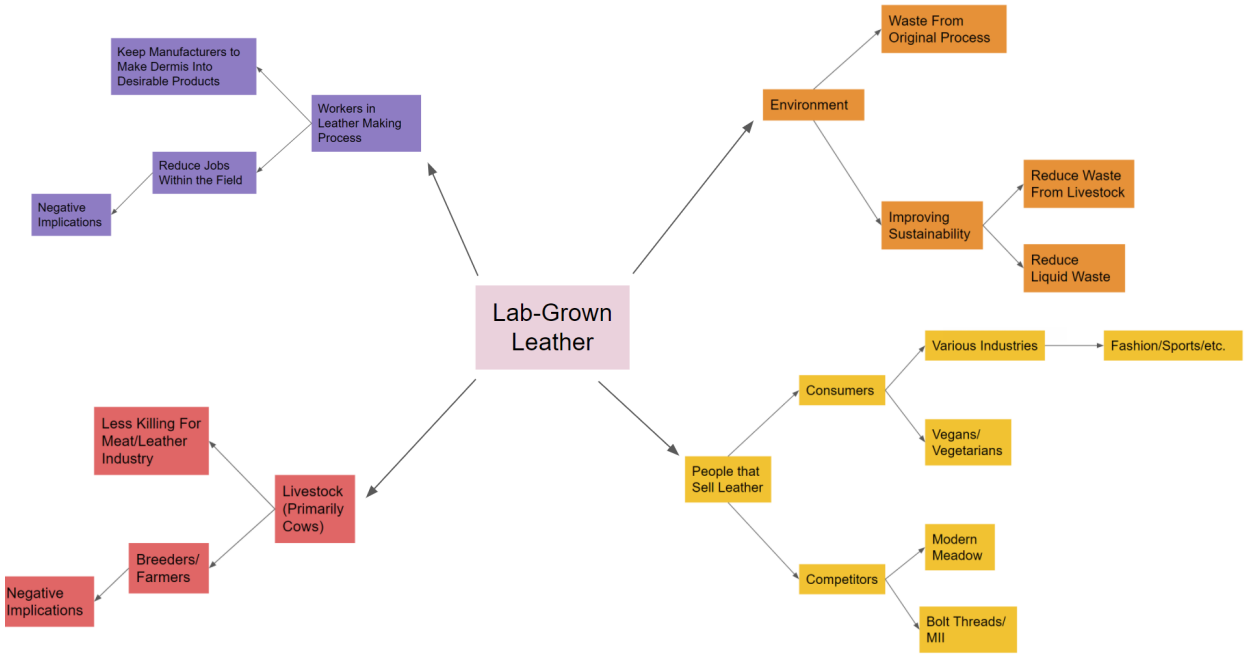
In simple terms, pleather is made by obtaining a base material such as cotton or polyester, formulating the plastic to bind the base fabric, binding the materials using PU or PVC, and cutting and preparing the fabric into the desired shape and size. The main applications are in

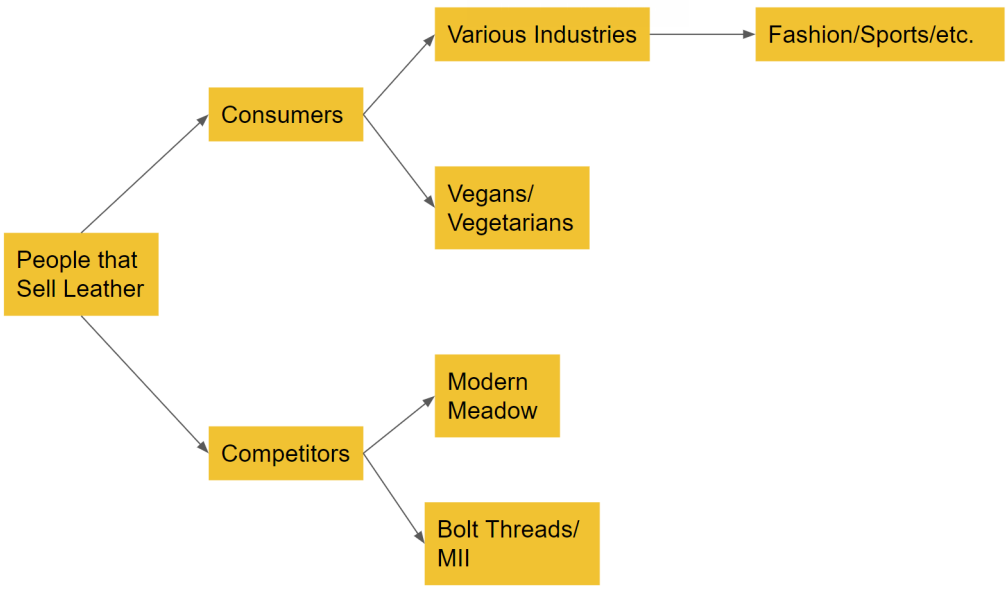
upholstery, apparel, and accessories such as watches, shoes, keychains, and many others. It is just as versatile and is less expensive and lighter compared to traditional leather. Pleather is also more ethical because it eliminates animal cruelty. There are ethical concerns when we explore the labor force. China is one of the main producers of pleather and the worker's rights are severely lacking when compared to the United States which raises some issues (Hodakel, 2020).

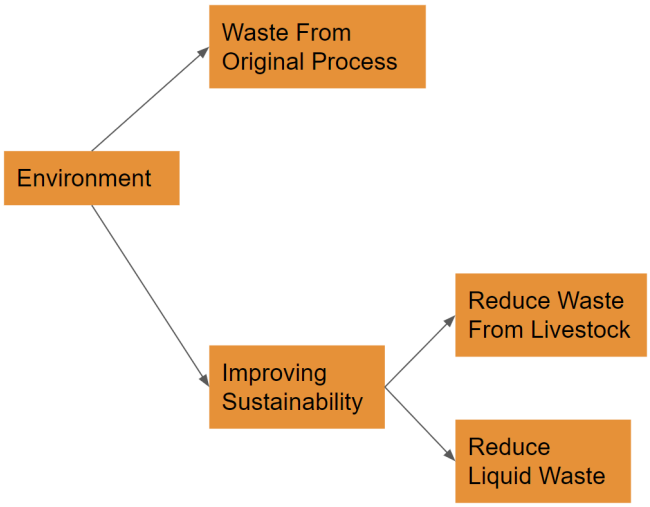
The binding material of the synthetic leather affects the properties of the pleather. For example, if PU is used, it is washable and allows for garment air flow. PVC pleather does not breathe and is difficult to clean because common cleaning solvents can make the material stiff. (Ujevic, 2009). During pleather production, fossil fuel derivatives such as ethylene are created. This introduction of toxic substances into the surroundings negatively impacts the environment. The plastic that is used in the pleather making does not allow for fast decomposition. There is some waste produced as well. Synthetic leather is not biodegradable, so it remains in the environment when discarded. For reference, it takes the materials approximately 200 to 500 years to degrade.

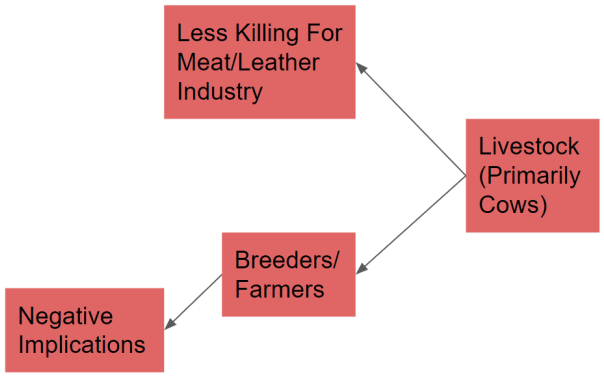
Appendix B: Project Strategy Supplemental Materials

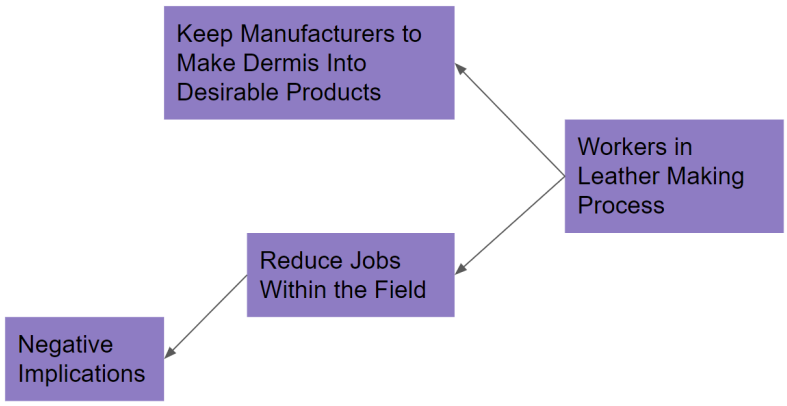
Appendix B.1 Stakeholder Map of Lab-Grown Leather Industry






Waste From Original Process
Environment
Improving Sustainability
Reduce Waste From Livestock
Reduce Liquid Waste


Less Killing For Meat/Leather Industry
Livestock (Primarily Cows)
Breeders/ Farmers
Negative Implications



Appendix B.2 Five Stage Prescriptive Model

Problem Definition

Customer and Need, Objectives

1. Clarify objectives
    a. Develop a new process for the creation of leather for a keychain that will reduce the usage of animal products in the current process
        i. Features & Behaviors we want the design to have:
            1. Reduce animal usage in producing leather
                a. Use tissue engineering to produce bovine dermis
                    i. Need cells to produce collagen
                    ii. Want the thickness of our final product to be approximately 2-3oz

1. Unsure how the tanning process will affect thickness. Final product of lab grown dermis may need to be thicker to accommodate this

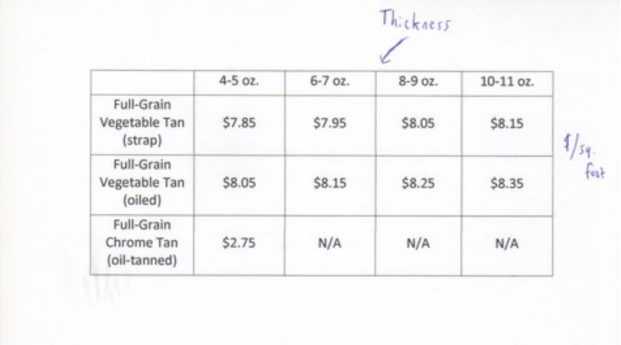
2. Reduce solid waste from process
    a. Function- eliminate/reduction of hair, epidermis etc.
        i. How much less do we want to produce? How much is feasible? - I think a good starting point could be producing only 10% of the amount of skin waste as the original process could be feasible
    b. Eliminate scar tissue, (only use the skin/ target area/area of interest)
        i. See above, again I believe 10% is a good starting point
        ii. 70% of untreated hide goes in the trash
3. Reduced liquid waste from process
    a. Water waste
    b. Cr iv (in tanning?) (I believe it is in a liquid?)
        i. “tanning one ton of hide typically results in 20 to 80 cubic meters of wastewater with Chromium concentrations around 250 mg/L and sulfide concentrations at roughly 500 mg/L” - to make this statistic more visual, find out how many products

can be made from 1 ton of hide and what a cubic meter of water looks like

ii. Looking into alternatives for chromium

4. Scalable
    a. Using spinach leaves may not allow the ability to create a larger cross-section than the size of the spinach leaf itself but looking into other different kinds of leaves could offer the ability to use ones with larger cross-sections
        i. Pineapple, Amazonian trees, etc.
5. User-friendly; no professional experience required, HS education level understanding
6. Reproducible
    a. Accurate and precise
        i. Will we be able to press a button to change the thickness (probably not – this could possibly be accomplished through layering pieces of dermis that are grown
7. More ethical
    a. “Ethical” is hard to describe currently
8. Cost effective

a. Current margins to create leather:



i. (Liberty, n.d)

b. Most companies use leather that costs ~$2.75/sqft to keep their large margins (same source as image)

9. Timely fashion

ii. Who is our customer?

1. People who aim to lessen their environmental impact
    a. Vegans/Vegetarians
    b. Environmentally Conscious
2. Companies that sell leather products
    a. Fashion/accessory industry
    b. Cars
    c. Instrument cases
    d. Sports (baseballs/footballs/basketballs/accessories)

2. Establish metrics for objectives
    a. Get into lab/start cell culture week of 11/16
    b. The specs of our final product at the end of cell growth:
    c. 2 in by 1 in cross-section with a thickness of approximately 3-4 mm

3. Identify constraints
    a. The overall process of getting pelts to use for creating leather is tied heavily to the meat industry. There isn't really any way with this project to minimize any negative environmental impact within livestock farming due to this
    b. If these happen, we fail
        i. Budget limit
            1. Materials
        ii. Time limit
            1. Lab accessibility
                a. Sterile Environment
            2. Project only spans for a year
4. Revise client statement
    a. The goal of this project is to develop a process for growing, tanning, and dyeing bovine skin to produce leather such that the methods of the process make significantly less of a negative environmental impact compared to the current leather-producing process.
    b. Revision: The goal of this project is to develop a process that will produce bovine dermis that can be tanned and dyed to create leather. This process will also hopefully produce significantly less waste and have less of a negative environmental impact than the current leather producing process.

Conceptual Design

5. Establish functions
    a. Produce lab grown leather

        i. **Produce bovine dermis and ~~collagen~~**

    b. Develop an environmentally conscious process for tanning and dyeing the bovine skin we grow

        i. If time allots, attempt to execute this process

6. Establish requirements (specifications)
    a. 2 in x 1 in minimum sheet for 1 keychain
    b. Find out sizes of incubators and cell culture plates – can they accommodate what we need them to?
    c. Thickness needed: See 8c
7. Establish means for functions
8. Generate design alternatives
    a. 6 in x 6 in minimum sheet for 6 keychains (too expensive to do, just an idealization)
    b. Producing multiple thin sheets of cells to layer in order to create the thickness desired
    c. **Thickness**
        i. Thickness needed: Best around 2-3 oz
        ii. O2 diffusion & nutrients (2000 microns) (constraint or function)
        iii. How far will perfusion go, leaf on multiple sides?
9. Refine and apply metrics to alternatives
    a. Verify that we need the number of materials we are using (spinach leaves, fibroblasts, fetal bovine serum, medium)
10. Choose a design

Preliminary Design

1. Model and analyze chosen design
    a. Decellularize spinach leaves to create a scaffold with vasculature
    b. Seed bovine fibroblasts onto decellularized spinach leaf (see process doc for more detailed info)
    c. Allow fibroblasts to proliferate and multiply
    d. Remove piece of skin from the scaffold
    e. Put the skin through a revised tanning and dyeing process (dependent on budget left and quality of skin created)
    f. With the remaining, create small keychains as shown in *image*
2. Test and evaluate chosen design
    a. Perform mechanical tests on some of the pieces of skin grown
    b. Perform mechanical testing on a couple of the spinach leaves we aren't planning on using for cell growth
        i. Using ASTM Standard test, using instron
3. Pt 2 Preliminary Design
    D. Dual scaffold – using a scaffold that can produce one cell on one side and another type on the right
    E. Thickness needed
        - i.e. 400 micron thick layer in x millimeters of thickness
        - Spinach leaf – do we want to use decellularized plants, add into constraints and functions
            - Perfuse the leaf to see how far the perfusion will go in.

- Oxygen diffusion
- What exactly do we need from the cells
    - How much collagen
    - How fast
- What factors do we need to deliver to produce more collagen

Appendix B.3 Budget

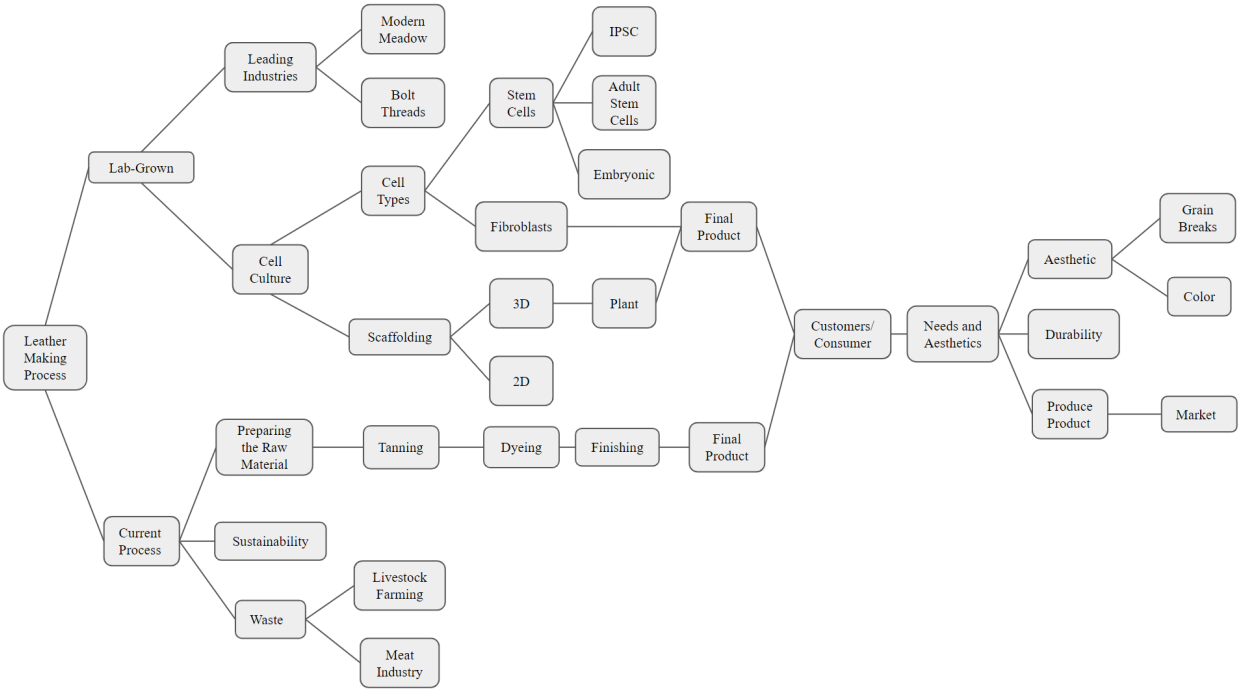
Cell Seeding and Decellularization Materials				
Item	**Cost**	**Quantity**	**Total Cost**	**Comments**
Spinach Leaves	$2.99	1	$3	1 lb - Purchased from Price Chopper
Cabbage Head	$2.34	1	$2	Purchased from Price Chopper
Fibroblasts		1	$0	
Ascorbic Acid	$66.40	1	$66	A4403-100MG
Lab Space Surcharge	$50.00	5	$250	Charged per student
Total Cost:			**$322**	

