WPI

Farm in a Lab: Cultivating A Lab-Grown Meat Solution

A Major Qualifying Project Report

Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the Degree of Bachelor of Science

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May 18, 2020

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Acknowledgements

The team would like to thank project advisor Dr. Glenn Gaudette for his guidance and support throughout the course of this project. The team would also like to thank the following students in Gaudette's Lab including Jordan Jones, Richard Thyden, Emily Robbins, William DeMaria, and Alex Rebello for their mentorship in the lab. A thank you to Scott Allan of the University of Bath and New Harvest for his guidance early in the project. Thank you to Lisa Wall for ordering supplies to help make our testing and prototype design possible.

Abstract

Animal agriculture accounts for many environmental, ethical, and economic concerns. Current practices in this industry are not sustainable due to limited resources, such as water and land, and the rapidly increasing population. Alternative solutions need to be considered in order to sustain the growing demand for meat production. One solution is cellular agriculture, which is the process of culturing muscle tissue in the lab. Lab-grown meat demonstrates promising results as an alternative option to traditional meat production. It provides a water-reducing, slaughter-free, decreased resource option for producing meat. Despite recent developments in lab-grown meat technologies, the process of producing cultured meat products remains expensive, time consuming, and difficult to scale. This project aimed to address this problem, by creating a bioreactor for large-scale bovine muscle tissue production utilizing decellularized plants as scaffolds. By changing the design from a stationary to rotating apparatus, the team unlocked a more scalable, time-efficient path to producing lab-grown meat. The results of this project demonstrate the effectiveness of the rotating bioreactor model for lab-grown meat production.

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Chapter 1. Introduction

The state of the meat industry today calls into question the issue of sustainability, and if we will be able to keep up with the growing demands. The meat industry relies heavily on natural resources which are limited in quantity, meaning there will ultimately be a time where these resources are no longer available as they are now. 450 gallons of water are required to produce one quarter-pound hamburger [1]. This number is exponentially larger than the amount of water required for the production of dairy products and fruits/vegetables. An increase in meat production in order to grow the industry with the population will lead to an increase in the amount of water allocated to this industry. Increasing water to the meat industry will mean taking it from other sectors such as the water provided to households for drinking and bathing. This will eventually lead to an increase in global water scarcity [2].

Another limited natural resource being consumed by the meat industry is land that is being used for livestock production. Currently, about 51% of U.S. land is being used for farmland [3]. This includes land used for cropland and for livestock grazing. It is important to consider how efficiently this land is being used. The production of one kilogram of meat requires 22 square meters of land, and a large quantity is contributed to growing the feed for livestock [2]. Each year, 13 billion hectares of forest land all over the world is converted to land to be used for agriculture [4, p. 59]. These statistics demonstrate that land is a finite resource and at some point we will reach the peak where we cannot grow the meat industry by traditional methods anymore. Same is the case with water; at some point we will no longer be able to increase the amount of water allocated to the meat industry.

The question that remains is how short will we actually fall from meeting that demand? In 2016, the world population was 7.4 billion, and by the year 2050, that number is expected to reach 9.2 billion, an increase of 23% [5]. Meat production will need to increase by 70% in order to sustain the growing population, but with the state of the meat industry today, we are only capable of a 43% increase [6], leaving a 27% gap that needs to be filled.

There are currently many different companies attempting to find solutions to filling this gap, all of which are steps in the right direction. One of the most noteworthy of these companies is Mosa Meat. Mosa Meat is a company working on the production of meat from harvested cow cells using cellular agriculture. They are marketing this as a “clean meat” alternative, which promotes a much more efficient process that requires less livestock for more meat. This alternative has a great impact in decreasing the environmental impact of the meat industry, while also creating a more reliable source of food that could satisfy the demand of an increasing population [7].

This project was aimed at finding solutions to the current major issues of lab-grown meat technologies. These issues specifically include scalability of the process, water usage and cost of production. To begin exploring solutions to these problems, the team looked to decellularized

spinach leaves as a cost-efficient, edible, and suitable scaffold for growing bovine myocytes, or muscle cells. The team developed a bioreactor model that allowed for cell attachment and proliferation, while also incorporating components to make it a scalable design that uses less water, and is a simple device in terms of user interaction.

The main concept of this final design is a rotating bioreactor. This design incorporates cells in suspension with decellularized spinach leaf scaffolds, in cell culture media. The solution was allowed to rotate at a 15 degree angle to the horizontal, creating a dynamic environment that promotes cell attachment. Through testing of this design and validation by Hoechst 33342 and Alexa Fluor Phalloidin-488, the team was able to confirm myoblast attachment to the plant scaffolds using this method, but also gathered evidence to support another concept related to the opportunity for scalability of this design; once the myoblasts attached and began to proliferate, they would “jump” from the original scaffolds to scaffolds that were added days later. This was a revolutionary discovery, and will prove as a useful system for making current lab-grown meat production processes much cheaper, more efficient, and scalable. Additionally, with projected calculations of producing one kilogram of meat using this bioreactor design, 86% less water is utilized in comparison to traditional agricultural methods.

Chapter 2. Literature Review

2.1 Environmental & Societal Considerations

Agriculture (more specifically livestock production) has significant environmental, economic and cultural impacts in our society. The following sections will discuss the most prevalent issues regarding agriculture to further understand the rapidly-growing demand for lab-grown meat. The issues to be discussed will detail climate change and greenhouse gases, animal rights, resources for meat production, job redistribution, and the economical impact, all in relation to meat production.

2.1.1 Climate Change & Greenhouse Gases

Greenhouse gas production is the driving force behind the increasingly alarming issue of climate change. It is a major subject of modern research. *Figure 2.1* shows an overview of how different sectors contribute to greenhouse gas production.

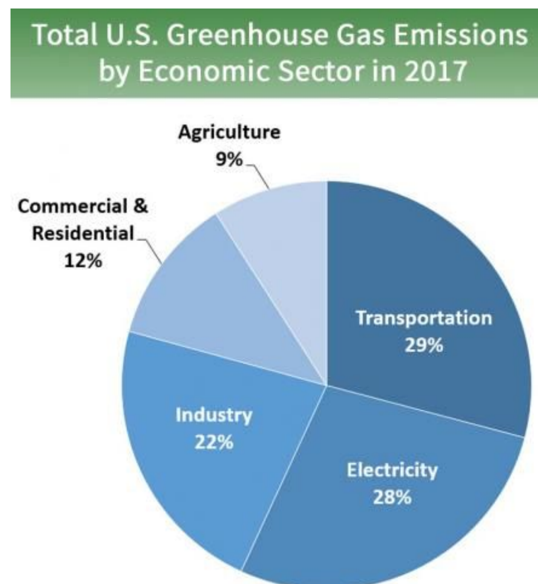


Figure 2.1: Breakdown of greenhouse gas emissions by economic sector [8]

This project seeks to address the contribution of the agricultural sector to greenhouse gas production, which accounts for 9% of all greenhouse gas production in the United States according to the Environmental Protection Agency (EPA) [8]. CO_2 outputs from within this sector can be further categorized as seen in *Figure 2.2* and *Figure 2.3*.