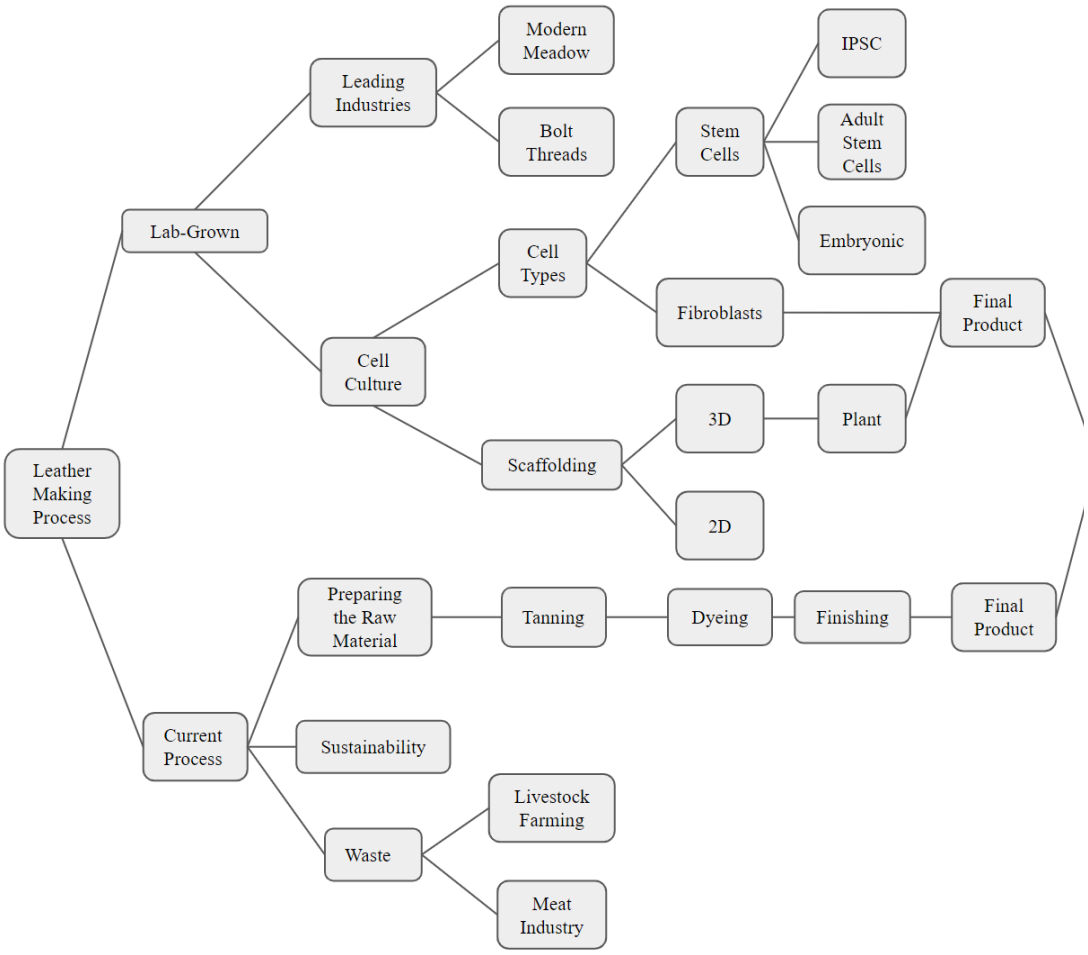
Mechanical Testing Materials				
Item	**Cost**	**Quantity**	**Total Cost**	**Comments**
Rice Paper	$5.95	1	$6	12 ounce pack of 22 cm round
Total Cost:			**$6**	

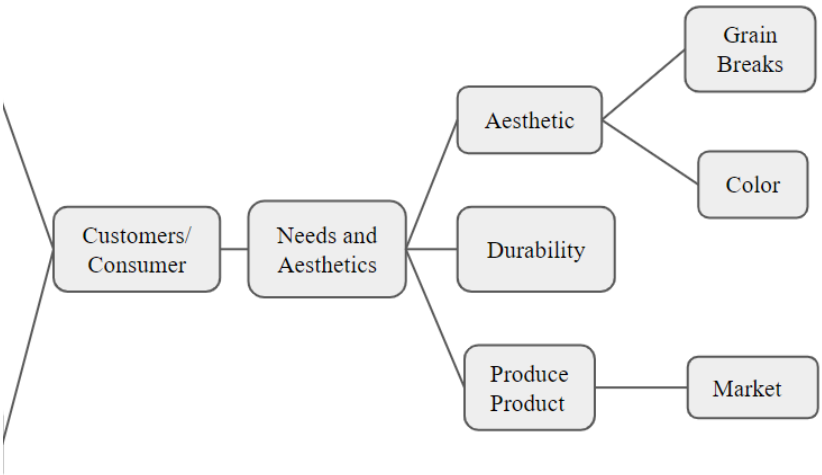
Device Materials (Including Arduino)				
Item	**Cost**	**Quantity**	**Total Cost**	**Comments**
3D Printing Materials	$50.00	1	$50	
Pressure Sensor	$16.00	1	$16	
pH Sensor	$20.00	1	$20	
Peristaltic Pump	$16.50	1	$17	
9V Battery	$11.99	1	$12	
9V Battery Connector	$5.99	1	$6	
Total Cost:			**$120**	

Overall Budget	
Cell Seeding and Decellularization Materials	$322
Mechanical Testing Materials	$6
Device Fabrication Materials	$120
Total Cost	**$448**

Appendix B.4 Project Flow Chart




Leather Making Process
Lab-Grown
Leading Industries
Modern Meadow
Bolt Threads
Cell Culture
Cell Types
Stem Cells
IPSC
Adult Stem Cells
Embryonic
Fibroblasts
Final Product
Scaffolding
3D
Plant
2D
Current Process
Preparing the Raw Material
Tanning
Dyeing
Finishing
Final Product
Sustainability
Waste
Livestock Farming
Meat Industry


Customers/ Consumer
Needs and Aesthetics
Aesthetic
Grain Breaks
Color
Durability
Produce Product
Market

Appendix C: Design Process

Appendix C.1 Calculations for Percent Waste Reduction

~ a cow hide typically weighs 55 lbs ≈ 25 kg. This weight is for a cow hide that possesses an area of 50-55 sq ft.

- according to Cortina Leathers, a 50-55 sq ft hide can use 6 panhels (2 ft x 2 ft) to make leather

50-55 sq ft
(2 ft x 2 ft) = 4 sq ft
4 sq ft (6 panhels) = 24 sq ft
50 - 24 sq ft = 26 sq ft wasted

$\frac{26}{50} = 0.52$ $\frac{24}{50} = 0.48$

Thus, 52% of material is wasted - 13 kg wasted
48% of material is used - 12 kg used

~ our project used a 6" by 8" sheet of cow skin

$A = 6" \times 8" = 48 in^2 = 0.333$ sq ft

mass of our piece: $\frac{x \text{ kg}}{25 \text{ kg}} = \frac{0.333 \text{ sq ft}}{52.5 \text{ sq ft}}$

$x = \frac{0.333 (25 \text{ kg})}{52.5}$

$x = 0.1587$ kg

- comparison: our project = 0.333 sq ft / 0.1587 kg
cow hide = 25 kg / 50-55 sq ft

Thus, we need to scale one process to compare

~ scale factor: $25 \text{ kg} = f(0.1587 \text{ kg})$
$f = 157.5$

~ our use of the 6" by 8"
- circular cut-outs of approximately 0.24 inches in diameter
~ in a 48 in^2 rectangle with 0.1 in spacing
- this gives 590 circles

$A_{circle} = \pi (0.24/2)^2 = 0.045 in^2$
$A_{all\ circles} = 590 [\pi \cdot (0.24/2)^2] = 24.43 in^2$

$A_{wasted} = 48 in^2 - (24.43 in^2) = 23.57 in^2$
$= 0.164$ sq ft

mass of A wasted: $\frac{x\ kg}{25 kg} = \frac{0.164\ sq\ ft}{52.5\ sq\ ft}$

$$x = \frac{0.164(25 kg)}{52.5}$$

$$x = 0.0782\ kg$$

scaled: 0.0782 kg (157.5) = 12.32 kg wasted

~ for our experiment - approximately 30 petri dishes were used
- 1 petri dish ≈ 14-15 g = 0.015 kg

30(0.015 kg) = 0.45 kg

Total solid waste = 12.32 kg + 0.45 kg = **12.77 kg**

Waste Reduction %: $\frac{12.77\ kg\ wasted}{13\ kg\ wasted} = 0.983$

1 - 0.983 = 0.0173 = **1.73 %**

* This only accounts for waste from a hide. It does NOT include fleshing and hair removal waste.

According to a recent study titled: Recovery and Utilization of proteinous wastes of leather making, the following is wasted.
unlanned waste: fleshing = 70 - 230 kg
raw trimming = 120 kg
for producing 200 kg of leather from a 1 ton hide (907.19 kg).

70 kg - 230 kg → Find fleshing amount proportioned to 25 kg hide

$$\frac{70 kg}{907.19 kg} = \frac{x}{25 kg} \rightarrow x = \frac{70(25)}{907.19}$$

$$x = 1.93\ kg$$

$$\frac{230 kg}{907.19 kg} = \frac{x}{25 kg} \rightarrow x = \frac{230(25)}{907.19 kg}$$

$$x = 6.34\ kg$$

Fleshing of 25 kg hide = 1.93 kg - 6.34 kg of waste

~ Liquid used in our process:

- Maintaining Cells - submerge in 10mL of media
  (every 3 days) ↳ for every petri dish
  - rinse with 10 mL of 70% ethanol to sterili

- Passaging Cells - rinse with 5 mL DPS twice
  (four total) ↳ 10 mL DPS

  - use 3mL Tripsan
  - use 2mL media

The experment went for 32 days, so cells were maintained about 11 times for approximately 15 petri dishes

waste from maintaining cells: 10 mL + 10 mL = 20 mL
20 mL × 15 samples = 300 mL
300 mL × 11 days = 3,300 mL

3,300 mL wasted

waste from passaging cells: 10 mL + 3 mL + 2 mL = 15 mL
15 mL × 4 passages = 60mL

60mL wasted

• Total liquid waste from cells: 3,300 mL + 60 mL = 3,360 mL

- Decell Process:
  ~ performed 3 batches for 21 leaves
  - for these batches, a 10X SDS solution was made
  ~ 1 L DI water used / mixed with SDS

~ single batch of leaves waste:

Days 1-2: 1x SDS mix was used
~ 900 mL DI mixed with 10X SDS solution
-replaced after 24 hours

⇒ 1800 mL wasted

Days 3-6: Bleach, Tween-20 solution
~ 960 mL DI H_2O
~ 30 mL bleach
~ 10 mL Tween-20

⇒ 1000 mL wasted

Day 7: submerged in 900 mL DI H_2O
⇒ 900 mL wasted

Day 8: Tris-Buffer Solution
~ 500 mL DI with tris-buffer powder

⇒ 500 mL wasted

Total Liquid Waste (1 Batch decell): 1800 mL + 1000 mL + 900 mL + 500 mL

= 4,200 mL

3 batches (4,200 mL) ⇒ 12,600 mL

Total Liquid Waste from Decell: 12,600 mL + 1000 mL

= 13,600 mL

⇒ Total Liquid Waste (our project):

13,600 mL + 3,360 mL = **16,960 mL**

~ According to data in Recovery and utilization of proteinous wastes of leather making: a review:

liquid waste for 200kg leather:

50 m^3 liquid effluent containing:
- other waste = 16 kg - post tanning
- 235-250 kg COD
- 100 kg BOD
- 150 kg suspended solids (SS)

} for pre-tanning

total = 516 kg

50 m^3 = 50,000 L

↳ half of above values are for pre-tanning, the other half is for after the tanning process

250 kg / 2 → 125 kg COD
100 kg / 2 → 50 kg BOD
150 kg / 2 → 75 kg SS

} 250 kg

$$\frac{250\text{ kg}}{x} = \frac{516\text{ kg}}{50{,}000\text{ L}}$$

$$x = \frac{250\,(50{,}000\text{ L})}{516} \longrightarrow x = 24{,}224.81\text{ L}$$

~ Need to scale for our project:
- leather piece that is 2in × 1in ≈ 0.15kg (150g)

200kg leather = f (0.15 kg leather)

f = 1,333.33

16,960 mL = 16.96 L

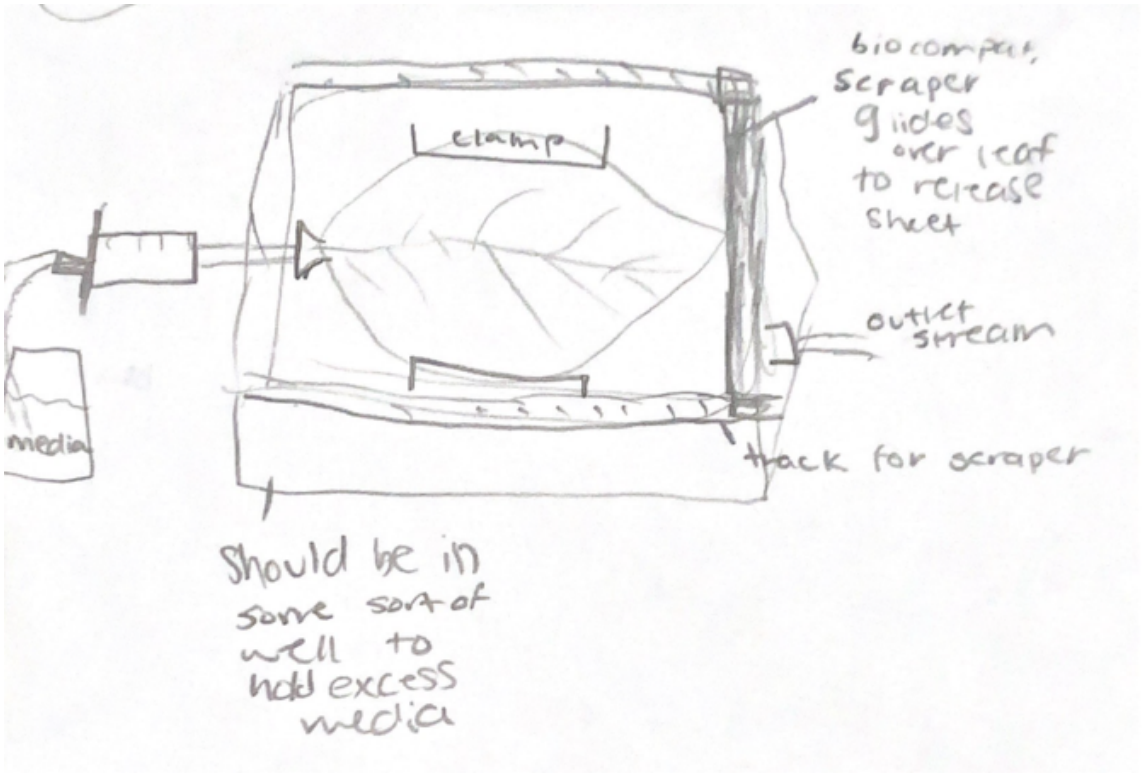
1,333.33 (16.96 L) = 22,613.33 L

$\frac{22,613.33\ L}{24,224.81\ L} = 0.933$

1 − 0.933 = 0.067 = **6.65%**

Appendix C.2 Individual Preliminary Designs

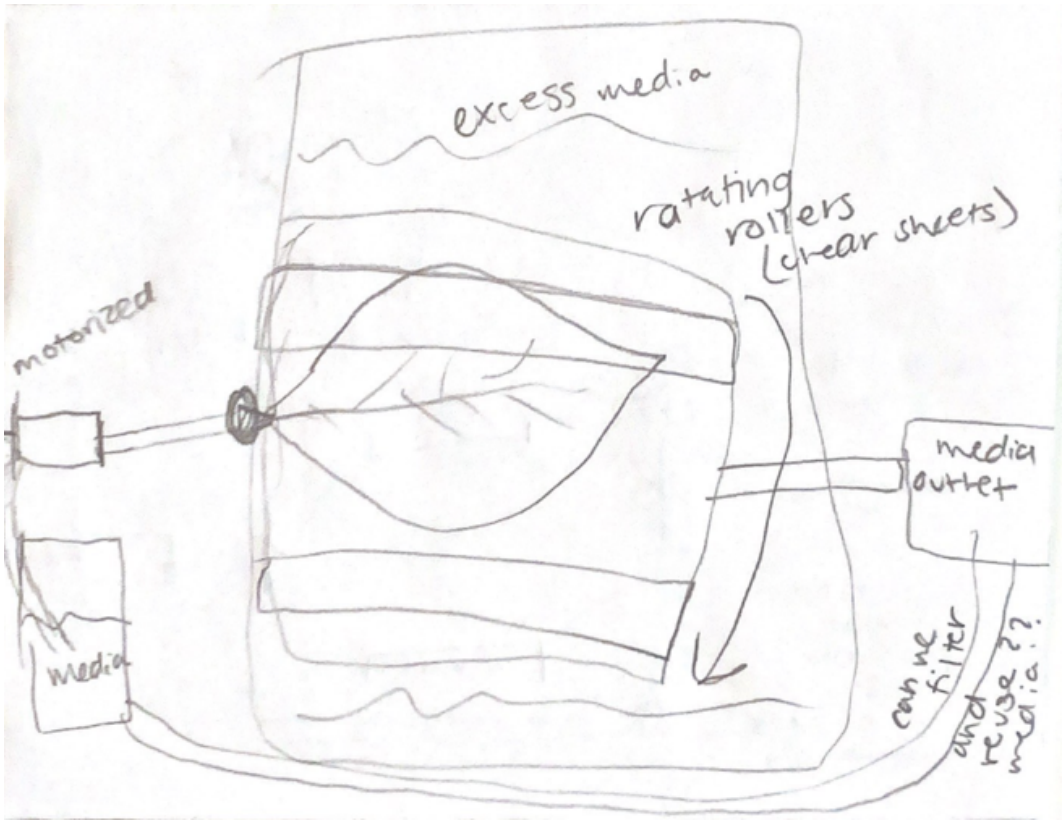
Appendix C.2.1 Alyssa's Preliminary Designs



Alyssa Preliminary Design #1

Pros: scraper idea to remove cells once they are fully grown into a sheet, the collection and reuse of excess media and preventing waste.

Cons: lack of automation, covering device

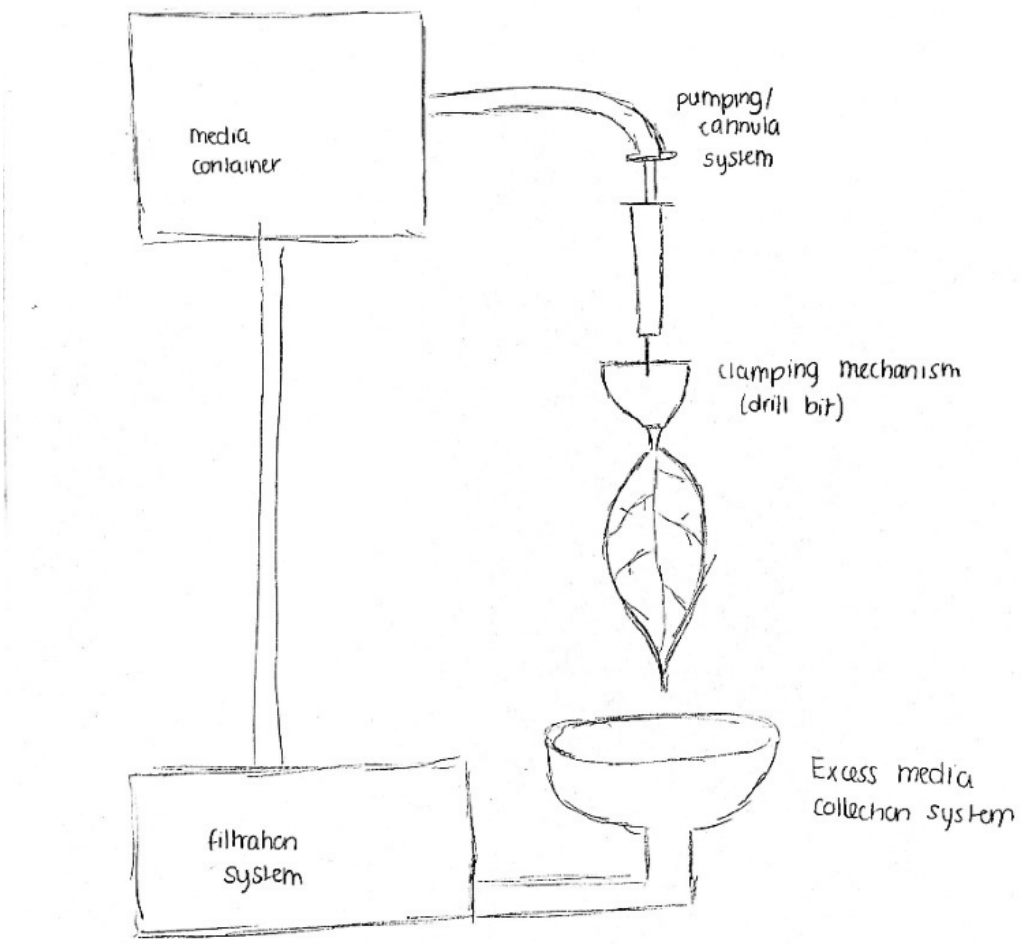


Alyssa Preliminary Design #2

Pros: refiltration of media, rollers to clear the cell sheets, the well for excess media, and automation

Cons: need clamp around the leaf, motorizing details, and seeding cells manually.

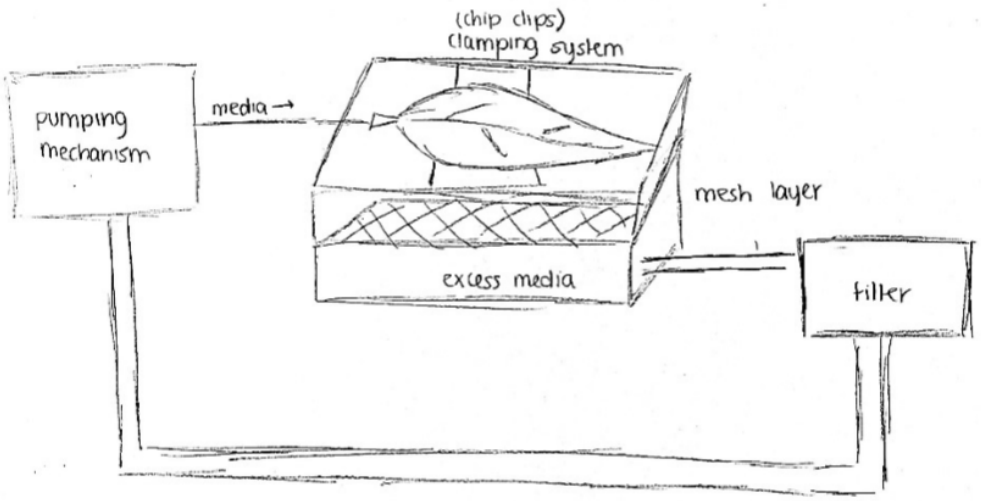
Appendix C.2.2 Samantha's Preliminary Designs



Samantha's Preliminary Design #1

Pros: filtration system, automates media into the scaffold in a closed system.

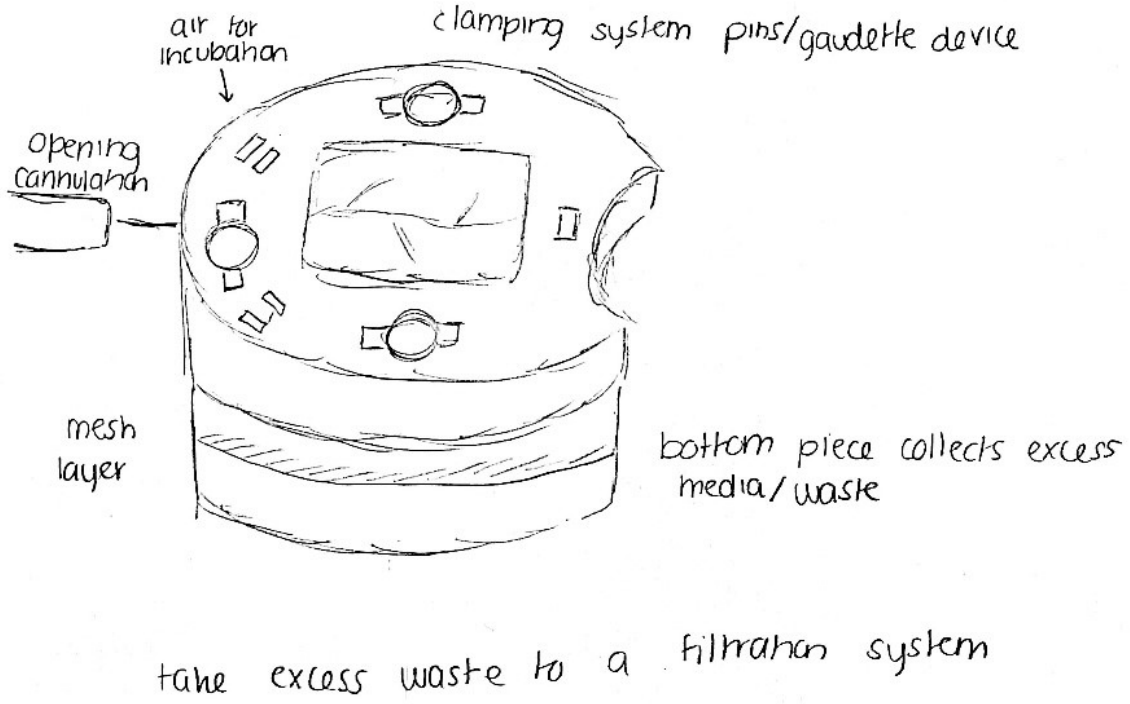
Cons: vertical cannulation, separating leaf for incubation



Samantha's Preliminary Design #2

Pros: filtration device, clamping mechanism

Cons: mesh replacement layer manual, leaf support on more than two sides

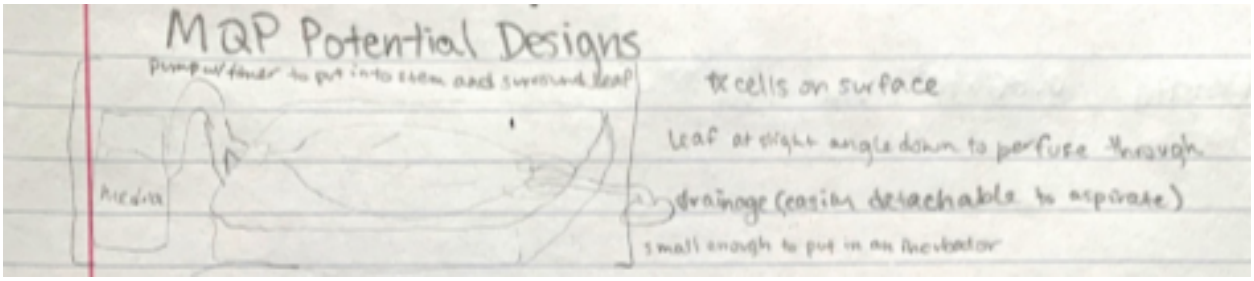


Samantha's Preliminary Design #3

Pros: works with incubators, media removal and storage

Cons: seal top component, filtration

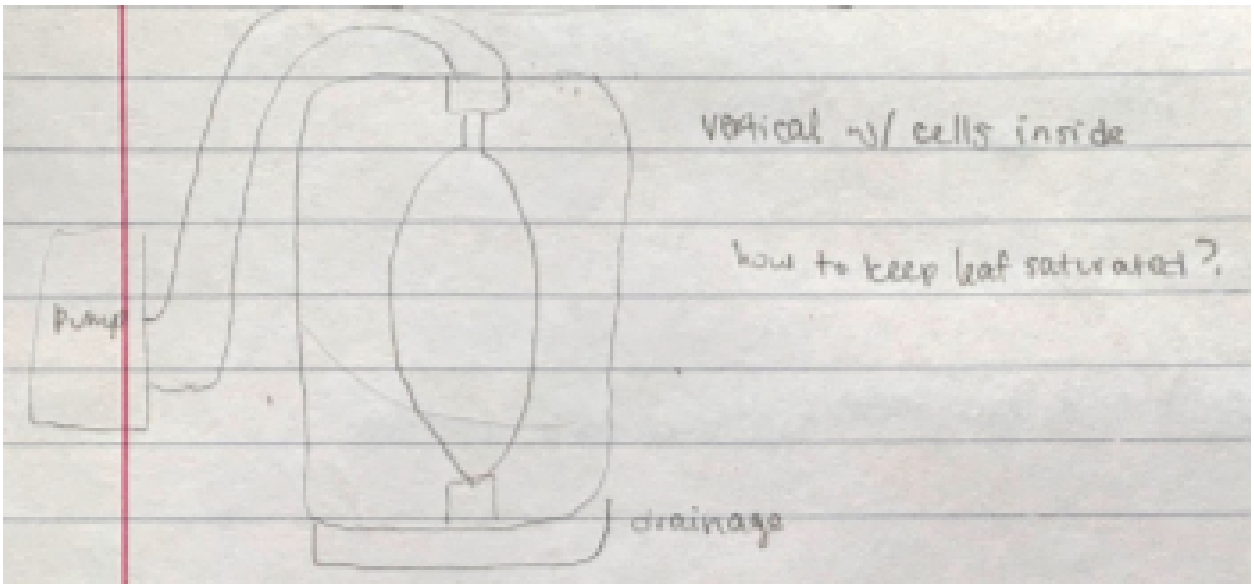
Appendix C.2.3 Leah's Preliminary Designs



Leah's Preliminary Design #1

Pros: horizontal seeding, angled perfusion, Y split for media and well plate, drainage, filter media

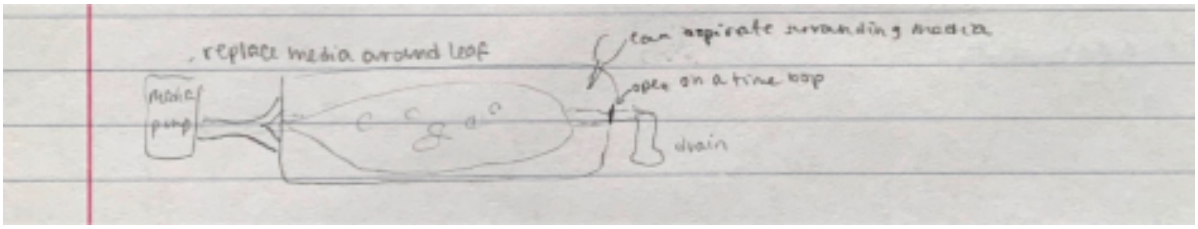
Cons: Cell dripping off (depending on angle) & not adhering, excess waste collection not included, pumping of excess liquid



Leah's Preliminary Design #2

Pros: vertical cannulation

Cons: difficult cell seeding

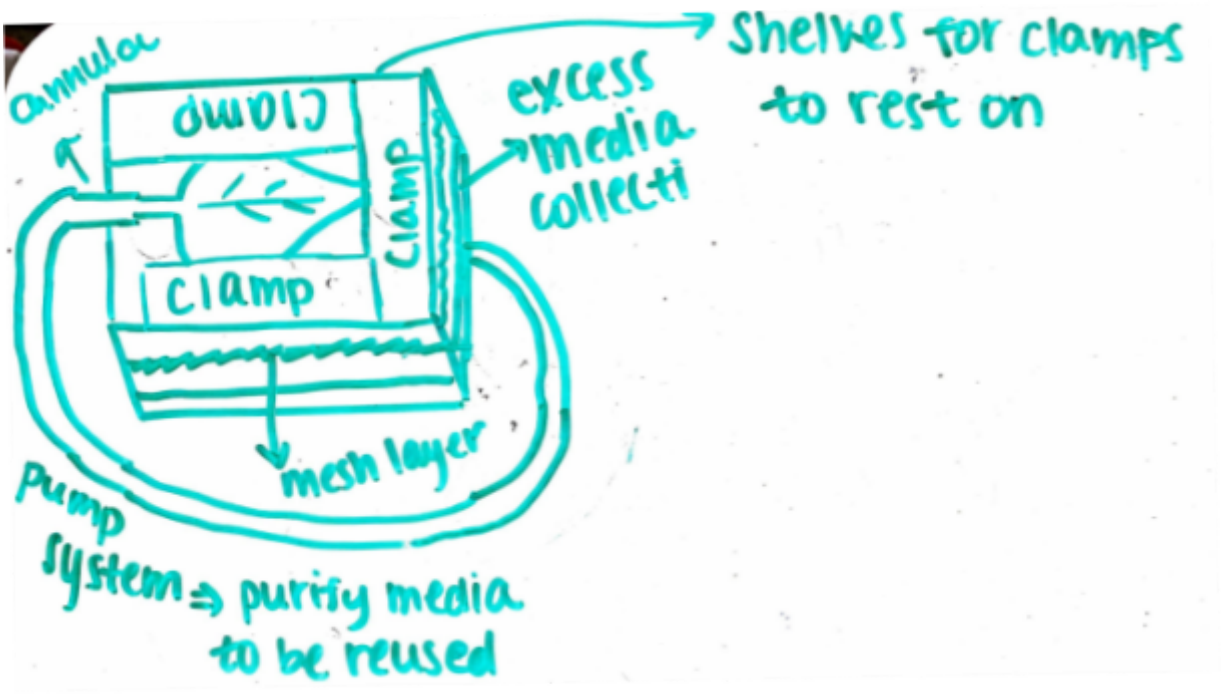


Leah's Preliminary Design #3

Pros: submerged in media, vacuum drain, filtrate excess, no cannula

Cons: open system needs cover, needs to be placed in incubator

Appendix C.2.4 Alexa's Preliminary Design

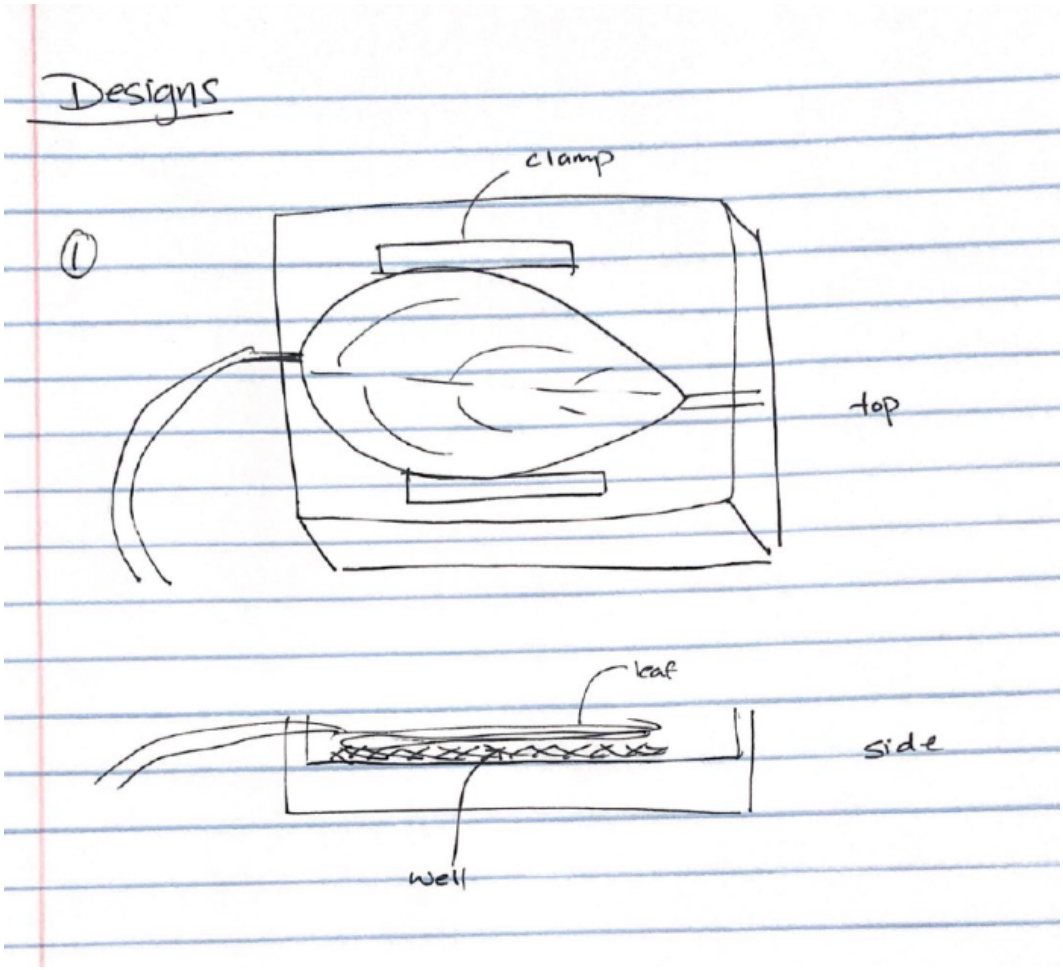


Alexa's Preliminary Design

Pros: user friendly, layered, excess media collection,

Cons: cover, incubation needs

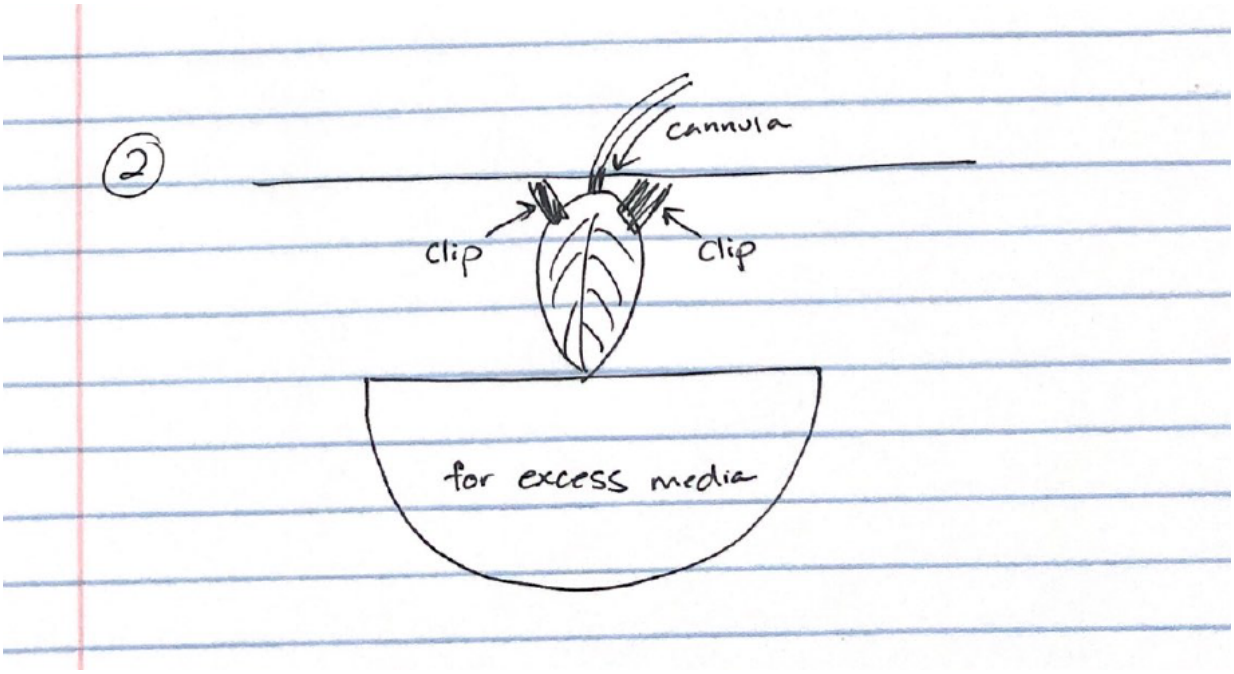
Appendix C.2.5 Julia's Preliminary Designs



Julia's Preliminary Design #1

Pros: clamps, mesh layer, reuse media

Cons: no easy cell removal, need 3 clamps



Julia's Preliminary Design #2

Pros: vertical cannulation

Cons: vertical cannulation, not sterile system, two clamps, no pump

Appendix C.3 Analysis of Design Elements

Appendix C.3.1 Decellularization Process

At Home Process:

- For this process, our team followed Professor Gaudette's protocol. However, given the pandemic and inability to go into the lab physically, our team had adapted the process to be performed at home. For this, we utilized 100 square centimeter petri dishes, spinach leaves, a 10% SDS mixture, deionized water, and clorox bleach.
- The at home procedure is outlined below.

- Create a solution consisting of 70 ml of deionized water, 8 ml of clorox bleach, and 0.5 ml of 10% SDS mix in a 100 cm^2 petri dish. Stir the solution using a plastic straw.
- Once stirred, place a spinach leaf within the mixture. Ensure that the spinach leaf is able to completely submerge. Let the spinach leaf sit for two days while occasionally stirring the mixture by moving around the petri dish. After two days, replace the bleach, deionized water, and SDS solution with a new one.
- Repeat this process until spinach leaves look visually clear and decelled.
- Once the spinach leaves are fully decelled, place the decelled leaves in deionized water. The leaves may be preserved on a normal lab bench in deionized water.