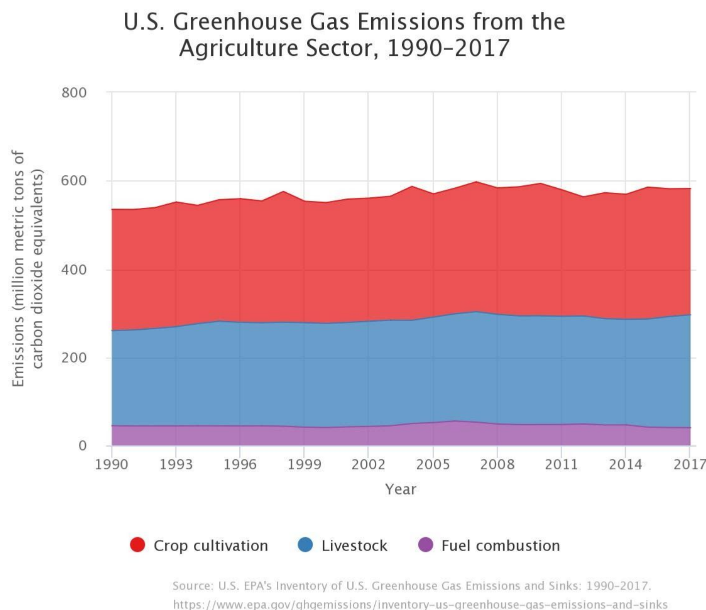


Figure 2.2: Emissions of the 3 major contributors (crop cultivation, livestock, and fuel combustion) in the agricultural sector from 1990-2017 [9]

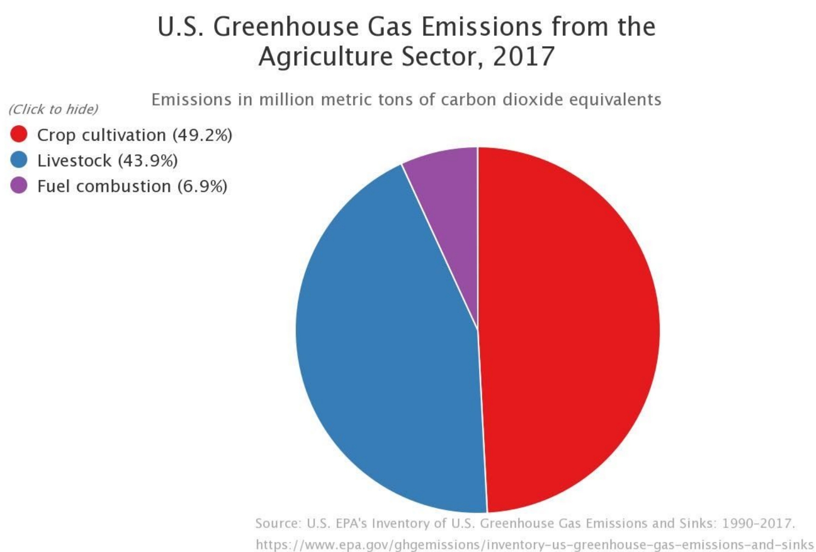


Figure 2.3: Emissions of the 3 major contributors (crop cultivation, livestock, fuel combustion) in the agricultural sector for 2017 [9]

This data shows that emissions from agriculture have not changed drastically in the past 27 years and that livestock make up a substantial portion of the overall contribution (about 44% according to Figure 3). In contrast, the EPA has identified a promising trend in CO_2 emissions in the electricity sector, showing a decrease over the past ten years, as seen in *Figure 2.4* [9].

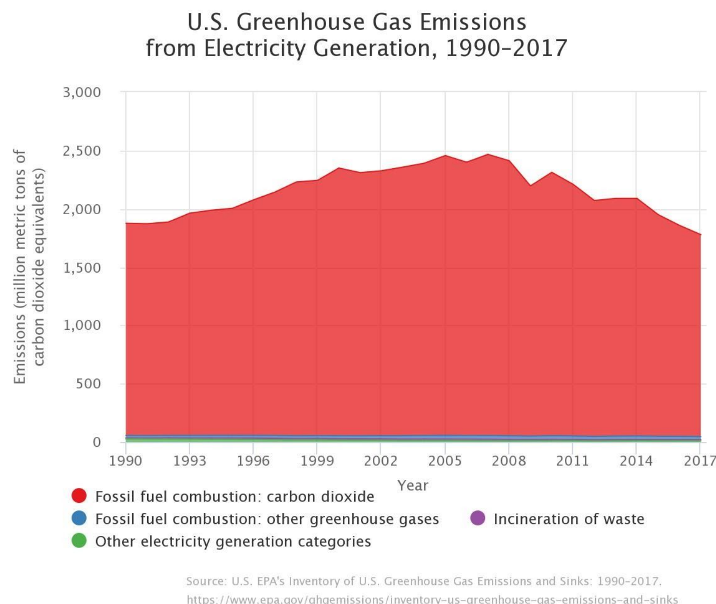


Figure 2.4: Greenhouse gas emissions in the electricity sector [9]

Lab-grown meat production as an alternative to traditional agriculture could mimic the promising trends of the electricity sector with efficient production practices, in reference to EPA's data.

2.1.2 Animal Rights

Cattle that are raised for beef production are one of the few animals that are still raised outdoors [10]. They are often sent to spend the end of their lives on a feedlot, a cramped area with hundreds to thousands of other cattle, where they are deprived of the vegetated pastures that they are used to. Additionally, they are typically fed a corn and soy diet which can lead to illnesses as this is not their native diet [10].

The ethics of beef cattle farming has prompted many Americans to consider a vegetarian-based diet. According to a study published by *Vegetarian Times*, which surveyed a representative population sample of the United States, 3.2 percent of American adults, 7.3 million people, have a vegetarian-based diet and about one million of these individuals are vegan [11]. One tenth of the U.S. adult population, or 22.8 million people, reported that they follow a vegetarian-inclined diet [11]. Despite these numbers, the quantity of meat used in the American diet continues to increase [12].

2.1.3 Resources for Meat Production

The meat industry demands many resources which include the animals themselves, land, energy, and food that goes into raising animals for their meat. To understand the potential impact

of lab-grown meat alternatives, it is important to first assess the current state of these resources in relation to the meat industry.