Laboratory Procedure:

- As the pandemic progressed and restrictions lightened, our team additionally decelled spinach leaves within a laboratory. In the lab small errors such as contamination and not properly mixing the solution could be avoided. The solution made was the same as the at home procedure, but now the team had access to resources such as a shaker table, sterile environment and conditions, and precise measuring tools. For instance, in the lab there are much more precise measuring devices such as pipettes and conical tubes. The shaker table was used to properly mix the solution together. The procedure that was followed in the lab may be seen below.
  - Protocol for Plant Decellularization
    - Materials:
      - 1X SDS solution in DI H2O

        - For 2L of solution mix 200 mL of 10x SDS with 1800 mL of DIH20
        - 2L of 10x SDS Solution
            - Mix 200 g of SDS powder in 2L DIH20 until there are no more visible SDS pellets
            - Can be stored at room temperature until needed
    - 1% Tween-20 with 3% bleach (or Cl tablets) in DI H2O
    - Tris buffer solution
        - 0 mM Tris Buffer (605.7 mg in 500 mL of DiH2O)
        - Buffered to pH 9.0

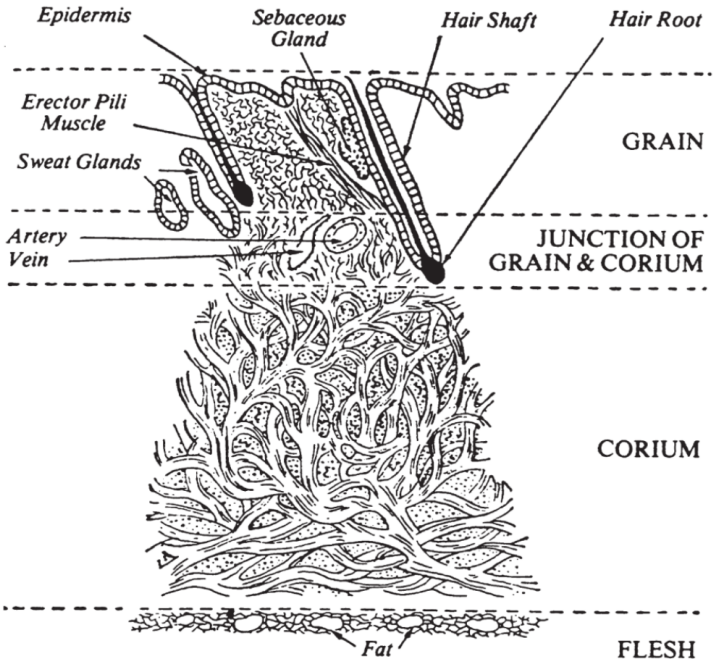
- Decellularization Protocol:
    1. Maintain submerged in 1x SDS for 2 days in order to decellularize plant material, until leaves and stems become more transparent in appearance. Replace solution after 24 hours. Green coloration at this step is normal and not indicative of an unsuccessful decellularization.
    2. Remove 1x SDS and add Tween-20/Bleach solution to set up.
    3. Maintain set up for 24 hours, can go longer if needed. Watch until leaves and stems have become clear/transparent. This solution should purge any remaining coloration from the plant matter.
    4. Remove solution and add DI H2O to set up. Submerge in DI H2O for 24 hours.

5. Wash decellularized leaves on the rotator in Tris buffer solution overnight.
    a. Replace the solution at least twice (usually after the first hour and then secondly in the morning)
    b. Tris buffer removes residual SDS that maybe left entrapped in the leaf
    c. Make sure the rotator moves the leaf gently
    d. Watch the stem as they become very fragile
6. Remove leaves and stems from solutions, freeze overnight in -20°C freezer
7. Lyophilize leaf/stem for 24 hours
8. Store at lyophilized leaf scaffold at room temperature until needed.
    a. If functionalized with RGDDOPA, store lyophilized leaves in refrigerator
9. See rehydration protocol for next steps

Appendix C.3.2 Grain Break Testing

Leather has specific mechanical properties that will need to be replicated as we attempt to grow the scaffolds in the lab in order to meet ASTM standards as well as consumer expectations. The most important feature of bovine dermis that gives leather its strength is the grain layer. The specific properties of this layer need to be appropriately replicated so the appearance and tensile strength of the final product are as similar as possible to real leather.

The grain layer of skin is the uppermost layer of the unhaired pelt, also known as the corium minor. The structure is fibrous, but the fibers are very fine that they appear to be solid. As seen in the figure below, the corium lies underneath the grain layer; it is the upper layer of the dermis that gives the leather its strength. The layer on the surface is called the grain enamel which is the most valuable part of the skin when producing leather, as it is the top, visible layer of leather that makes it so desirable.

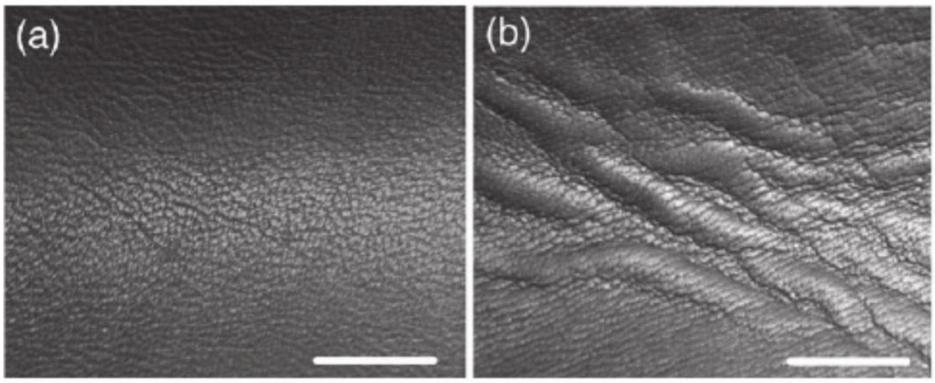


Cross-section of bovine skin layers (Covington, 2009).

It provides the most pleasing appearance if the grain is tanned and processed correctly. Before tanning, most cattle hides must undergo splitting because they are very thick. This is a process that thins out the skin to make it easier to work with while tanning. Prior to splitting, however, the starting thickness of the grain-corium junction must be examined as it determines the strength of the material afterwards. A value known as the grain-corium thickness ratio must be considered – where a smaller ratio equals a stronger piece of leather. Grain layer has about

20% of the strength of the corium. This is due to the density of the fibers in the layer and is associated with the greater interaction of the corium fiber's structure, which reduces risk of tear. The corium also contains a high measure of fibrous collagen which increases the strength as well (Covington, 2009).

As the grain layer is the most prevalent aesthetic aspect of the leather, the consumer-needs demand the smoothest possible surface. A physical imperfection known as grain break occurs at the grain-corium junction. Damage to the enamel reveals underlying fibers which reflect more light and can appear dull compared to the rest of the surface (Covington, 2009). All leather experiences grain break, however, it's the difference between a coarse break or a fine break, as shown in the figure below. Coarse breaks occur in loose leather and fine breaks occur in more tight leather. A fine break is more desirable as it shows fewer wrinkles in the leather compared to a coarse break. In loose leather, there are larger gaps in the collagen fibers that are generally aggravated during the tanning process (Wells, 2016). These breaks can be measured and analyzed through various forms of testing.

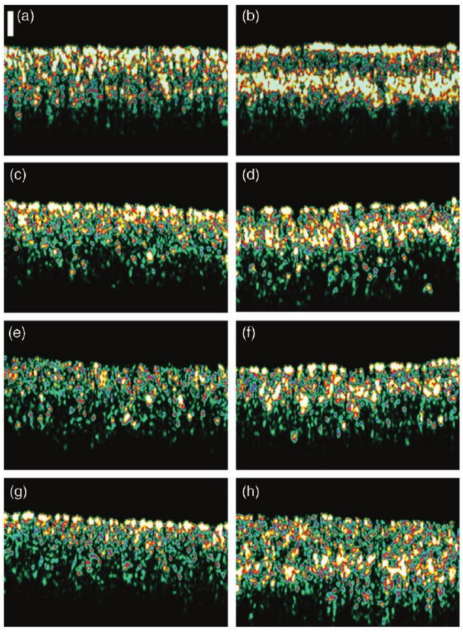


Tight leather (left) with a break of 1 and loose leather (right) with a break of 7 (scale bar=2mm) (Wells, 2016)

The standard ASTM test involves folding the leather grain-side in and quantifying the number of wrinkles on a standard break scale. Two operators measured break scale and the

results were averaged. The leather is considered tight if there are less than three breaks and loose if there are more than five. Anything between three and five breaks are discarded. Another method is the tear and tensile test. Standard methods for double-edge tear testing were performed after samples were stored at 20^{o} Celsius and 65% humidity for 24 hours. Thickness of the leather was measured after folding -- three measurements taken parallel and three perpendicular to the line of the backbone. These tests determined that loose leather was much stronger than tight leather with tensile strength of 130 (σ = 61) N mm^{-1} vs 73(σ = 10) N mm^{-1}. A third test is using Airborne Ultrasonic (AU) Waves to measure the grain break (Wells, 2016). This test can categorize mechanical properties of leather without disturbing the material. This test consists of two ultrasonic transducers about 3 cm apart. A transmitter with a diameter of 38 mm pulses a tone burst through a power amplifier to a receiver with a diameter of 25 mm. This is connected to a preamplifier mounted on a computer-controlled X-Y scanner using software that allows the transducer and receiver array to be moved over the entire surface of the crust. Time of Flight (TOF) values were recorded which represent the time it took ultrasonic waves to reach max peak passing through the transmitter or during a time period of 150-250 microseconds. Strong correlations are seen between the mechanical properties of leather and the corresponding AU parameters based on the distribution of the transmission time (time of flight) through leather. Grain break is shown to have a negative correlation with both $TOF_{200\text{-}250}$ and $TOF_{210\text{-}220}$ which is opposite to the behavior shown for Young's modulus, which has a positive correlation with both $TOF_{200\text{-}250}$ and $TOF_{210\ \text{–}220}$. The lower TOF value indicates a shorter transmission time of ultrasonic waves through the leather samples, this reflects a less dense and flexible fibrous structure, softer leather, which results in better break evaluation. When analyzing scans from AU testing on the grain-corium junction, loose leather shows a band of reduced intensity below the

top layer in the grain/grain-corium boundary, which is seen in the figure below. This is because it's less dense and there is more space between fibers. The middle of the grain layer in loose leather has little reflected ultrasonic signal and reflection is very intense with tight leather (Liu, 2015).



Ultrasonic images of tight leather (a, c, e, g) and loose leather (b, d, f, h) (Liu, 2015).

Appendix C.3.3 Instron Setup & Use

Procedure:

IF YOU HAVE BIOLOGICAL SPECIMEN, FOLLOW THESE STEPS. IF NOT, SKIP TO STEP 8:

A. Cut out a piece of blue drape (in the corner of the lab)
B. Follow the instructions on the wall, make sure to cover the bottom of the machine to avoid contamination
C. Place sticky rubber squares over the clamps

1. Put specimen between the clamps
2. Adjust them accordingly to the size of the test specimen
3. Once secure, open your particular test. Double click on it, it should open.
4. Once open, go to “browse” and put it in a file that you will remember to find it in. The Instron will put it in a very hard to navigate location otherwise.
5. Hit “next” in the top right corner. Hit next again. A page with the dimensions inputs will open. Measure your piece and type the dimensions.
6. Move the “mechanical stop” on the machine to the right under the top bracket and the “stop” above the bracket slightly above the anticipated stopping point
7. Place the shield around the machine. Align the hole with the emergency stop button
8. Zero the extension and balance the load on Bluehill
9. Hit “start” on Bluehill. Once the piece rips, you will have to manually hit “stop” on Bluehill because it will not stop on its own
10. Once stopped, hit “next” and the Bluehill procedure will start over, minus the calibration.
    a. If done, hit “Finish,” then “Finish” again. Make sure to *finish the specimen.* It will ask you if you want to “restart the same test with the same specimen.” Hit “No.” The tests will save to the computer. Zip the file and send them to yourself in an email.

Appendix C.3.3.1 At Home Mechanical Test

1. Collect all the necessary materials

2. Record the mass of the binder clip and zip lock bag being used to better understand the load that a decellularized spinach leaf is holding. In this case, the zip lock bag used played no important role in how strong the decellularized spinach leaf was.
3. Attach the first binder clip to the decellularized spinach leaf after the leaf has been hydrated in deionized water. Attach the binder clip where the stem is. Ensure that there is a secure grip.
4. Attach the second binder clip to the zip lock bag and to the bottom portion of the spinach leaf.
5. Next fill the zip lock bag with water recording how much milliliters of water that it is carrying. In this case, the design team utilized a food scale to measure the amount of water that was placed into the zip lock bag.
6. After filling the zip lock bag and recording the amount of water that it held, lift the system from the first binder clip at the stem. Begin by lifting 1 milliliter of water and then continue by adding 1 milliliter increments of water. Lift the system, until the spinach leaf breaks.
7. Record the amount of water where the spinach leaf breaks. Repeat these steps for the other two spinach leaves.

Appendix C.3.3.2 Mechanical Testing of Rice Paper

Before Testing:

- Before performing any mechanical test with the Instron, make sure the Instron is properly set up. Follow the procedure titled “Instron Setup” before continuing.

Materials

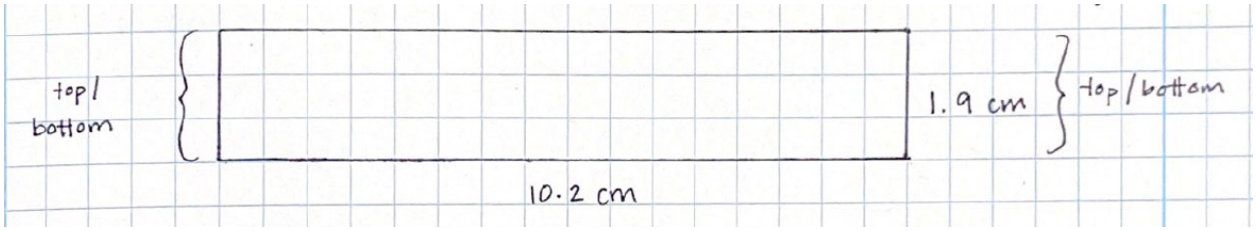
- Rice paper
- Graph paper (from the lab notebook)
- Ruler with centimeter markers
- Rectangular pyrex tupperware (any flat well that can hold a shallow amount of water and half a sheet of rice paper)
- Meat cleaver
- Cutting board
- General lab materials (lab coat, safety glasses, gloves, mask, face shield)
- Instron 5544
- Flat tip forceps x2
- Duct tape

Procedure

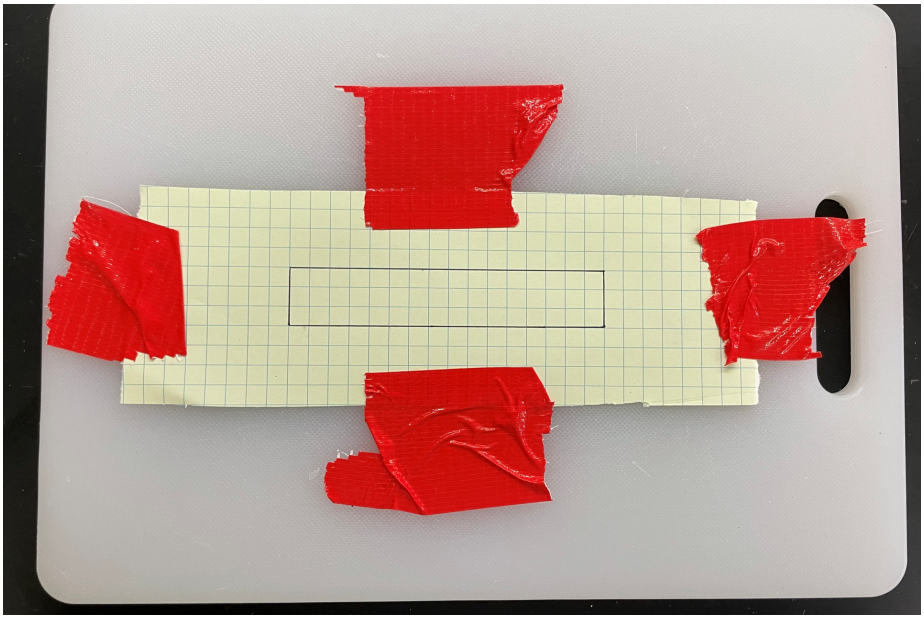
1. Follow the “Instron Setup” procedure before starting and put on necessary lab gear.

2. Fill the Pyrex with water. It should be filled enough where the bottom is covered evenly and completely

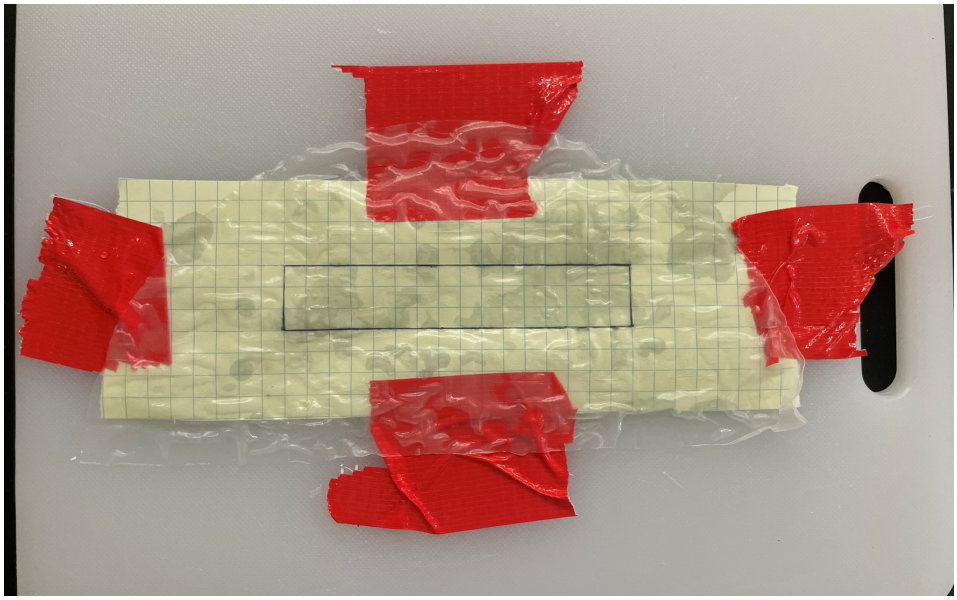
3. Take a sheet of graph paper and draw a rectangle with dimensions of 16 squares horizontally and 3 squares vertically. The measurements of these dimensions is 10.2 cm x 1.9 cm.



4. Secure the paper to the cutting board with duct tape.



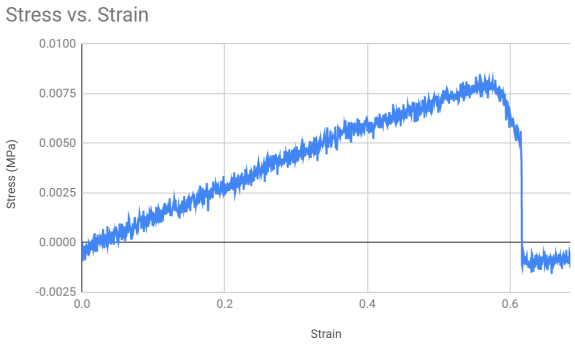
5. Take a sheet of rice paper and snap it in half over a trash can to avoid projection of shards onto the floor.
6. Submerge one of the halves of the rice paper into the water so that both sides are coated. **It should only be in the water for around 1 second.** ***Immediately*** place the wet rice paper over the graph paper guide on the cutting board.

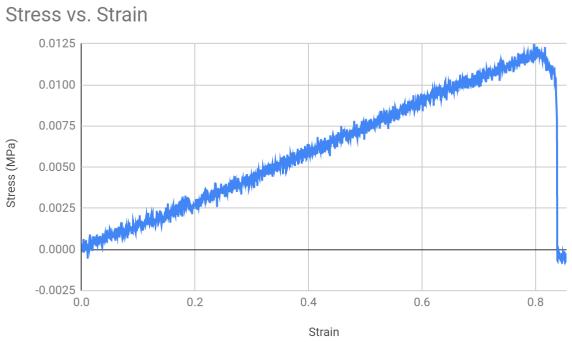


7. Take the meat cleaver and cut along the lines of the rectangle. This **MUST** be done in **ONE FLUID MOTION** (from tip to tail of the blade in one single cut) per edge. This must be done **immediately**, before the rice paper has a chance to soften and stick to the paper. Carefully remove excess paper from the cutting board with forceps.
8. Take both pairs of forceps. Use one pair to lift a corner of the rice paper off the cutting board, then take the second pair and slide it under the short edge via the corner that was just lifted. Make sure the forcep has a full, flat grip on the whole edge. It is a **MUST** to avoid creating folds in the paper.
9. Carefully bring the sheet of rice paper and both pairs of forceps over to the Instron. Lower the bottom short edge into the bottom set of clamps. Tighten the clamps with the knobs.
10. Jog the top clamp up or down to a point where the entryway of the clamp is accessible and lay the top edge of the rice paper on one of the clamp surfaces. Use the second pair of forceps to secure the rice paper while you remove the first pair from underneath.

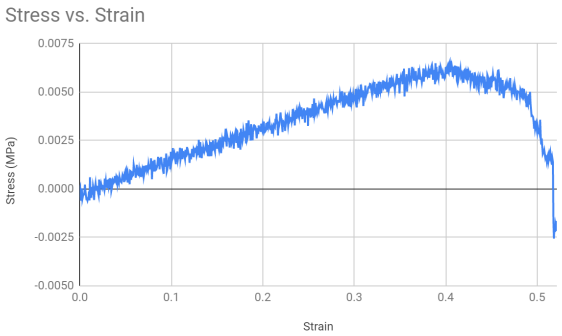
11. Secure the top clamp. The paper should be able to stick freely as the clamp gets closer together. Hold the rice paper with the forceps until the clamp reaches them.

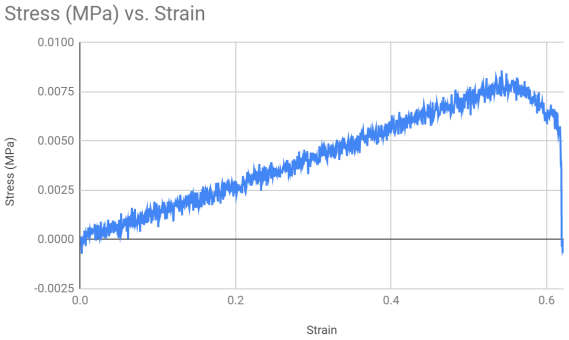
12. Cover the Instron with the acrylic shield and make sure the balance and extension are set to zero.
13. Hit start and record the data after the paper rips.



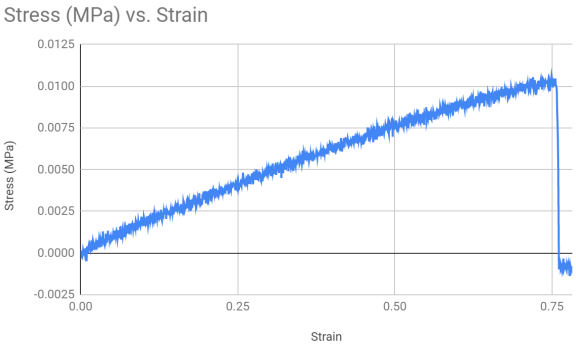


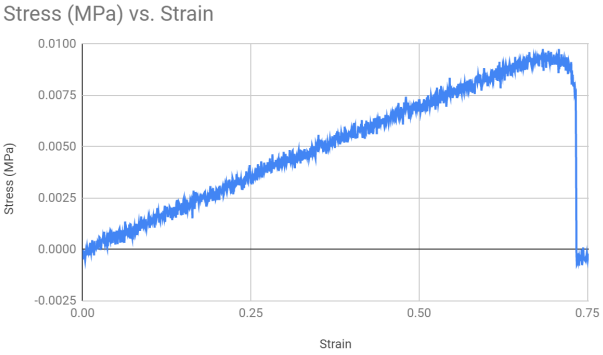
Tests 1 and 2



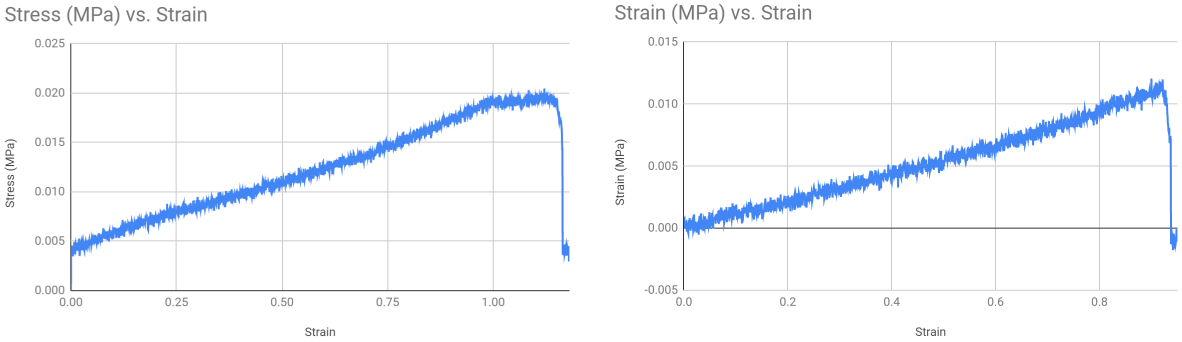


Tests 3 and 4

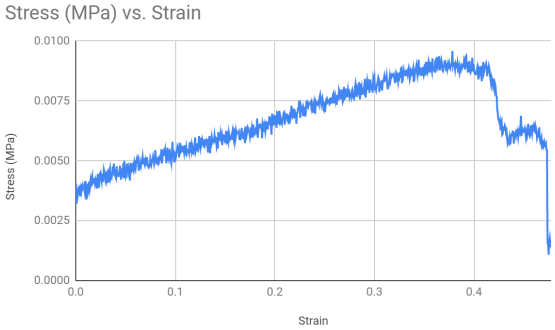




Tests 5 and 6



Tests 7 and 8



Test 9

Appendix C.3.4 Bovine Fibroblast Isolation

1. Before Starting

1. Sterilize scissors and forceps with 70% ethanol.
2. Place a small magnetic stirrer inside a 30 mL beaker, cover with two layers of foil, and autoclave.
3. Prepare a 28 Wunsch units/mL stock solution of Liberase Blendzyme 3 in sterile water. Make 0.5 mL aliquots and freeze at -20°C. Thaw a new aliquot before every use. The solution may appear cloudy after thawing. Vortex the solution until it becomes clear.
4. Warm up the cell culture media.

2. Animal Sample Preparation

1. Pick up sample from provider
2. Place sample on ice during transport
3. Prepare hood space using the materials listed above
    a. Fill petri dish with 30mL rinse media
    b. Place sample in hood
    c. Label sample appropriately
4. Rinse skin sample

3. Extracting Cells

1. Transfer the tissue fragments into a 10 cm tissue culture dish using sterile scalpel. Do not transfer too much PBS with the sample.
2. Cut the tissue into ~1 mm pieces using two scalpels. Use two blades using a scissor action starting from the center and pulling apart. Keep the tissue balled up, do not cut piece by piece. When the cutting is sufficient the skin resembles putty, it will not separate into pieces but will stretch thin. The lung tissue is easier to cut, and it will separate into tiny pieces.
3. Using a scalpel, transfer the cut tissue into a sterile 30 mL beaker with a sterile stir bar. Wash the plate used for cutting tissue with 10 mL of DMEM/F12 media with 0.14 Wunsch units/mL Liberase Blendzyme 3, and 1X antibiotic/antimycotic, and add the solution to the 30 mL beaker with tissue fragments.
4. Cover the beaker with sterile foil, and incubate at 37°C, stirring slowly, for 30 to 90 minutes. The length of the incubation depends on the species and tissue type. Take care

not to over-digest the tissue. Best yields are obtained when tissue fragments are still present at the end of the digestion. Skin from large animals takes longer to digest. Check the digestion after 30 min, and then every 10 min. When the skin digestion is complete, the media becomes cloudy and skin fragments separate from each other, and the edges of the pieces become "fuzzy".

5. Pipet the solution containing tissue fragments up and down to break the clumps. Transfer the solution to a sterile 50 mL tube. If the fragments move easily through the 10 mL pipette cutting and digestion was done well. Rinse the beaker 3 times with 10 mL of warm DMEM/F12 media with 15% FBS, 1X antibiotic/antimycotic and add the media to the 50 mL tube with tissue fragments. Close the 50 mL tube and mix by inversion a few times. The FBS in the media will stop Liberase digestion.
6. Spin at 524 g in a swinging bucket tissue culture centrifuge for 5 min. Remove the supernatant. Resuspend the pellet in 10 mL of warm DMEM/F12 media with 15% FBS, 1X antibiotic/antimycotic. Pipet the suspension with maximum force to break the tissue pieces.
7. Add another 30 mL of DMEM/F12 media with 15% FBS, 1X antibiotic/antimycotic, mix and centrifuge at 524 g in a swinging bucket tissue culture centrifuge for 5 min. Repeat one more time to remove the traces of Liberase.
8. Resuspend the pellet in 10 mL of DMEM/F12 media with 15% FBS, 1X antibiotic/antimycotic and transfer to a 10 cm tissue culture dish and place in a tissue culture incubator at 37°C, 5%CO_2, 3%O_2.
9. After 15 min incubation, take media and replate into new plate, add 10ish mL of media into tissue culture plate w fibroblasts that culture within 10 min, replate

10. Check the plates every day for fibroblasts and media color. If the isolation was successful, fibroblasts crawl out of the tissue fragments and attach to the plate (Figure 1). The fibroblast starts to exit tissue fragments within 2-5 days.
11. If the media changes color to yellow, this indicates potential contamination or overcrowding of cells. Examine the plates under the microscope at high magnification. If bacteria, fungi, or worms are present, discard the plates (this is rarely an issue with laboratory animals, however may occur when the samples are collected from the wild). If no contamination is present, but the media changes color, this is caused by either too many cells or too many tissue fragments placed in the same dish. If more than 60% of the plate is covered by attached fibroblasts, change the media on the plate and transfer the tissue fragments to a new plate with the new media. If not many fibroblasts are attached to the plate, change the media and split the tissue pieces to 2-4 plates.
12. After 7 days, if the media had not been changed earlier, change the media and transfer the tissue fragments to a new plate with new media.
13. Incubate the cells and tissue fragments for an additional 7 days. By day 14 all viable fibroblasts have exited the tissue fragments.
14. After 14 days from the beginning of the cell isolation, discard the old media and tissue fragments, harvest the cells and plate them on a new plate at $5x10^5$ cells/plate EMEM with 15% FBS, 1X Penicillin/Streptomycin, non essential amino acids, and sodium pyruvate. EMEM media will support growth of fibroblasts only and other cell types will die or stop proliferating.
15. After the cells reach 80-90% confluence, freeze an aliquot of cells for future use.

16. Continue culturing the cells by splitting them at $5x10^5$ cells/plate when the cells reach 80-90% confluence.

Appendix C.3.5 Freezing & Passaging Cells

Freezing Cells:

- Trypsinize and resuspend cells as described under Cell Passaging
- Count cells and calculate the total number of cells in the suspension (density in cells/ml x total volume of suspension in mL)
- Centrifuge cells @200g for 5 minutes
- Prepare freezing solution A (DMEM with 10% FBS) and B (DMEM with 10% FBS, 20% DMSO)
- Aspirate supernatant and resuspend cells in half the final volume in freezing solution A and resuspend cells thoroughly.
- Add an equal volume of freezing solution B slowly while mixing the contents gently.
    - Freezing medium generally consists of cell culture medium + 10% DMSO + 10% serum at final concentrations. DMSO prevents ice crystal formation during freezing.
- Cells are typically resuspended to 0.5 to 2 million cells/ml in freezing medium and pipetted into cryovials. Aliquot 1 ml into each cryovial. Tighten the caps well. Do not overfill cryovials!
- Place cryovials into the freezing container and transfer the container into the -80°C freezer overnight, then transfer vials to liquid nitrogen for storage
    - **NOTE: Cells should be frozen slowly and thawed quickly!**

Passaging Cells:

- When cells are 70-80% confluent, aspirate medium and wash cells twice with 1X DPBS by adding and then aspirating PBS. (Confluency refers to the area of cell coverage on the plate. If cells are covering ~ 80% of the surface of the plate, they are 80% confluent).
- Use 1⁄2 the volume of PBS to rinse as there was medium in the dish (e.g., a 100 mm dish is fed 10 mL medium, therefore use 5 mL PBS per rinse).
- Trypsinize cells by adding 1X trypsin EDTA (~ 3 mL for a 100 mm plate) for 3-5 mins. Plates may be returned to the incubator while trypsinizing.
- Add fresh media to plate, pipette cells up and down to resuspend and break up clumps of cells. Serum in culture medium inactivates trypsin.
- Add a volume of resuspended cells to fresh medium in a new plate (e.g., add 2.5 ml cell suspension to 7.5 ml fresh medium in a new 100 mm dish = a 1:4 split/passage). Alternately, seed a specific number of cells in the total volume prescribed for a particular culture dish.
- Check growth each day; passage as necessary.

Appendix C.3.6 Perfusion Analysis

- Perfusion Model:
    - Perfusion is the delivery of solutes through diffusion. We will be dealing with an extremely small Reynold's number. This means that we are dealing with laminar flow for a microfluidics application.
    - Spinach leaves, like any other plant scaffold, have various veins that make up its intravascular network. This network of veins is what makes spinach leaves an ideal option for perfusing media for cell growth.

- The governing equation for this perfusion analysis is the change in pressure is equivalent to the flow through the area of this vasculature.

$$\Delta P = AQ$$

P = Pressure

A = Area

Q = Flow

- The pressure and flow will be linear and the flow will be constant. This flow will divide among the various veins of the spinach leaves. The flow through these different veins may be treated as resistors in series and parallel.
- Not only is there resistance when the media disperses amongst the different veins, but through the whole mesh of the leaf itself. The flow and resistance of this process can produce a pressure and flow curve.
- Diffusion additionally occurs through the veins into the rest of the leaf. Diffusion can be modeled through mass flow rate. Mass flow rate is equal to the area multiplied by viscous coefficient of the fluid. That is then multiplied by the concentration divided by the thickness. This can be seen in the equation below.

$$\dot{m} = \mathcal{M}A*(\Delta C/\Delta x)$$

$\dot{m}$= mass flow rate

$\mathcal{M}$ = viscous coefficient

A = Area

C = concentration

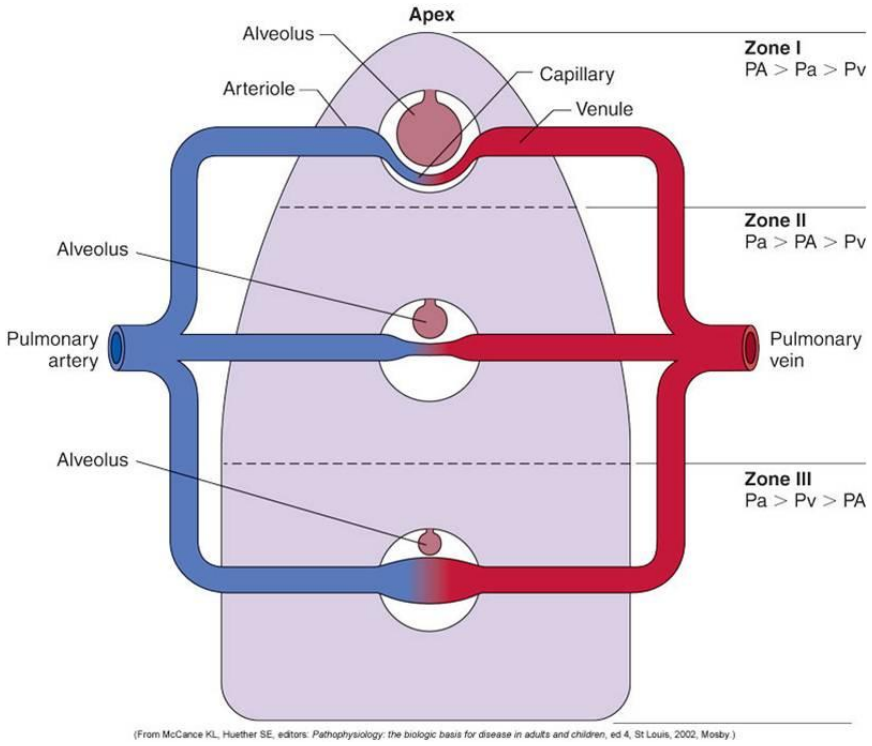
x = thickness

- This perfusion process can be modeled as delivery of oxygen to a capillary in lungs or heart muscles. Where at one concentration there is a large presence of oxygen which diffuses through a layer that is a couple of microns thick in order to deliver to a capillary. The concentration will depend on the media used.
- Most people do not attempt to perform these calculations. Therefore, instead of performing these calculations they calculate a perfusion rate.

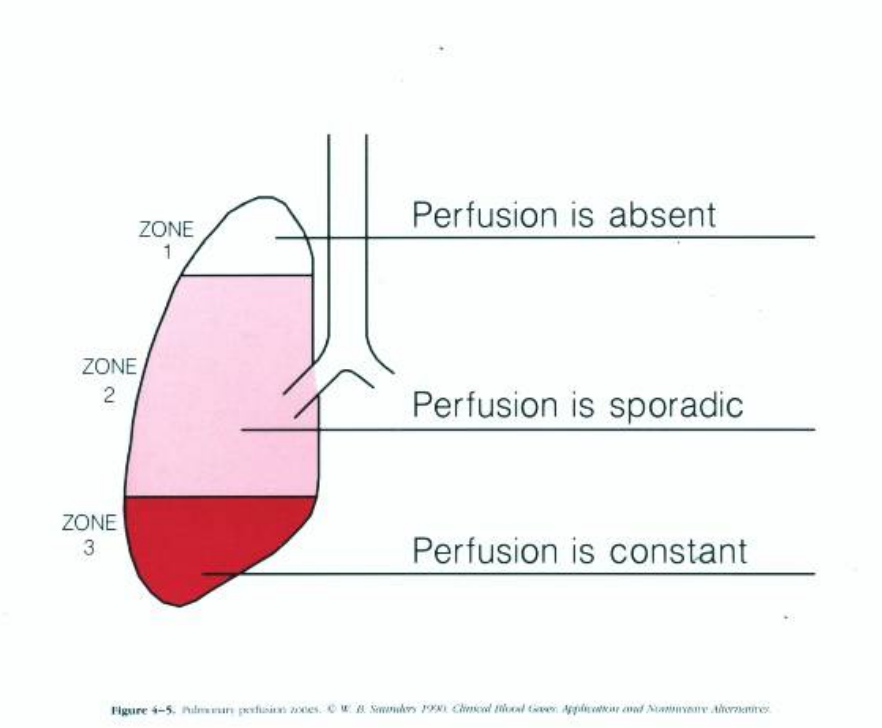
- Perfusion Rate:
  - Perfusion rates are measured in milliliters per cubic meter multiplied by time in seconds. These units may be seen below. For lungs and heart muscles, it is the delivery of oxygen divided by the volume of the tissue that it is passing through.

$$mL/m^3s$$

  - Perfusion rates are proportional to pressure (P). Let's look at lungs for instance. More flow is delivered with greater pressures. In the body, we investigate the perfusion versus the need for oxygen and its use. In a vertical position, gravity and pressure changes affect the perfusion model. With the two into play, more perfusion occurs at the bottom portion of the lung. This process may be seen in the image below.



Lung Perfusion Diagram (Medic Tests, 2021).



Lung Ventilation to Perfusion Relationship (RT116 Cardiac Review, Circulation and Hemodynamics, 2010)

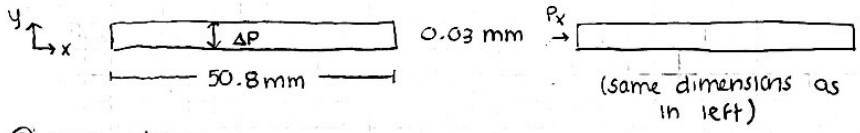
- It is also important to note that arteries are distensible, meaning that the veins will expand and not remain rigid when fluid passes through them. In order to account for distension, we look at the change in diameter of the veins. A change in size indicates and results in a change in flow, resistance, and pressure.
- As seen from the images above, perfusion is more distensible at the bottom and all values are single measured variables.

Appendix C.3.6.1 COMSOL Calculations, Initial Values, and Boundary Conditions

COMSOL Pre-Simulation - Assumptions and Initial Conditions:

① Problem Description:

~ The objective of this problem is to model the pressure drop across a decellularized spinach leaf.