2.1.3.1 Land Usage

About 51% of land in the United States is used for agriculture. This includes use for cropland, pastures, and land for livestock grazing [3]. *Figure 2.5* depicts the land usage for farming in the United States.

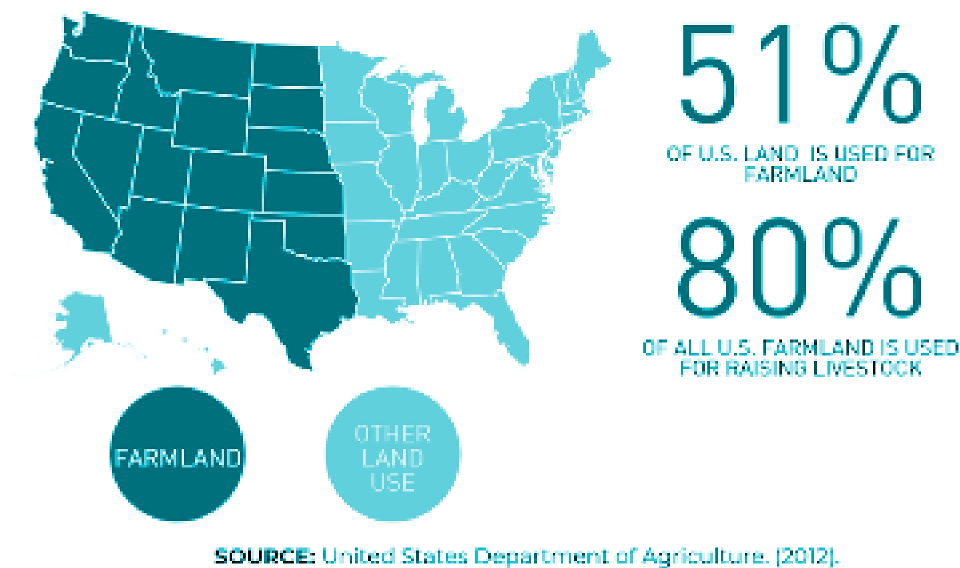


Figure 2.5: Farmland use in the United States [3]

Not only is agricultural land used for raising livestock, it is also used for the production of grain or other food to feed livestock. The process of growing animal feed itself proves to be an inefficient process in itself, as it creates a significant loss of calories per land used [13]. The production of one kilogram of meat requires the use of 22 square meters of land, mostly due to the large quantity of feed required to grow livestock [2].

As the global population continues to rise, so does the total necessary production of meat in the world to keep up with the increasing demands. Each year, about 13 billion hectares of forest land all over the world is converted to land to be used for agriculture. Land is a limited resource, therefore there will be a point where the amount of land required to keep up with the meat production demands will not exist. It is also important to note that as the amount of open land to be converted for agricultural use decreases and livestock density increases, there will be an increased amount of agricultural land closer to human-populated areas. This brings up issues related to human health, as many emerging diseases originate and are carried by livestock. There

is also the concern of the effect of hormones and antibiotics used in livestock production, and these chemicals being utilized in close proximity to communities [4].

2.1.3.2 Water Allocation

Water allocation for meat production is a major concern related to this sector. *Figure 2.6* summarizes the amount of water used to produce one kilogram of food in various categories. Ox meat, which refers to bovine meat, is the food that requires the most amount of water for the production of one kilogram of meat, around 15,500 liters [1]. This is more water than the average domestic household uses over a ten month span [2].

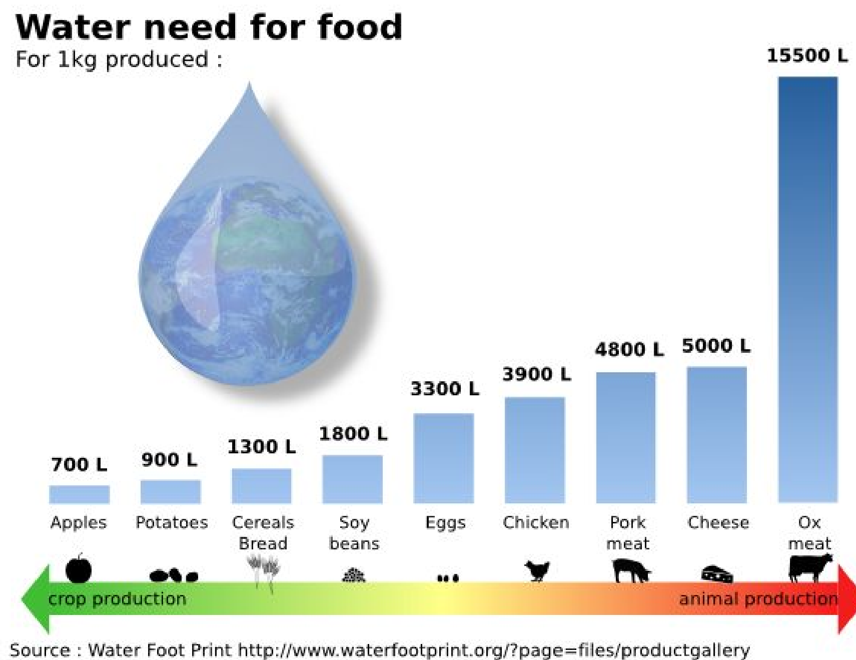


Figure 2.6: Amount of water to produce one kilogram of food [1]

Similar to land use, water is a limited resource, and currently, about 8% of global freshwater is being used for livestock production. This percentage increases when considering the amount of water needed to produce grain for livestock feed. As meat production continues to scale up, so will the water usage for meat production, which will lead to increased global water scarcity [2]. In a world where climate change is also an increasing issue, there may come a point in time when we need to reconsider the way water as a resource is allocated currently, and there are more necessary sectors than meat-production, especially when an alternative production process exists.

2.1.3.3 Energy Efficiency

As a final point of concern in terms of resources, it is important to consider the idea of energy efficiency in the meat production process. Meat production is a very energy-inefficient process. The process to produce meat for human consumption includes:

- the energy to produce the grain to feed the cattle/other livestock
- the fuel to transport the livestock to slaughter, then again to bring them to the market.
- the post-production energy that goes into cooking/preparing the meat

It takes nine times more energy to produce meat for human consumption in comparison to grain, when looking at the energy required to produce one calorie of grain versus one calorie of meat [14].

2.1.4 Job Redistribution

Agriculture is one of the most important sectors of the American industry, with farming and its related industries adding over one trillion dollars in gross domestic product (GDP) between the years of 2015 and 2017, or over 5% of the annual GDP [15]. Farms and livestock only contribute to a small fraction of this sector, less than 200 billion dollars in GDP as seen in *Figure 2.7*, the remaining industries rely on the agricultural industry to be able to produce goods and services.