② Assumptions:

~ 2D problem
~ thickness = 30 μm = 0.03 mm
~ media used will be treated as water

③ physics, eqs:

$\Delta P = AQ$
$\dot{m} = \mu A * (\Delta C / \Delta x)$
$\Delta P = QR$

P = Pressure, A = area, Q = flow, R = resistance
μ = viscous coef., C = concentration, x = thickness

④ Materials:

~ Patm = 101,325 Pa = 14.69 psi
~ 100 psi pressure transducer, P_{xi} = read from sensor
~ spinach leaf with dimensions in ①

⑤ Initial and Boundary Conditions:

y = 0 (top): P = P_i
y = H (bottom): P = Patm
x = 0, P = P_x
x = L, P = Patm

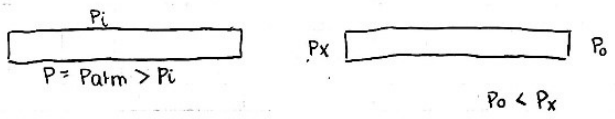
⑥ applied loads:

~ no applied loads in problem.

⑦ Results:

~ We will discover the amount of pressure needed to move 50.8 mm in the x-direction using $\Delta P = AQ$

~ gravity impacts the bottom portion of the leaf



Mass Flow Rate:

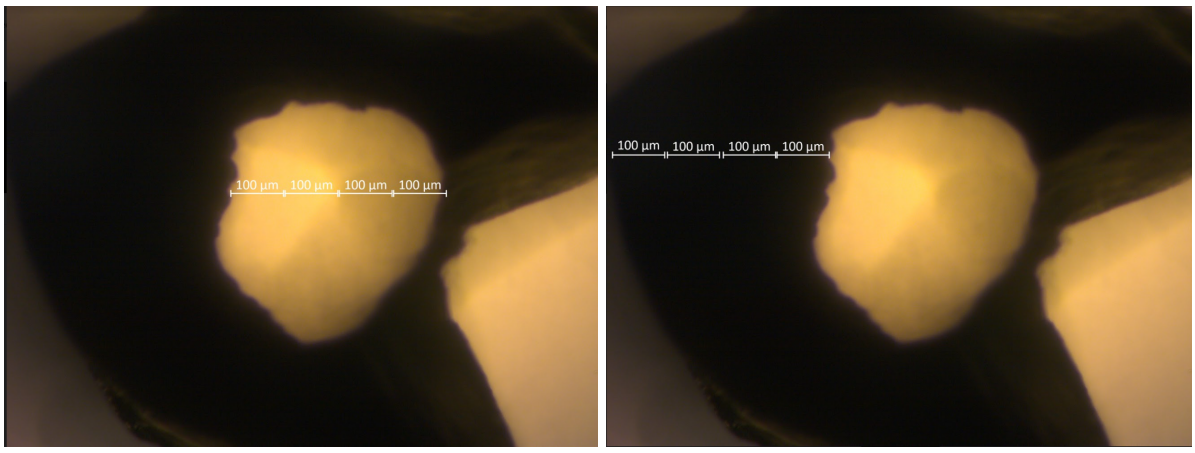
Calculated the mass flow rate by running the pump into a conical tube. We measure the time it took to fill 5 milliliters of water. Thus, mass flow rate was found in three trials and averaged to be used in the COMSOL computation.

Trial Number:	Amount of Water (mL):	Time (s):	Mass Flow Rate (mL/s):	Mass Flow Rate (kg/s):
1	5	18.14	0.276	0.000276
2	5	17.44	0.287	0.000287
3	5	17.19	0.291	0.000291

The average of these three trials is 0.285 mL/s or 0.000285 kg/s. This value will be used in our calculations and COMSOL simulation.

Spinach Leaf's Stem Dimensions:

The wall thickness and diameter of a spinach leaf was estimated using scales from the microscope. Three samples were measured under the microscope. Images showing this process as well as a table of results is shown below.



Stem Tested:	Outer Diameter (µm):	Inner Diameter (µm):	Wall Thickness (µm):
1. Small	1,190	390	400
2. Medium	1,360	440	460
3. Large	1,790	450	670

Taking the average of the three samples, for the COMSOL Multiphysics analysis we will assume that the outer diameter is equal to 1,446.67 µm. The inner diameter is assumed to be 426.67 µm, and the wall thickness is 510 µm.

COMSOL Computations - Initial Values:

~ inlet velocity: $\dot{m} = \rho A V$

$\dot{m} = 0.285\ mL/s$

$= 0.000285\ kg/s$

$A = \pi r^2$

NOTE: $D_{spinach\ leaf\ stem} = 1,146.67\ \mu m$

$= 0.00114667\ m$

$A = \pi (0.0014667/2)^2$

$= 1.6422 \cdot 10^{-6}\ m^2$

ρ = density of water

$= 997\ kg/m^3$

$$V = \frac{\dot{m}}{\rho A} = \frac{0.000285}{997\,(1.6422 \cdot 10^{-6})} \quad \frac{kg}{s} \cdot \frac{m^3}{kg} \frac{1}{m^2} = \frac{m}{s}$$

$$\boxed{V = 0.1741\ m/s}$$

~ Reynold's Number: $Re = \frac{\rho V D}{\mu}$

μ = dynamic viscosity at room temperature

$= 8.90 \cdot 10^{-4}\ Pa \cdot s$

$$Re = \frac{997\,(0.1741)\,(0.0014667)}{8.9 \cdot 10^{-4}} \quad \frac{kg}{m^3} \frac{m}{s} m \cdot \frac{s^2 m}{kg} \frac{1}{s}$$

$$\boxed{Re = 286.05}$$

~ Max Pressure (thin walled cylinder):

$\sigma_\theta = \frac{Pr}{t}$ $\quad \sigma_\theta$ = hoop stress

$\sigma_\theta = \sigma_y$ = yield stress → from stress vs. strain curve of decelled spinach leaves

$\sigma_y = 0.008\ MPa = 8 \cdot 10^3\ Pa$

$r = 0.00114667/2\ m$

$= 5.73 \cdot 10^{-4}\ m$

t = thickness $= 510\ \mu m$

$= 0.00051 m$

$$P = \frac{\sigma_y (t)}{r} = \frac{8 \cdot 10^3\,(0.00051)}{(5.73 \cdot 10^{-4})} \quad \frac{Pa\ m}{m} = Pa$$

$$\boxed{P_{max} = 7,116.26\ Pa} \approx 1.032\ psi$$

COMSOL Velocity Increase Reasoning - Calculations:

In the Bernouli's equation, the inlet pressure (P_1) in addition to the kinetic energy or density (ρ) multiplied by the inlet velocity (v_1) squared divided by two is equal to the outlet pressure (P_2) in addition to the density (ρ) multiplied by the inlet velocity (v_2) squared divided by two.

Bernoulli's Equation: $P_1 + ½ \rho v_1^2 = P_2 + ½ \rho v_2^2$

We assumed to neglect the potential energy given that there is no change in height

$0.12\ [psi] + ½\ (997\ kg/m^3)(0.1714\ m/s)^2 = 0.1\ [psi] + ½\ (997\ kg/m^3)(v_2^2)$

$827.371\ [Pa] + 14.645\ [kgm^2/m^3s^2] = 689.476\ [Pa] + 498.5\ [kg/m^3](v_2^2)$

$827.371\ [kg/ms^2] + 14.645\ [kg/ms^2] = 689.476\ [kg/ms^2] + 498.5\ [kg/m^3](v_2^2)$

$842.016\ [kg/ms^2] = 689.476\ [kg/ms^2] + 498.5\ [kg/m^3](v_2^2)$

$152.54\ [kg/ms^2] = 498.5\ [kg/m^3](v_2^2)$

$(v_2^2) = 0.306\ [kg/ms^2] / [kg/m^3]$

$v_2 = sqrt(0.306)\ [m^2/s^2]$

$v_2 = 0.553\ m/s$

Appendix D: Design Verification

Appendix D.1 Mechanical Testing

Appendix D.1.1 Mechanical Testing of Spinach Leaves

Before Testing:

- Before performing any mechanical test with the Instron, make sure the Instron is properly set up. Follow the procedure titled “Instron Setup” before continuing.

Materials

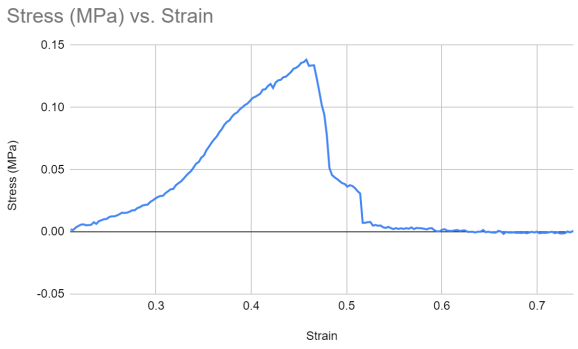
- Decellularized spinach leaves in petri dishes
- Calipers
- Meat cleaver
- Cutting board
- General lab materials (lab coat, safety glasses, gloves, mask, face shield)
- Instron 5544
- Flat tip forceps x2
- Allen wrench

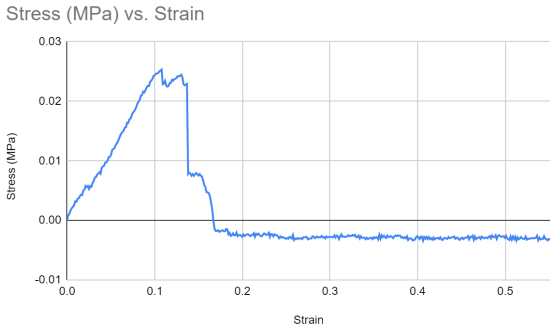
Procedure

1. Follow the “Instron Setup” procedure before starting and put on necessary lab gear.
2. Carefully remove the spinach scaffold from the dish and place on the cutting board.
3. To remain consistent with the previous mechanical tests, take the meat cleaver and cut spinach leaves to test the yield strength with a rectangular scaffold. Make sure to cut

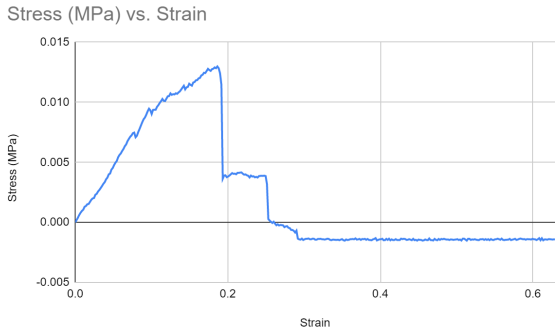
along the veins and not across them since spinach is not isotropic. This **MUST** be done in **ONE FLUID MOTION** (from tip to tail of the blade in one single cut) per edge.

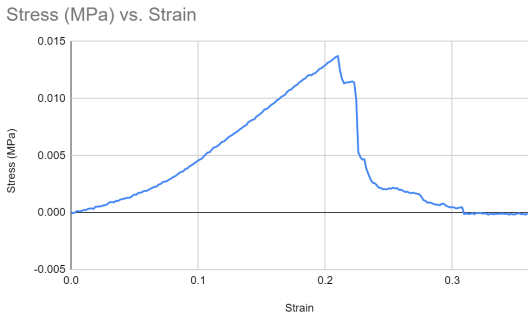
4. Use calipers to measure the dimensions of the spinach.
5. Take both pairs of forceps. Use one pair to lift a corner of the spinach off the cutting board, then take the second pair and slide it under the short edge via the corner that was just lifted. Make sure the forcep has a full, flat grip on the whole edge. It is a **MUST** to avoid creating folds.
6. Bring the spinach over to the Instron. Lower the bottom short edge into the bottom set of clamps. Tighten the clamps with the knobs.
7. Jog the top clamp up or down to a point where the entryway of the clamp is accessible and lay the top edge of the spinach on one of the clamp surfaces.
8. Use an allen wrench to secure both the clamps and ensure the spinach will not pull out.
9. Cover the Instron with the acrylic shield and make sure the balance and extension are set to zero.
10. Hit start and record the data after the spinach rips.



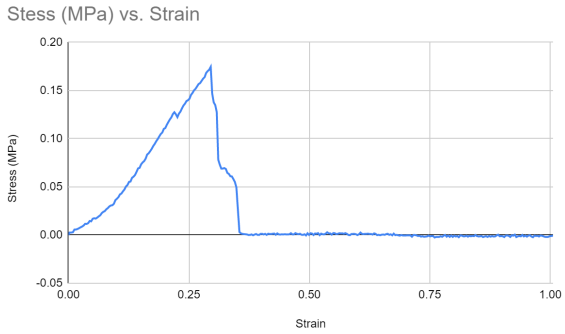


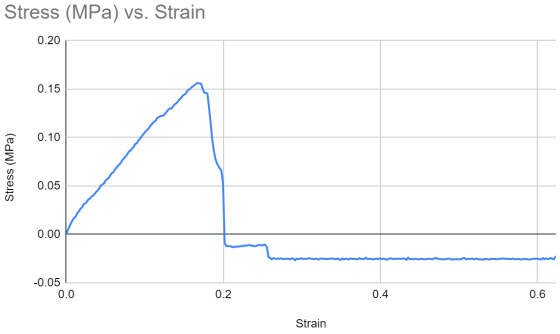
Tests 1 and 2



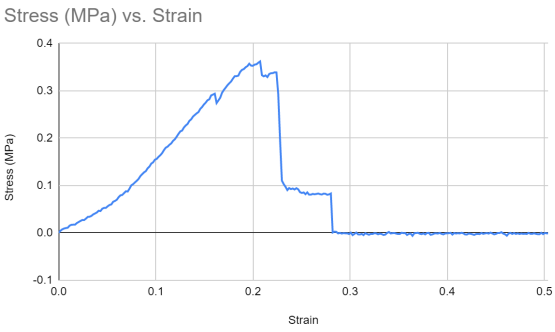


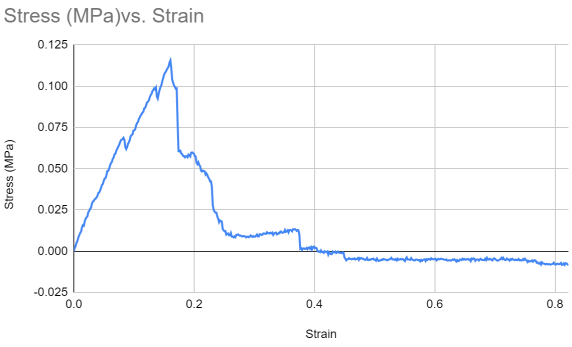
Tests 3 and 4



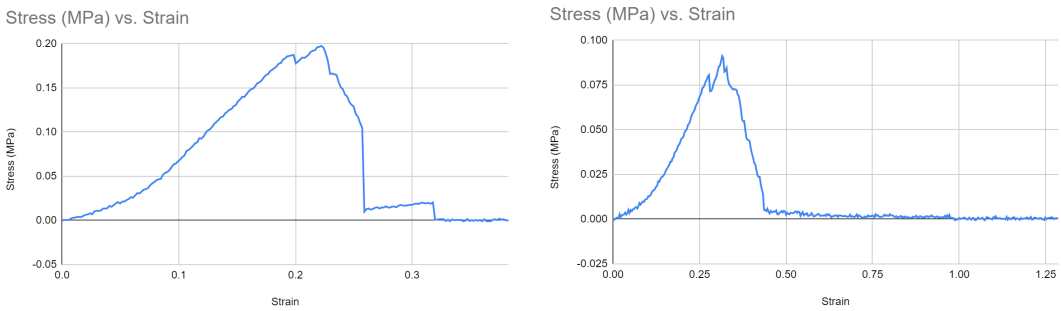


Tests 5 and 6





Test 7 and 8



Tests 9 and 10

Appendix D.1.2 Mechanical Testing of Leather

Before Testing:

- Before performing any mechanical test with the Instron, make sure the Instron is properly set up. Follow the procedure titled “Instron Setup” before continuing.

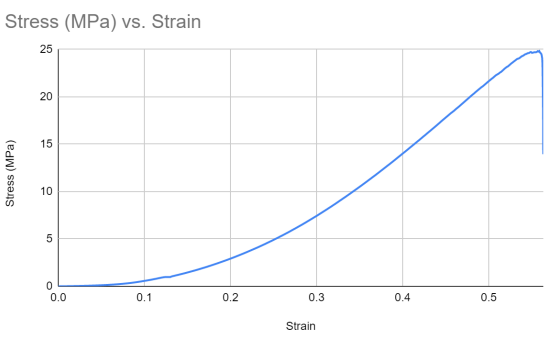
Materials

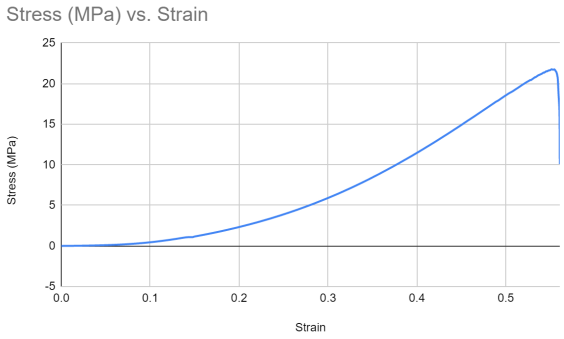
- Leather scrap
- Calipers
- Scalpel with blade
- Cutting board
- General lab materials (lab coat, safety glasses, gloves, mask, face shield)
- Instron 5544

Procedure

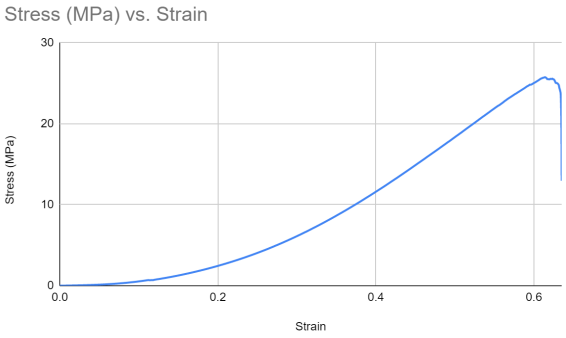
11. Follow the “Instron Setup” procedure before starting and put on necessary lab gear.
12. Place the leather on the cutting board.

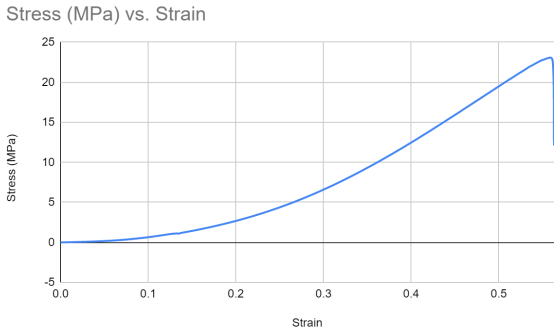
13. Take a scalpel and cut the leather into dog-bone shapes. Make sure they are symmetrical along the long side.
14. Use calipers to measure the dimensions of the leather. With a dog-bone shape, the dimensions that are necessary is the inside of the bone, not the edges. This will be the gauge length and width.
15. Carefully bring the scaffold over to the Instron. Lower the bottom edge into the bottom set of clamps. Tighten the clamps with the knobs.
16. Jog the top clamp up or down to a point where the entryway of the clamp is accessible and lay the top edge of the leather on one of the clamp surfaces.
17. Secure the top clamp.
18. Cover the Instron with the acrylic shield and make sure the balance and extension are set to zero.
19. Hit start and record the data after the scaffold rips.