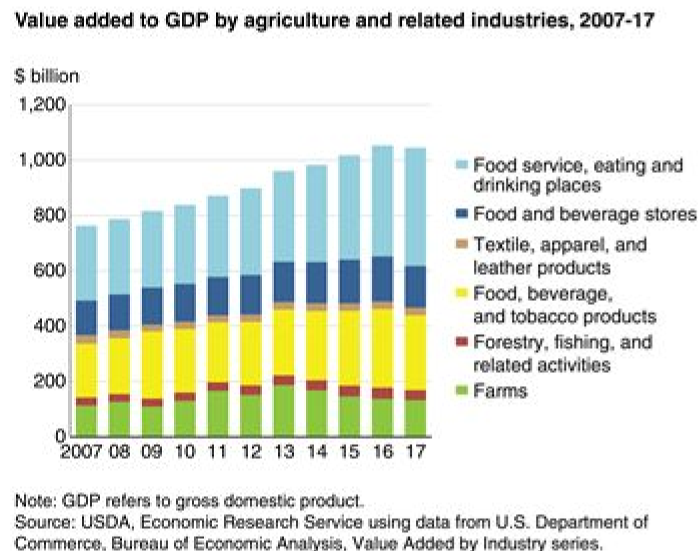


Figure 2.7: GDP of Agricultural and related fields, 2007-2017 [15]

In addition to the large portion of the GDP that is produced by farming, raising livestock, and its associated professions, the agricultural sector and its related fields provide work for

millions of Americans. According to the Bureau of Labor Statistics, 876,300 "agricultural workers," or people who work directly to maintain crops and tend to livestock, were employed during 2018 [16]. According to the Economic Research Service of the US Department of Agriculture, 21.6 million jobs or 11% of the total employment in the United States was directly in or closely related to the agricultural field, as seen in *Figure 2.8* [15].

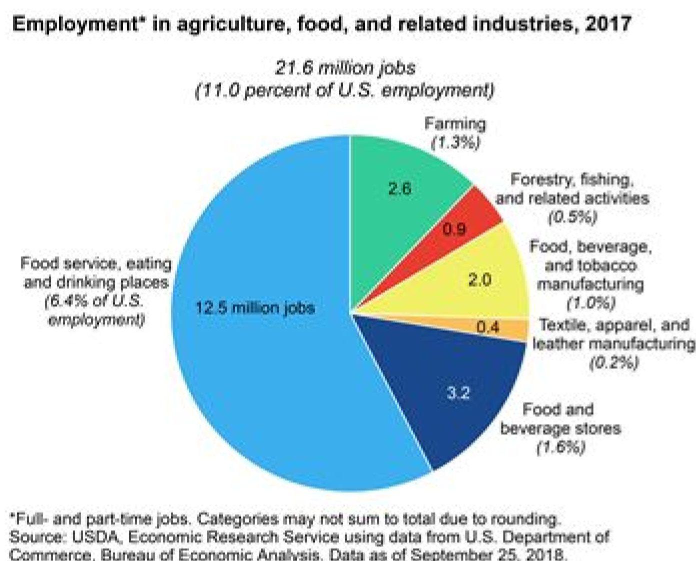


Figure 2.8: Breakdown of employment in the agriculture, food, and other related industries [15]

While the GDP produced by agricultural and related fields should remain relatively constant due to the high demand and integration into the U.S. economy, the hundreds of thousands of farming jobs that provide employment to blue collar workers around the country are threatened by the production of lab-grown meat products. If the market shifts to favor heavily against the commercial raising of livestock for food and instead favor lab-grown meats, these entry level jobs will become unavailable to almost one million unskilled laborers that they employ and instead be provided to those with the capabilities of obtaining a college education or enough training to run the sophisticated lab equipment required to produce lab-grown meat [15], [16].

2.1.4.1 Political Ramifications

Because of the large investment that traditional meat production methods have in the U.S. economy, attempting to modify production methods from farming to lab based cellular growth would have wide-ranging political ramifications. The cattle industry lobbying group has an influence over a small number of key senators in the U.S. government and are fiercely opposed to this switch, due a potential loss of profits jobs [17]. In order to gain a favorable view in the eyes of traditional farming lobbyists, numerous concessions and restrictions on the products

would have to be agreed to to provide lab-grown beef a viable economic market, of which many of these concessions would be difficult to predict until products are closer to full distribution.

2.1.5 Economical Impact

The meat industry has a large impact on the U.S. economy as it accounts for $832.4 billion dollars annually, which is equivalent to just less than six percent of the total GDP [18]. When evaluating alternatives to traditional meat, we must consider the impact it may have on jobs and the economy. However, with the population expected to increase, the demand for food across the nation will also rise.

2.1.5.1 Population Growth

As of 2017, the world population was up to 7.6 billion people and is expected to increase by another billion in 2030 [5]. In the same year, the annual meat production is predicted to be 376 million tons [6]. The rate of meat consumption is increasing year over year as a result of population growth and increase in average income, it is important to consider if this can be sustained [19]. Urbanization, especially in developing countries, could lead to a situation where the demand for meat will not be met by its production [20]. Looking to *Figure 2.9*, we can see that the required increase in meat production by the year 2050 in order to feed the growing population would need to be 70%. With the limited resources such as water and land, that capacity is actually only an increase of 43% [19], [20]. To fill this gap, we must consider alternatives, like lab-grown meat, to be able to provide enough products to meet the rising demand.

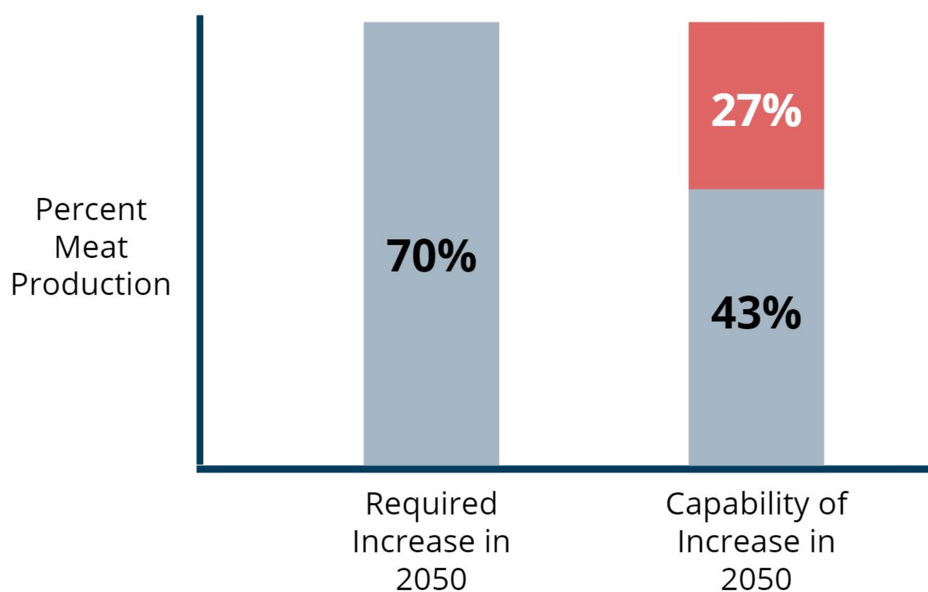


Figure 2.9: Project meat production and capacity in 2050 [19], [20]

2.2 Possibility of Lab-Grown Meat Alternatives

In order to meet the demand for meat production in the future, alternative methods must be considered. Promising alternatives are currently being explored in the lab-grown meat sector.

2.2.1 Media

One of the most vital ingredients necessary to successfully culture cells in vitro is the cellular growth media. Media is used to provide oxygen and nutrients to the cells while growing and used to remove waste when it is removed from the system. One of the most widely and commonly used media for cellular cultures is Fetal Bovine Serum, which is derived from the clotted blood of a calf.

2.2.1.1 Fetal Bovine Serum

Fetal Bovine Serum's (FBS) main component is bovine serum albumin, which is used as a primary nutrient and source of protein for cells in culture. FBS also contains small amounts of amino acids, sugars, lipids, hormones as well as a number of growth factors that promote cellular growth [21].

Unfortunately, even though FBS is widely used in cell cultures, the solution is expensive, with prices frequently ranging from $350 to well over $500 per 500 mL bottle of FBS [22].

2.2.1.2 Non-Animal Alternatives

Another option available for use in cell cultures is known as animal-free media [22]. This media contains all the nutrients required for cell growth while containing no components directly sourced from animals, specifically it is serum free. However, animal-free media poses its own challenges for cell culturing lab-grown meat, as many nutrients and recombinant proteins found in animal-free media options are derived from human or animal cells and components [23]. In addition to the costs associated with the special media, the consumption of meat products grown with media sourced from humans is subject for ethical concerns.

2.2.2 Bioreactors

For this project, our team will be focusing on the design of a bioreactor for tissue engineering. One source describes these vessels as "...in-vitro culture systems...", used "...to mature and guide the development of tissue engineered constructs" [24]. Since bioreactors cover such a broad range of industries, they can be classified in a number of ways In this section, we will be focusing on bioreactor classifications that fit best with what the initial and refined client statements in *Chapter 3*.

2.2.2.1 Mixing Bioreactors, Miniature Bioreactors, & Examples

The first major classification that Blose describes in *Bioreactors for Tissue Engineering Purposes* is the mixing bioreactor. The author describes the importance of mixing bioreactors by stating "[mixing bioreactors] alleviate both the concerns of supplying nutrients and removing waste by consistently circulating the media, whether through stirring, rocking, or perfusion" [24]. The author also describes multiple different examples that fit into the classification of mixing bioreactors, including perfusion, rocking, or stirring bioreactors.

Another important consideration for this project is the relative size we can achieve for our bioreactor, while still providing scalability. One paper we explored defined these types of bioreactors as Miniature-Bioreactors, or MBRs [25]. The author describes how these reactors "can be used in conjunction with automated robotic systems and other miniature process units to deliver a fully-integrated, high-throughput (HT) solution for cell cultivation process development" [25].

The authors also provided several examples of MBRs, along with the type each reactor falls into. Since our project may fall into the stirring reactor type, we focused on a few with that classification. A prototype of a MBR developed by the authors is shown below in *Figure 2.10*.

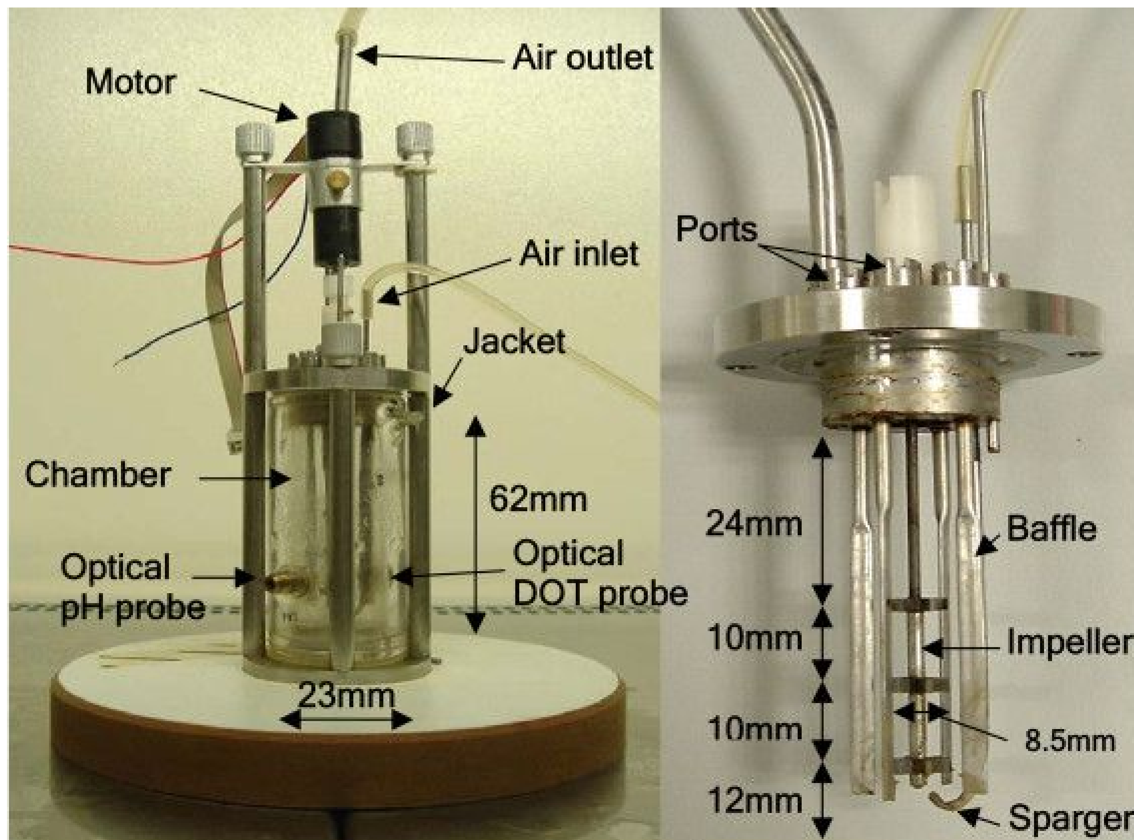


Figure 2.10: Prototype reactor, made by Betts & Baganz [25]

Figure 2.10 provides insight to an example design of a bioreactor, as commercial bioreactors provide less information in specific design. In addition to the design, the authors also specified that the pH probe and DOT (dissolved oxygen) probes can be monitored online [25].