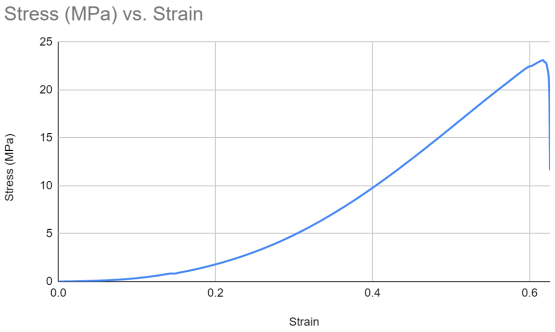


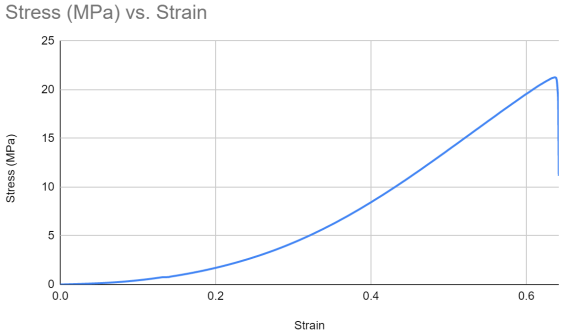
Tests 1 and 2



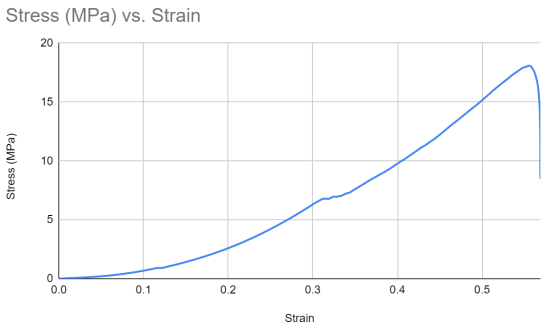


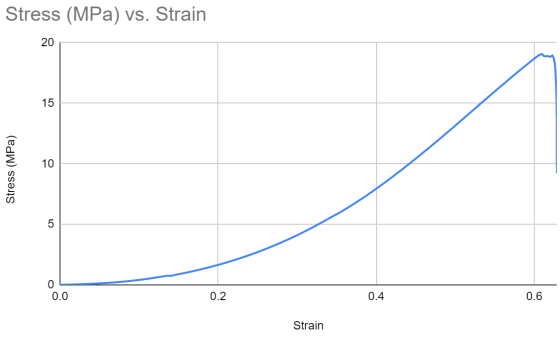
Tests 3 and 4



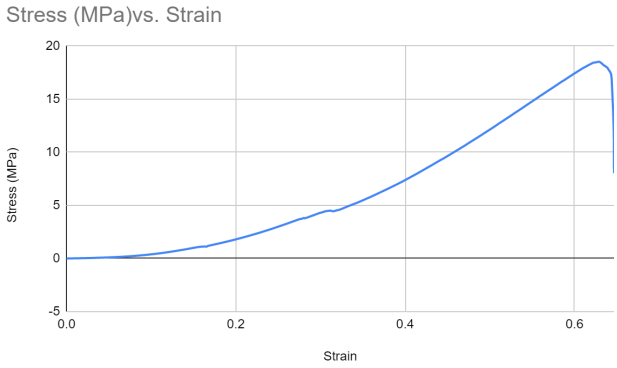


Tests 5 and 6





Test 7 and 8



Test 9

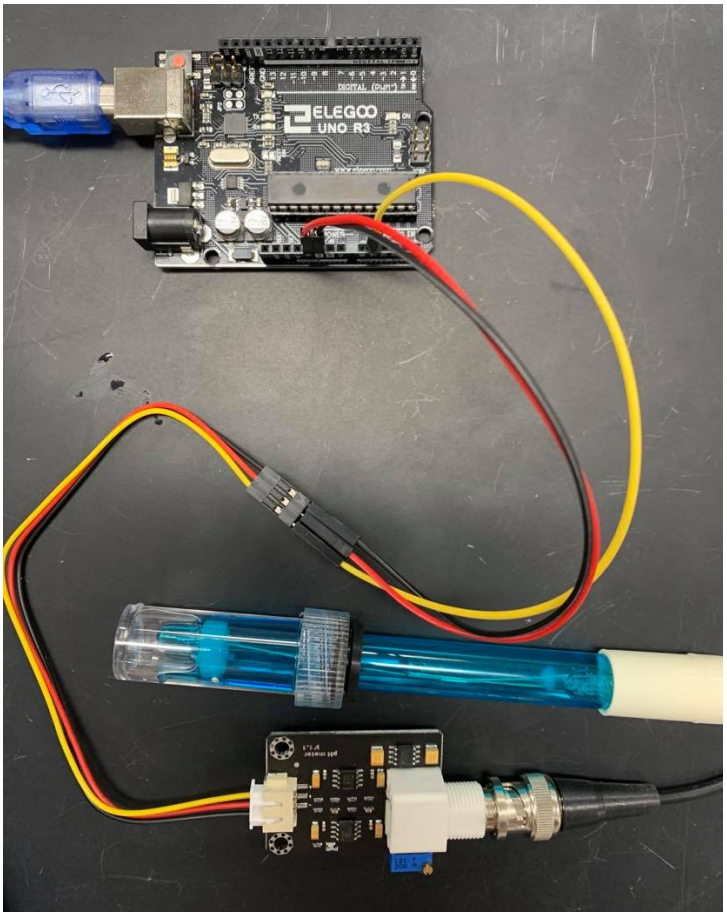
Appendix D.2 Cell Seeding onto Spinach Leaf in our Device with Cloning Wells

1. On the device, ensure that the 96 cloning well piece is properly inserted into the device and is making contact with the decellularized spinach leaf
2. Take the device into the biosafety cabinet and retrieve the bovine fibroblasts from the incubator, or thaw frozen fibroblasts.
3. Take a sterile serological pipette and begin seeding fibroblast cells into the cloning wells that make contact with the decellularized plant scaffold.
4. Place the device into an incubator with and allow cells to adhere (about 2 days)
5. Then the device procedure in Section 6.1.4 can be followed.

Appendix E: Arduino Sensor Procedure

A. pH Sensor

a. For this experimental set up, the pH sensor possessed a control BNC board that was connected to the Arduino. The pH sensor interface circuit utilized three connections to the Arduino. The first pin labelled V+ was connected to a 3.3 volt source. The pin labeled G on the interface connected to ground on the Arduino. Finally, the analog voltage output, or the pin labeled P_o on the interface, connected to A_1 on the Arduino. The circuitry for this system can be seen in Figure 1 below.



Circuit Diagram for pH sensor

Documented Source Code for pH Sensor (Fahad, 2020)

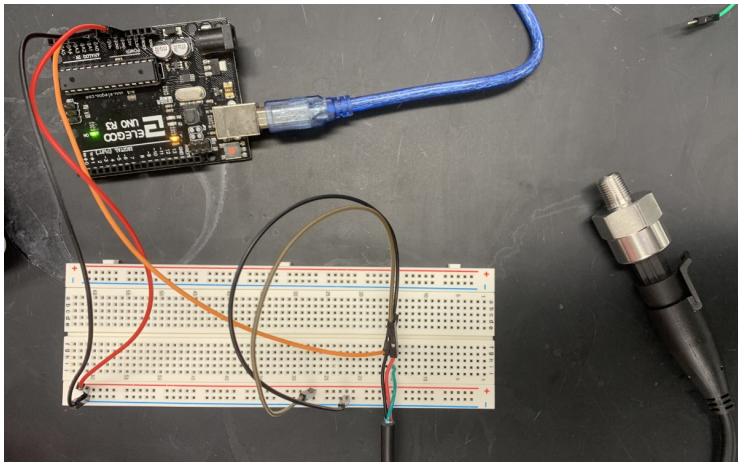
```
#define SensorPin 0          // the pH meter Analog output is connected with the Arduino's Analog
unsigned long int avgValue;  //Store the average value of the sensor feedback
float b;
int buf[10],temp;

void setup()
{
  pinMode(13,OUTPUT);
  Serial.begin(9600);
  Serial.println("Ready");    //Test the serial monitor
}
void loop()
{
  for(int i=0;i<10;i++)       //Get 10 sample value from the sensor for smooth the value
  {
    buf[i]=analogRead(SensorPin);
    delay(10);
  }
  for(int i=0;i<9;i++)        //sort the analog from small to large
  {
    for(int j=i+1;j<10;j++)
    {
      if(buf[i]>buf[j])
      {
        temp=buf[i];
        buf[i]=buf[j];
        buf[j]=temp;
      }
    }
  }
  avgValue=0;
  for(int i=2;i<8;i++)                      //take the average value of 6 center sample
    avgValue+=buf[i];
  float phValue=(float)avgValue*5.0/1024/6; //convert the analog into millivolt
  phValue=3.5*phValue;                      //convert the millivolt into pH value
  Serial.print("    pH:");
  Serial.print(phValue,2);
  Serial.println(" ");
  digitalWrite(13, HIGH);
  delay(800);
  digitalWrite(13, LOW);

}
```

```
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
pH:7.40
```

Serial Monitor Readings for pH

B. Pressure Transducer:

a. In order to calibrate and set up the pressure transducer sensor, first wire the circuitry setup seen in Figure 3 below. For this setup, the pressure sensor which has three connections that attaches to the Arduino. The black wire of the pressure sensor connects to ground. The red wire connects to a 5 volt source, and the green wire connects to A_0 of the Arduino. The pressure sensor needs to be calibrated through the use of existing equations for the code. The mathematical calculations used to calibrate the sensor may be found in Figure 4 below. These calculations are used to establish the code seen in Table 2. This code will successfully operate and run the pressure transducer.



Circuit Diagram for Pressure Sensor

Pressure Transducer Calculations:

~ analog read [analogRead();]

- This function takes the analog voltage value from a pin and converts it to a number. From the Arduino, 0-5 V will read 0 - 1,023 numbers.
- 1,024 possible values
- From product sheet: 0.5 V is where 0 psi is read
  4.5 V is where 100 psi is read

① $\frac{X}{1,024} = \frac{0.5V}{5V}$

$X = 1,024\left(\frac{0.5}{5}\right)$

$X = 102.4$ (zero pressure)

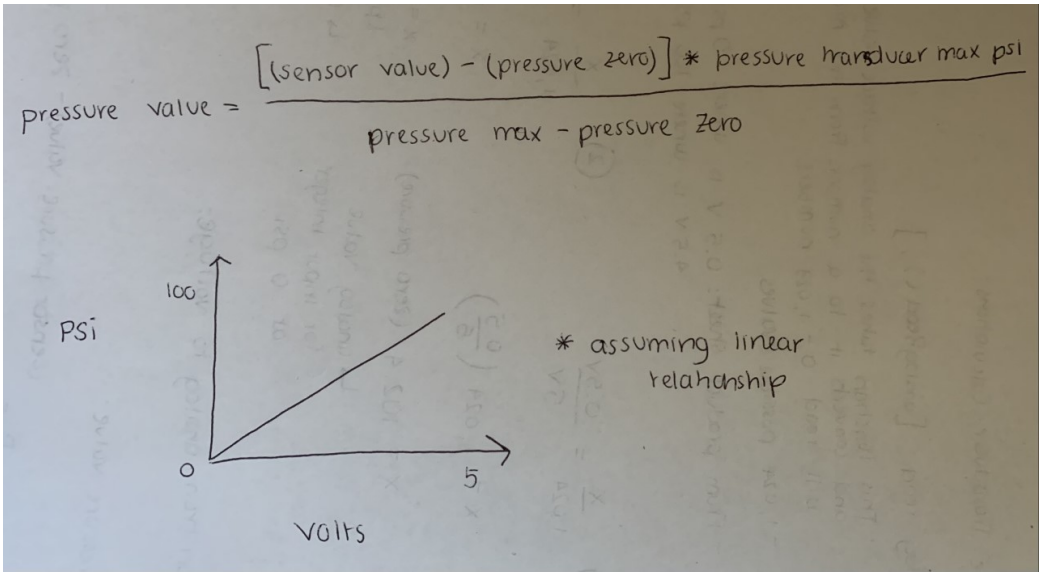
↳ analog value for input integer at 0 psi.

② $\frac{X}{1,024} = \frac{4.5V}{5V}$

$X = 1,024\left(\frac{4.5}{5}\right)$

$X = 921.6$ (pressure max)

↳ analog value for 100 psi



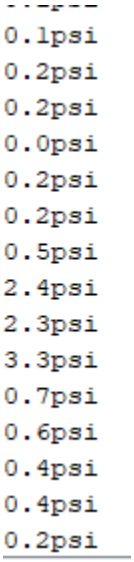
Mathematical Calculations for Coefficients of the Pressure Sensor Source Code (Ovens Garage, 2020)

Documented Source Code for Pressure Sensor (Ovens Garage, 2020)

```
#include <Wire.h> //allows communication over i2c devices
// #include "LiquidCrystal_I2C.h" //allows interfacing with LCD screens

const int pressureInput = A0; //select the analog input pin for the pressure transducer
const int pressureZero = 102.4; //analog reading of pressure transducer at 0psi
const int pressureMax = 921.6; //analog reading of pressure transducer at 100psi
const int pressuretransducermaxPSI = 100; //psi value of transducer being used
// const int baudRate = 9600; //constant integer to set the baud rate for serial monitor
// const int sensorreadDelay = 250; //constant integer to set the sensor read delay in milliseconds

float pressureValue = 0; //variable to store the value coming from the pressure transducer

// LiquidCrystal_I2C lcd(0x3f, 20, 4); //sets the LCD I2C communication address; format(address, columns, rows)

void setup() //setup routine, runs once when system turned on or reset
{
  Serial.begin(9600); //initializes serial communication at set baud rate bits per second
  pinMode(pressureInput, INPUT);
 // lcd.begin(); //initializes the LCD screen
}

void loop() //loop routine runs over and over again forever
{
  pressureValue = analogRead(pressureInput); //reads value from input pin and assigns to variable
  pressureValue = ((pressureValue-pressureZero)*pressuretransducermaxPSI)/(pressureMax-pressureZero); //conversion equation to convert analog reading to psi
  Serial.print(pressureValue, 1); //prints value from previous line to serial
  Serial.println("psi"); //prints label to serial
  //lcd.setCursor(0,0); //sets cursor to column 0, row 0
  //lcd.print("Pressure:"); //prints label
  //lcd.print(pressureValue, 1); //prints pressure value to lcd screen, 1 digit on float
  //lcd.print("psi"); //prints label after value
  //lcd.print("    "); //to clear the display after large values or negatives
  delay(500); //delay in milliseconds between read values
}
```

b. Once calibrated, test the sensor by blowing on it. This action should cause a slight increase in pressure and the serial monitor would show a result such as that seen

in Figure 5. This figure shows that the pressure slightly increases from 0.1 psi for the room to about 2 or 3 psi.

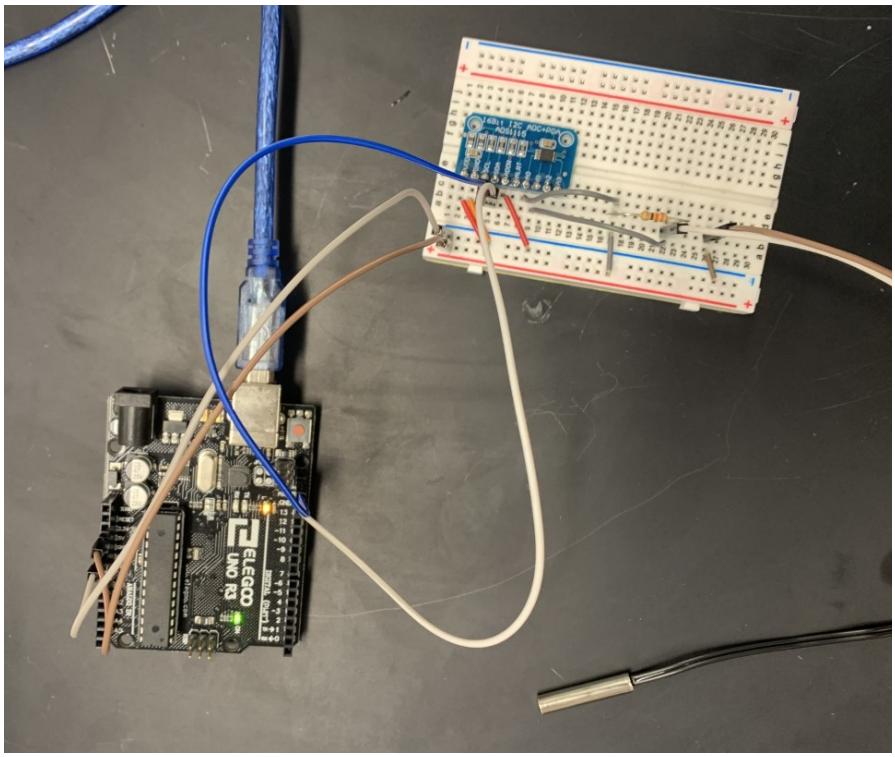


Serial Monitor Readings for Pressure Sensor

C. Thermistor:

a. In order to set up and calibrate the NTC 10K Thermistor, wire the circuit as seen in Figure below. For this experiment, a circuit was wired containing the NTC liquid thermistor and an ADS1115 analog to digital converter (ADC) to an Arduino. For the ADC, connect the VDD pin to a 5 volt source on the Arduino. Connect the GND pin of the ADC to ground on the Arduino. Connect the SCL pin of the ADC to the SCL port of the Arduino. Next, connect the SDA pin of the ADC to the SDA port of the Arduino. Connect the ADDR of the ADC to ground. Pins A_0 and A_1 of the ADC connect to a 330 ohm resistor. The 330 ohm resistor connects to the thermistor on one end and to the voltage source on the opposite

end. Finally, connect the other side of the thermistor to the 5 volt power source. This circuit setup may be seen below.



Circuitry Diagram for NTC 10K Thermistor

b. Before using the thermistor, it needs to be calibrated. The thermistor may be calibrated using a regression model. Shown in table 3 below is the final calibrated source code.

```
# include <Wire.h>
#include <Adafruit_ADS1015.h>
Adafruit_ADS1115 ads(0x48);
int16_t rawADCvalue;
float volts = 0.0;
float voltMax = 4.096;
float a0 = -15.53, a1= 558.36, a2 = -2029.9;
float a3 = 4162.2, a4 = -4085.7, a5 = 1514.7;
float TempDegC=0;
unsigned long StartTime = 0;

void setup(void)
{ Serial.begin(9600);
ads. setGain(GAIN_ONE);
ads. begin();
StartTime = millis(); }

void loop(void)
{rawADCvalue = ads.readADC_Differential_0_1();
volts = (rawADCvalue/32767.0) * voltMax;
TempDegC = a0 + a1*volts + a2*pow(volts,2) + a3*pow(volts,3) + a4*pow(volts,4) + a5*pow(volts,5);
unsigned long CurrentTime = millis();
float ElapsedTime = (CurrentTime-StartTime)/1000.0;
Serial.print("Time (sec)"); Serial.print(ElapsedTime,3);
Serial.print(", Volts Measured = "); Serial.print(volts,2);
Serial.print(", Temp. (deg C) = "); Serial.print(TempDegC,2);
Serial.print(", (deg F) = "); Serial.println(TempDegC*1.8+32.2);
delay(500); }
```

Source Code for Calibrated NTC 10K Thermistor

c. The serial monitor while the thermistor is exposed to room temperature should appear as shown in Figure below.

```
Time (sec)4.751, Volts Measured = 0.10, Temp. (deg C) = 23.25, (deg F) = 74.05
Time (sec)5.277, Volts Measured = 0.10, Temp. (deg C) = 23.25, (deg F) = 74.05
Time (sec)5.804, Volts Measured = 0.10, Temp. (deg C) = 23.25, (deg F) = 74.05
Time (sec)6.331, Volts Measured = 0.10, Temp. (deg C) = 23.25, (deg F) = 74.05
Time (sec)6.857, Volts Measured = 0.10, Temp. (deg C) = 23.25, (deg F) = 74.05
Time (sec)7.385, Volts Measured = 0.10, Temp. (deg C) = 23.25, (deg F) = 74.05
Time (sec)7.911, Volts Measured = 0.10, Temp. (deg C) = 23.78, (deg F) = 75.00
Time (sec)8.437, Volts Measured = 0.10, Temp. (deg C) = 24.30, (deg F) = 75.93
Time (sec)8.965, Volts Measured = 0.10, Temp. (deg C) = 24.81, (deg F) = 76.85
Time (sec)9.491, Volts Measured = 0.11, Temp. (deg C) = 25.81, (deg F) = 78.65
Time (sec)10.017, Volts Measured = 0.11, Temp. (deg C) = 26.29, (deg F) = 79.53
Time (sec)10.546, Volts Measured = 0.11, Temp. (deg C) = 26.78, (deg F) = 80.40
Time (sec)11.073, Volts Measured = 0.11, Temp. (deg C) = 26.78, (deg F) = 80.40
Time (sec)11.601, Volts Measured = 0.11, Temp. (deg C) = 27.25, (deg F) = 81.25
Time (sec)12.129, Volts Measured = 0.12, Temp. (deg C) = 27.72, (deg F) = 82.09
Time (sec)12.656, Volts Measured = 0.12, Temp. (deg C) = 28.17, (deg F) = 82.91
```

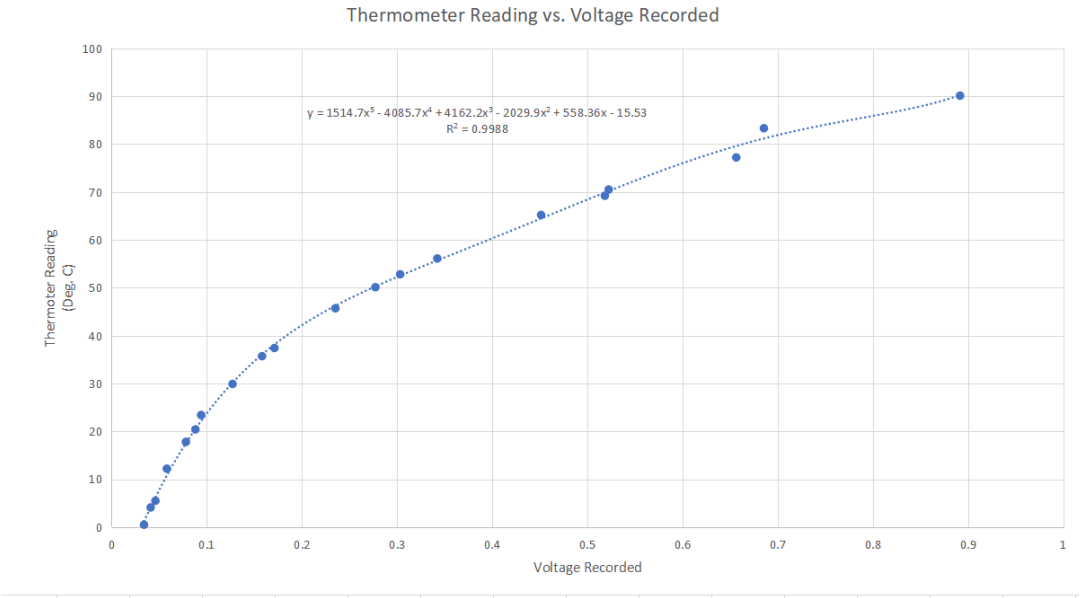
Serial Monitor Readings for NTC Thermistor

d. The coefficients of A_0 to A_5 from the source code in the table were adjusted and used by comparing results of a thermometer reading to the voltage reading of the thermistor. The voltage reading was used and transformed into a temperature. The data that recorded the thermometer and thermistor readings may be found in Table below.