A more commercial example is the BioXplorer 100 from HEL, which is an MBR for fermentation and cell culture [26]. This reactor claims to be fully-automated, with options for both monitoring parameters and data exploration. The website also emphasized scalability and integration with other robotic processes, and data collection and visualization.

All of these bioreactor designs show that there is a possibility to create a self-sustaining, scalable bioreactor for the purpose of tissue engineering applications.

2.2.3 Plant Scaffolds

Currently, cultured meat is being produced amorphously as the current scaffolding material is not suitable for meat production as they do not promote cell alignment, do not allow for easy removal after cell proliferation, and are not edible as a food product. Plant scaffolds provide an opportunity to address these issues. Plants, such as spinach, can be decellularized, which is the process of removing all plant cells, yet maintaining the integrity of the leaf, the vasculature landscape of the plant, and the cellulose structure. It has been shown that human endothelial cells can be seeded on a plant scaffold and utilize the vasculature of the plant to adhere to the scaffold [27]. More recently, a team proved that muscle cells can be seeded on a plant scaffold, observations of myocyte development, multinucleation, and cellular alignment were also noted in these experiments (*Figure 2.11*) [28].

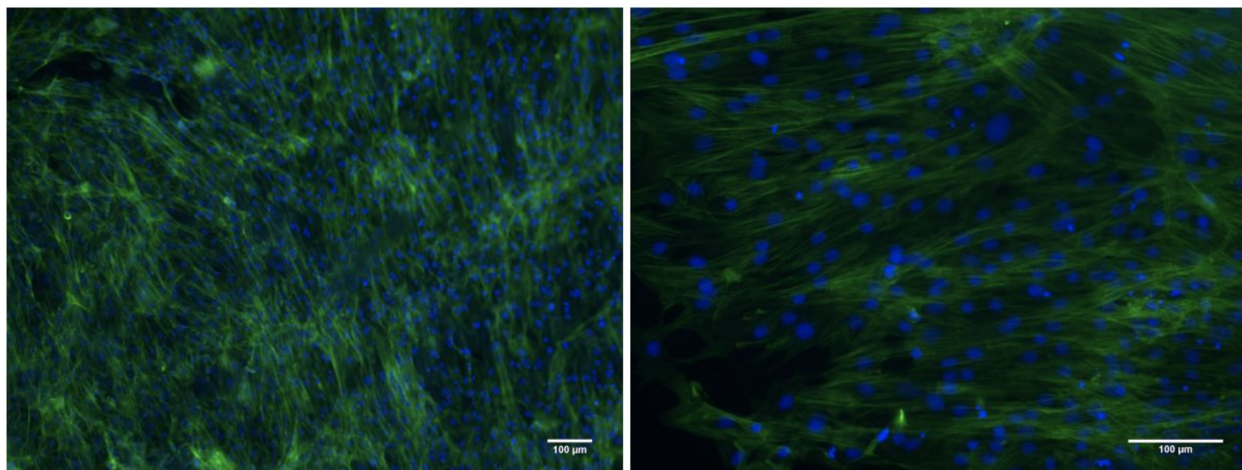


Figure 2.11: Images of muscle cells seeded on decellularized plant scaffolds [28]

2.2.4 Absorbance and pH Usage for Media Viability

An important portion of cell culturing is monitoring the viability and toxicity of media with pH [29]. In this project's case, the ideal pH is around 7.4 [30]. However, pH probes are often

large and expensive. To combat this, the team researched ways to use absorbance to estimate the pH value of the cell culture medium.

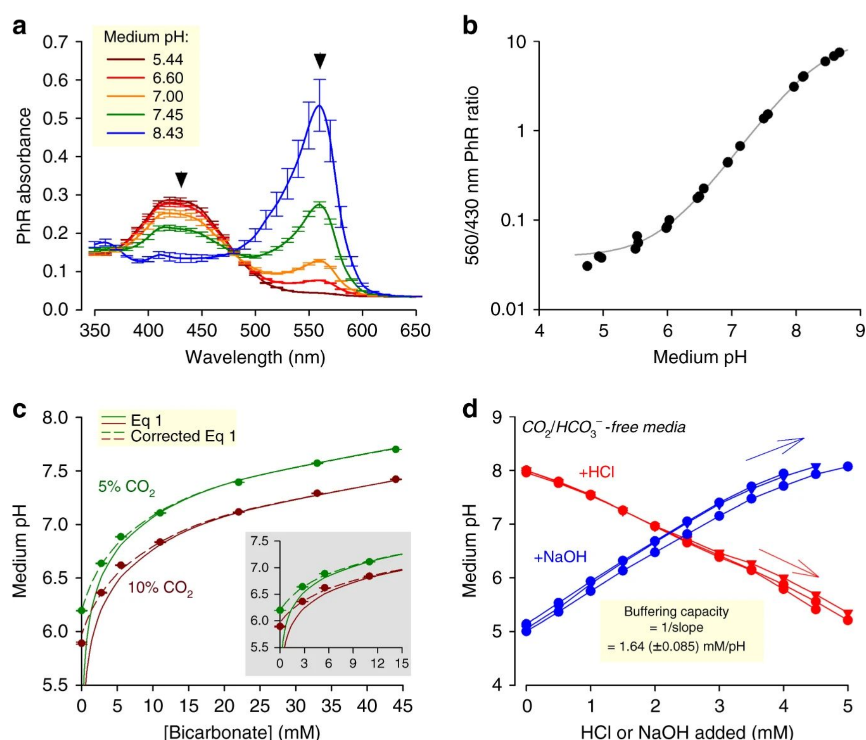


Figure 2.12: Absorbance vs pH research from "Evidence-based guidelines for controlling pH in mammalian live-cell culture systems" [29]

In particular, the team was interested in plot b from *Figure 2.12*. The 560/430nm ratio is very similar to the color range of the particular dye used in the Gaudette Lab, Phenol Red [31]. If two LEDs of those wavelengths were combined with a transimpedance amplifier circuit (seen in *Figure 2.13* below), a miniature spectrophotometer could be constructed to effectively estimate the pH of the cell culture media [32]. See the *Conclusions and Recommendations* chapter for more on how this could be incorporated.

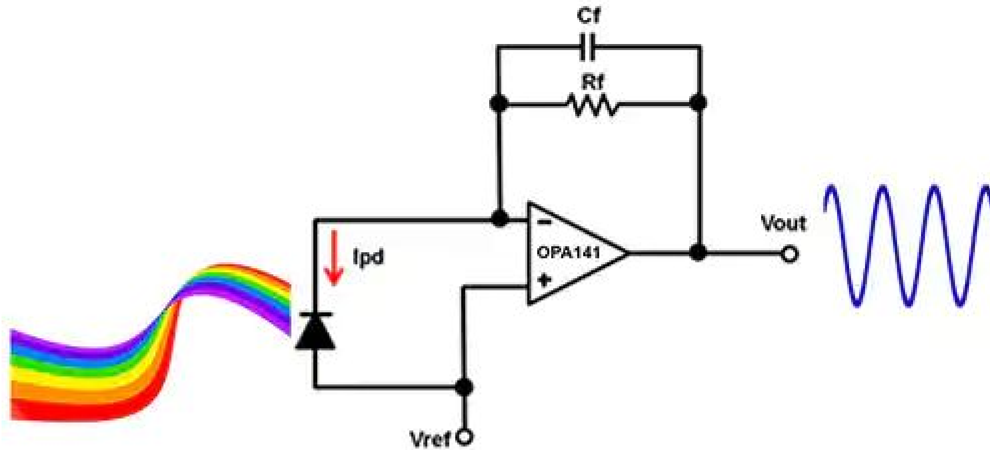


Figure 2.13: Transimpedance Amplifier (from Digi-Key). A photodiode outputs a current depending on the light levels in the area, which then is proportional to Vout [32]

2.2.5 Current Solutions for Meat Alternatives

There are many current options and groups working towards creating alternatives to meat. Specifically focusing on the hamburger, the following sections will look at a few examples of these alternatives, including what is available now and company-based technologies that are in development.

2.2.5.1 Alternative Burger Solutions

The first and arguably most popular meat alternative is the vegetarian “veggie” burger. Veggie burgers are a plant-based meat alternative that have gained popularity in recent years. One of the main motivating factors for the production of veggie burgers is that they eliminate the use of any animals in burger production. There are also environmental benefits. Veggie burgers require less land and water in comparison to hamburgers, but they do not eliminate them all together [33]. For this reason, the upscale of food production that will be required to sustain the increasing population may not be attainable with this meat alternative.

More recent developments in the plant-based meat alternatives include the Impossible™ Burger by Impossible Foods and a beef alternative produced by Beyond Meat. These companies aim to create a meat-like alternative to motivate people to switch to plant-based versions of their favorite foods while not sacrificing their taste. Beyond Meat is motivated by a plant-based burger alternative to decrease the negative health and sustainability effects of the meat production industry, as well as the animal welfare considerations [34]. Impossible Foods markets their work in decreasing the environmental impacts of meat-production with a plant based substitute, in comparison to making burgers from traditional livestock systems. *Figure 2.14* depicts the statistics that they claim is the difference in environmental impact for the production of one Impossible™ Burger versus the production of one burger made from a cow [35].

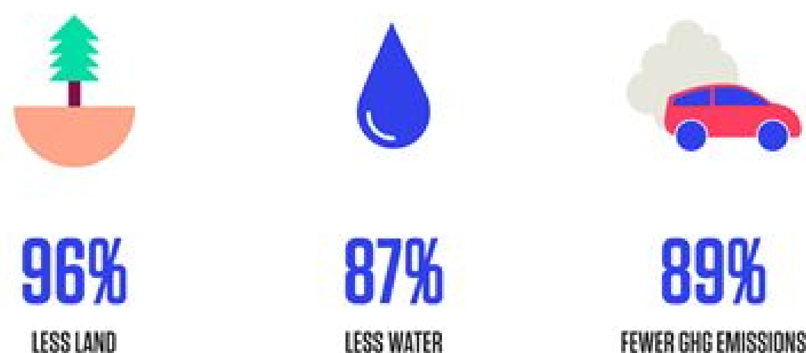


Figure 2.14: Environmental impact of the Impossible™ Burger versus a cow-based burger [35]

Plant-based alternatives do greatly decrease the environmental impact of meat production, but scaling up these processes to a point where they can produce enough to replace what is now made by the meat industry may still prove to be problematic, and still use a significant amount of resources. This is the motivation behind many lab-grown meat alternatives and ongoing research into these technologies today.

2.2.5.2 Company-Based Technologies for Lab-Grown Meat

Aside from plant-based meat alternatives, there is also extensive ongoing research into lab-grown meat as an alternative. Many companies are currently investigating the production capabilities of these meat-alternatives, with their main issues being cost and scale of production. These are promising technologies as they aim to provide a meat product without the slaughtering of an animal, but there is still more research to be done before these substitutes make their way to our tables.

Mosa Meat is a company working on the production of meat from harvested cow cells. They are marketing this as a "clean meat" alternative, which is a much more efficient process that requires less livestock for more meat. This alternative will have a great impact in decreasing the environmental impact of the meat industry, while also creating a more reliable source of food that could satisfy the demand of an increasing population [7]. In 2013, one burger cost 250,000 Euros to produce, so the main focus of Mosa Meat now is finding a way to scale up production while bringing the cost of their product down to make it a more attainable substitute [36].

Two companies that are currently developing similar technologies include Memphis Meats and Aleph Farms. Both of these companies are growing meat from cultured animal cells, and have a focus on developing a meat alternative that significantly decreases the environmental impact of meat production [37], [38]. Finless Foods is a similar company, the difference is that they are focusing their research on creating cell-culture based seafood alternatives. Their

motivation is to create a cleaner and healthier fish, while reducing the need for fossil-fuel producing fishing fleets [39].

The main challenges these companies are facing is producing these meat-alternatives at a reasonable price. Some of these price-related issues may be resolved by upscaling the process, but this introduces the problem of finding a sustainable and affordable source of media and nutrients for growing the muscle cells to make lab-grown meat. This is one of the main roadblocks holding back the large-scale, affordable production of lab-grown meat alternatives [40].

2.2.5.3 Experimental and Research-Based Technology for Lab-Grown Meat

Many labs and other organizations are also focusing on this new area of research. Some of the most notable are described in the sections below.

2.2.5.3.1 Gaudette Lab

Dr. Glenn Gaudette's research lab is a prime example of a lab-grown meat product that is currently being researched and developed into a marketable product. Dr. Gaudette and his team have previously performed research regarding the use of decellularized spinach leaves and other vegetables as a scaffold for meat growth. Previous work from this lab demonstrated that despite the atypical surface presented for cells to adhere to that cellular adhesion of myocytes was possible to decellularized plant matter [28].

2.2.5.3.2 Ellis Labs

Dr. Marianne Ellis from the University of Bath is another example of experimental and research-based techniques for culturing lab-grown meat products. One of her most recent papers discusses the use of bioreactors for the culturing of cells for meat production and how to best design the reactors to maximize their effectiveness and efficiency, both in terms of output and economic viability. Some of Dr. Ellis' other related works on lab-grown meats include studies of particular scaffold materials and their uses, as well as the study of how to create 3D tissues to achieve a designated purpose [41], [42].