Thermometer Temperature (Deg. C):	Voltage Reading:	Thermistor Rading (Deg. C):
0.5	0.034	130.177
4.1	0.041	128.721
5.5	0.046	127.702
12.2	0.058	125.09
17.8	0.078	121.291
20.4	0.088	119.312
23.4	0.094	118.12
29.9	0.127	112.246
35.7	0.158	107.148
37.4	0.171	105.101
45.7	0.235	95.927
50.1	0.277	90.583
52.8	0.303	87.528
56.1	0.342	83.485
65.2	0.451	73.339
69.2	0.518	68.311
70.5	0.522	68.052
77.2	0.656	60.005
83.3	0.685	58.555
90.1	0.891	49.643

Recorded Data for Thermometer and Thermistor

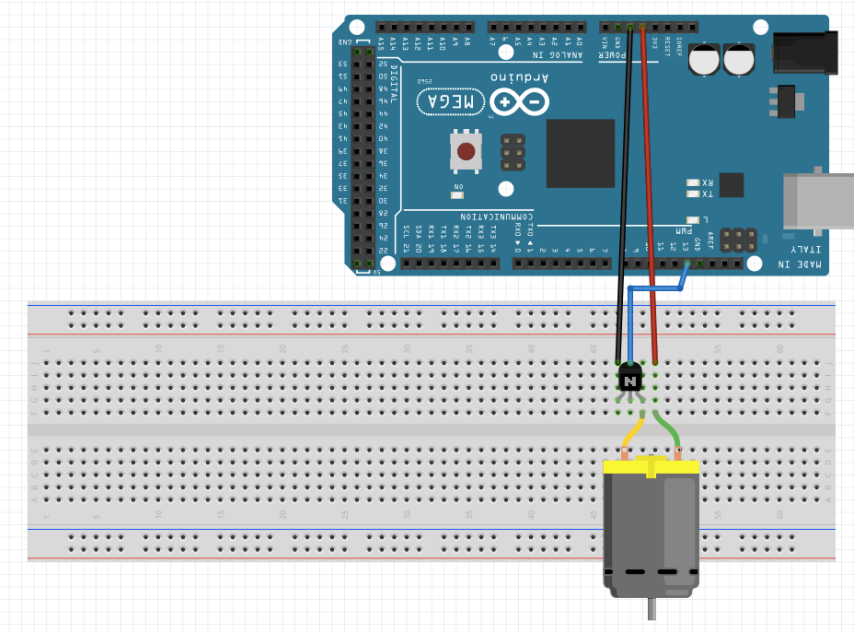
e. The data from Table was plotted as a regression model. The regression model mimicked a fifth degree polynomial function. The corresponding coefficients seen in the source code of the thermistor comes from this regression model. The coefficients of each power corresponds to its variable. For instance, the coefficient for x to the fifth power is A_5, the coefficient of x to the fourth power is A_4, and so on and so forth.



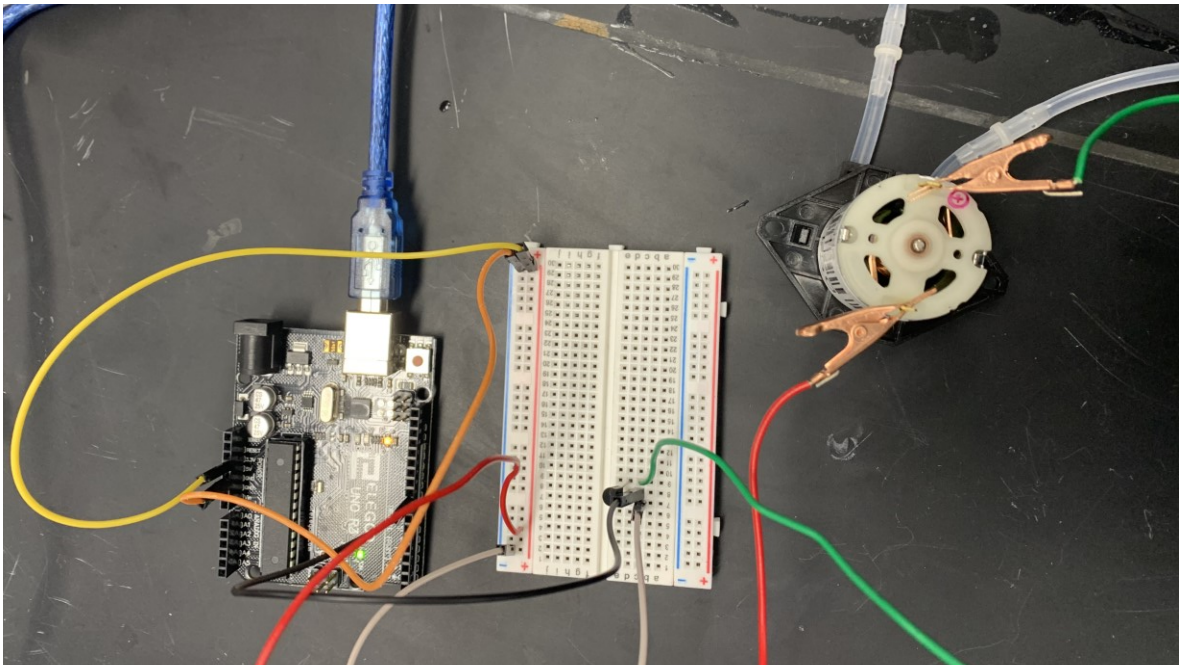
Regression Model for NTC Thermistor

D. Peristaltic Pump:

a. The circuit for a peristaltic pump is similar to the setup of a DC motor, with the exception that no motor controller is needed. Additionally, for the peristaltic pump another component is needed. The extra component is an NPN transistor PN2222. A transistor is a current driven semiconductor which can be used to control the flow of electric current. For this, the base lead controls a much larger current between the collector and the emitter. Essentially, a transistor adds current to operate the peristaltic pump. The pump is wired similar to the DC motor circuit as shown in Figure. For the set up, one end of the pump connected to a 5 volt source on the Arduino. The other end of the pump connected to the first prong of the transmitter. The second, or middle prong, of the transistor is connected to pin 8 of the Arduino. The last prong of the transistor is connected to a ground pin on the Arduino. The set up of the peristaltic pump assembled is shown in Figure below.



Circuitry Diagram for DC Motor and Transistor



Circuitry Diagram for Peristaltic Pump and Transistor

b. Using the wire setup, students will then be able to successfully run the peristaltic pump. The pump will operate with the code shown in the Table below. Using the

below source code, students will successfully be able to move water from one location to another using silicon tubing. In order to adjust the speed at which the pump moves water the PWM function is utilized. This code is additionally seen in the table. For this project, students are working with microfluidic applications. This means that the flow is minimal and laminar. Thus, an extremely small Reynold's number is at play.

```
const int pump = 8;


void setup() {
 pinMode(pump, OUTPUT);
}

void loop() {
 digitalWrite(pump, HIGH);
 delay(500);
 digitalWrite(pump, LOW);
 delay (500);
 }
```

Source Code for Peristaltic Pump (ElectronicsLearning, 2015)

```
int motorSpeed = 30;
int pump = 8;

void setup(){
}
void loop(){
analogWrite(motorPin, motorSpeed);
delay(1000);
}
```

Source Code for Peristaltic Pump with PWM

Appendix F: Recommendations

Appendix F.1 Cell Viability - Live Dead Assay for Bovine Fibroblasts and Staining

Once cells are seeded, future teams can determine that cells properly attach to the spinach leaf scaffold. The live/dead assay and staining protocols can allow the team to see the living and dead cells which can be analyzed as well as determining that the cells are only fibroblasts. The protocol is as follows:

Live/Dead Assay for Plates and Seeding Experiments

Solution 1:

- 1.0 mL Serum Free DMEM
- 2.0 µL Ethidium Homodimer-1
- 0.5 µL Calcein AM

Solution 2:

- 1.0 mL Serum Free DMEM
- 2.0 µL Ethidium Homodimer-1
- 0.5 µL Calcein AM
- 0.5 µL Hoechst Dye

Concentrations:

- 200 µL/96 Well plate
- 1 mL/12 Well plate

Process:

1. Incubate dead controls in 70% Ethanol for 30 minutes prior to experiment
2. Mix solution 1 within 1 hour of use
3. Incubate cells with solution 1 for 15 minutes
4. Mix solution 2 within 1 hour of use
5. Incubate cells in solution 2 for 15 minutes
6. Wash cells with 1x PBS 3 times
7. Fix cells in 4% Phosphate buffered formaldehyde for 10 minutes
8. Mount and coverslip cells on an uncharged microscope slide

Phalloidin/Hoechst Staining:

Reagents:

- Phosphate Buffered Saline
- 4% Paraformaldehyde (Only needed for tissues/cells that have not been fixed);
- 0.25% Triton-X
- 0.25% V/V Triton-X in PBS
- **10 µL** Triton-X in 3990 µL PBS
- 1% BSA
- 1% V (W)/V BSA in PBS
- **40 µL** in 3960 µL PBS
- Phalloidin (AF 488 Phalloidin A12379 or FITC Phalloidin, Invitrogen)
- 2.5% V/V Phalloidin in PBS
- **50 µL** in 1950 µL
- Hoechst
- 0.0167% Hoechst dye in PBS

- **0.5 µL** in 3000 µL PBS

For unfixed cells:

1. Rinse in PBS x2
2. Fix in 4% Paraformaldehyde for 10 minutes
3. Rinse in PBS x2 4. Follow directions for fixed cells

For fixed cells:

1. Rinse with PBS x2
2. Triton-X solution for 10 minutes
3. Rinse with PBS x2
4. Block with BSA solution for 30 minutes
5. Phalloidin solution for 30 minutes
6. Rinse with PBS x2
7. Hoechst solution for 3-5 minutes (typically 3)
8. Rinse with PBS x2
9. Cytoseal and coverslip
10. Store frozen at -20 degrees C.

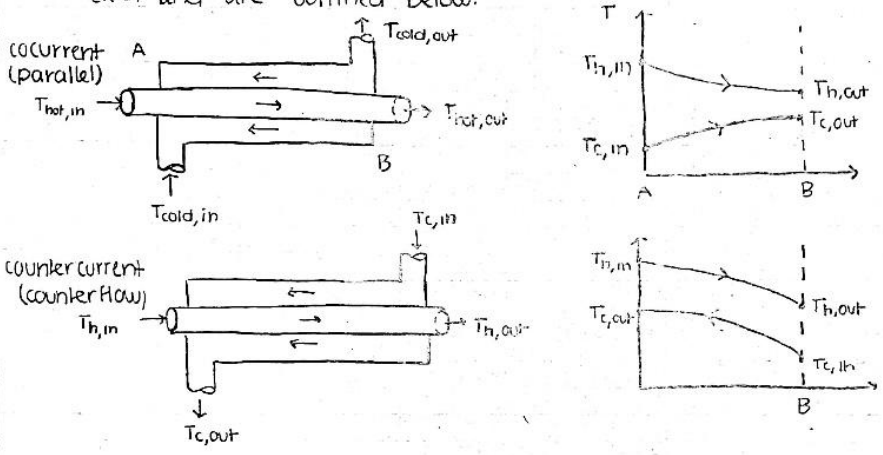
Results:

- F-actin is stained green if you used 488, red if you used FITC
- Nucleus is stained Blue

Appendix F.2: Heat Exchanger Analysis

Final HEX analysis:

~ The team will enhance the current design by heating media from fridge temperature to incubation temperature
~ The two fluids involved will be the media and water.
- the water will be the hotter fluid and warm the colder media
~ A counterflow HEX will be used. Two counterflow HEXs exist and are outlined below.



~ For our enhancement, we will use a countercurrent counterflow HEX.

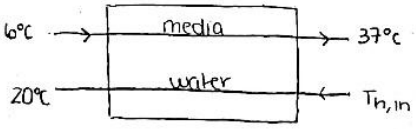
~ Assumptions:
- media will be treated as water
- mass flow rate of hot fluid in = $1 \cdot 10^{-4}$ kg/s
- Hot fluid comes out at room temperature

~ Known:

① Media: $T_{c,in}$ = refridgerator temperature = 6°C
$T_{c,out}$ = incubator temperature = 37°C
c_p = same as water = 4.19 kJ/kg K
$\dot{m}$ = measured from pump = 0.285 mL/s
$\approx 2.85 \cdot 10^{-4}$ kg/s

② Water: $\dot{m} = 1 \cdot 10^{-4}$ kg/s
c_p = 4.19 kJ/kg K
$T_{h,out}$ = 20°C (room temperature)
$T_{h,in}$ = unknown

solve for $T_{h,in}$
or how hot the water needs to be to heat media from 6°C to 37°C



$Q = UA(LMTD)$

↳ log mean temperature difference

$Q = \dot{m}_c C_c (T_{c,out} - T_{c,in})$
$= \dot{m}_h C_h (T_{h,in} - T_{h,out})$

$Q = \dot{m}_c C_c (T_{c,out} - T_{c,in})$
$= 2.85 \cdot 10^{-4}\ kg/s\ (4.19\ kJ/kg\ K)(37°C - 6°C)$

$= 2.85 \cdot 10^{-4} (4.19)(31)\ \frac{kg}{s} \cdot \frac{kJ}{kg\ K} \cdot K \rightarrow \frac{kJ}{s}$

$\boxed{Q = 0.03702\ kJ/s}$

$0.03702\ kJ/s = \dot{m}_h C_h (T_{h,in} - T_{h,out})$
$0.03702\ kJ/s = 1 \cdot 10^{-4}\ kg/s\ (4.19\ kJ/kg\ K)(T_{h,in} - 20°C)$

$0.03702\ kJ/s = 1 \cdot 10^{-4}(4.19)(T_{h,in} - 20°C)\ \frac{kg}{s}\ \frac{kJ}{kg\ K}$

$0.03702\ kJ/s = 4.19 \cdot 10^{-4}\ \frac{kJ}{s\ K}\ (T_{h,in} - 20°C)$

$0.03702\ \frac{kJ}{s} \div 4.19 \cdot 10^{-4}\ \frac{kJ}{s\ K} = T_{h,in} - 20°C$

$88.35\ \frac{kJ}{s} \cdot \frac{s\ K}{kJ} = T_{h,in} - 20°C$

$88.35\ K = T_{h,in} - 293.15\ K$

$T_{h,in} = 381.50\ K$

$\boxed{T_{h,in} = 108.35°C}$

Appendix References

Christian, S. (February 10, 2016). Vegan Leather Isn't As Ethical As You Think. Vocativ Retrieved from: https://www.vocativ.com/281599/vegan-leather-isnt-as-ethical-as-you-think/

Covington Anthony, D. Tanning chemistry: The science of leather. Royal Society of Chemistry. 2009.

ElectronicsLearning. (2015, June 18). *Peristaltic Pump Circuit Controlled By an Arduino* [Video]. YouTube. https://www.youtube.com/watch?v=4W7tiLa5zGU

Fahad, E. (2020, December 8). pH meter Arduino, pH Meter Calibration, DIYMORE pH Sensor Arduino Code. Electronic Clinic. https://www.electroniclinic.com/ph-meter-arduino-ph-meter-calibration-diymore-ph-sensor-arduino-code/

EPA. (2020, September 8). *Learn about Dioxin*. EPA. https://www.epa.gov/dioxin/learn-about-dioxin.

J.R. Gershlak, S. Hernandez, G. Fontana, L. R. Perreault, K. J. Hansen, S. A. Larson, B. Y. Binder, D. M. Dolivo, T. Yang, T. Dominko, M. W. Rolle, P. J. Weathers, F. Medina-Bolivar, C. L. Cramer, W. L. Murphy, and G. R. Gaudette, "Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds," Biomaterials, vol. 125, pp. 13–22, 2017. https://www.sciencedirect.com/science/article/pii/S0142961217300856

Liberty Leather Goods. (n.d.). *The Leather Industry - An Overview of Fascinating Facts*. Liberty Leather Goods. https://www.libertyleathergoods.com/leather-industry/.

Liu, Cheng-Kung & Latona, Nicholas & Taylor, & Eble, & Ramos, M. (2015). Characterization of mechanical properties of leather with airborne ultrasonics. J. Am. Leather Chemists Assoc. 110(3):88-93. 2015. Liu, C.-K., Latona, N. P., and Taylor, M., Eble, C., and Ramos, M. L. Journal- American Leather Chemists Association. 110. 88-93.

Medic Tests. (2021). Ventilation/Perfusion (V/Q) Ratio and Mismatch. Retrieved from https://medictests.com/units/ventilation-perfusion-v-q-ratio-and-mismatch

Ovens Garage. (2020, April 6). *Pressure Sensor - Arduino* [Video]. https://www.youtube.com/watch?v=UrqPxwsPWGk

Realeather. (2020, February 28). Real Leather is Greener than Imitations. Retrieved from https://www.realeather.com/projects-and-ideas/Category/7/About-Leather/Article/59/Real-Leather-is-Greener-than-Imitations

RT116 Cardiac Review, Circulation and Hemodynamics. (2010). Ventilation – Perfusion Relationships. Retrieved from https://media.lanecc.edu/users/driscolln/RT127/Softchalk/Heart_Perfusion/Heart_Perfusion8.html

Wells, H.C., Holmes, G. and Haverkamp, R.G. (2016), Looseness in bovine leather: microstructural characterization. J. Sci. Food Agric., 96: 2731-2736. https://doi.org/10.1002/jsfa.7392