2.2.5.3.3 Pelling Labs

Dr. Andrew Pelling from the University of Ottawa is a final example of research that can be taken and applied to products on today's market. Pelling's lab is interested in tissue growth on scaffolds and his research can be applied to lab-grown meat production by applying the techniques used to help obtain aligned muscle fibers on the scaffold, resulting in a better product [43], [44].

2.2.5.4 Other Promising Technologies

Lab-grown meat is not the only industry that is looking into creating food alternatives. The following sections will discuss other technologies for engineering food products.

2.2.5.4.1 Perfect Day

Perfect Day is a California-based company that is working to produce dairy products without the use of a cow. This team utilizes dairy flora, which is created by adding milk genes to a microflora. Microflora has the ability to produce a large quantity of protein and is derived from nature. Dairy flora is then fermented to convert this plant sugar to milk proteins, specifically whey and casein. This creates flora-based dairy protein which is then used to create a variety of dairy products such as cheese and ice cream [45].

2.2.5.4.2 JUST

JUST is a company that is known for creating an egg product from the mung bean, which has properties that allow it to cook and gel like an egg. Since its founding in August 2019, JUST has sold the equivalent of ten million eggs. This product has proven to be more ecologically-friendly than typical egg product as it "uses 98% less water emits 93% less CO2, and uses 86% less land" which is the equivalent of "361 million gallons of water, 1.46 million kg of CO2, and 2.5 million square meters of land" [46]. JUST has also committed to innovating and producing cultured Wagyu beef by using cells from Toriyama cows located in Japan by using their cellular agriculture methods and applying it to this area of food production [47].

2.2.5.5 Advocating Organizations

Although these companies do not directly work with alternative food techniques, the companies listed below do provide support to people who do. New Harvest is a nonprofit organization that funds academic research in the field of cellular agriculture such as lab-grown meat, eggs, and milk [48]. Another example is the Good Food Institute, which helps newly formed companies with early-stage development in the lab-grown meat and plant-based tech sectors [49]. In addition, they find opportunities for professionals looking to work in the clean-meat sectors.

In addition to lab-grown meat advocacy, there are also organizations that actively advocate for the traditional method of meat production.Two of these organizations include the United States Cattlemen's Association and the National Cattlemen's Beef Association, which both support the traditional method of producing beef [50], [51].

Chapter 3. Project Strategy

3.1 The Initial Client Statement

Our team aimed to identify a sector of lab-grown meat that is relatively unexplored and in need of experimentation. After initial background research, our team discovered that lab-grown meat production processes are slow, inefficient, and time-consuming. With this in consideration, the following initial client statement was identified:

Lab-grown meat is a new and exciting technology that has the potential to solve countless environmental issues. However, several aspects of the current technology inhibit the scalability of lab-grown meat processes. Our team's goal is to design a system that increases the overall efficiency of culturing techniques, while taking advantage of the plant scaffold technology of previous projects.

3.2 Design Requirements for the Bioreactor System

When looking further into how to fulfill the needs of the initial client statement, the team decided to base the system design on a bioreactor model. Bioreactors are commonly used in industry for the large-scale production of biological processes. The following requirements were identified to fulfill the initial client statement and outline what the revised client statement should incorporate. Eventually, these requirements shaped what experiments the team chose to pursue to determine final design criteria. In addition, since this project focused on both tissue and instrumentation the following section was broken up based on those two areas.

3.2.1 Tissue Engineering Requirements and Constraints

The team defined requirements for the bioreactor to ensure adequate cellular growth and the delivery of a viable meat product. Additionally, the cells cultured in the bioreactor and their scaffolds must remain within certain constraints to ensure that the entire setup remains viable. These include:

1. Ensure constant non-zero oxygen supply to all cells via direct cannulation, spraying of media, or other methods [52].
2. Maintain a temperature of 36-37°C to comply with current laboratory procedures for mammalian cell growth [30].
3. Actively remove waste products from cell culture media or provide an opportunity for media to be replaced to remove waste products.
4. Maintain a pH of 7.0 to 7.4 and a CO_2 concentration of 5 to 7% [30].

To ensure that the cell culture is scalable and efficient for commercialization, our chosen cell culture scaffolds must satisfy the following constraints:

1. The specified scaffold must be able to be decellularized by a bulk decellularization process, to ensure scalability [28].
2. The scaffolds must not inhibit or prevent nutrient transfer to cells growing on the scaffold surface.
3. The scaffolds are scalable with respect to the available incubation space designed into our team's bioreactor.
4. Whether the scaffold is synthetic or organic, all components must remain edible according to FDA standards [53].
5. The process can be easily manipulated. One scaffold type must be viable in multiple bioreactor designs.

3.2.2 Instrumentation Requirements and Constraints

In order to outline exactly what is needed from an instrumentation design perspective, it is important to understand the minimum requirements and restrictions of the system itself. Some important requirements the team has identified through background research include:

1. Monitoring key growing environmental/media properties via sensors, including but not limited to temperature, oxygen/CO_2 levels, pH, and pressure [25].
2. Monitoring sterility of both the growing environment and the growth media.
3. Data acquisition and logging for long-term analysis of growth.
4. Live data monitoring and visualization.

In addition to these requirements, some important constraints will help the team outline the final design of the bioreactors instrumentation. These constraints include:

1. Size of the bioreactor: confined growing environment will require efficient use of space for instrumentation.
2. Cost: research of powerful yet efficient parts will be key to a successful design within our given budget.
3. Water usage: controlling the amount of water used throughout the media delivery system will be a major restriction.

3.3 Standard Requirements for the Bioreactor System

The team's design will merge biotechnology and food production so it is important to consider both segments of regulation for the project. The biotechnology design component will

include the understanding of ISO standards, Good Manufacturing Practices (GMP) and Good Laboratory Practices (GLP). ISO standards will outline the standards for the bioreator's compliance to safety and cleanliness. GMP and GLP are important considerations throughout the design and experimentation of the project as they ensure safety and limit contamination. The food production aspect will require the understanding of both USDA and FDA standards for meat production as it relates to product quality, safety, and ultimately, retail sale.

3.3.1 Standards for Bioreactor Design

There are important standards that the team must comply with for the bioreactor model in order for it to be considered a viable design. Safety is a key factor as all components of the product must be safe to consume for the final product and the device must be safe to operate. Additionally, the bioreactor must be sterile and have a proper cleaning process for usability. The team will comply with the following standards throughout the design process:

- ISO 22000:2018: Food safety management systems — Requirements for any organization in the food chain
- ISO 11737-2:2009: Sterilization of medical devices — Microbiological methods — Part 2: Tests of sterility performed in the definition, validation and maintenance of a sterilization process
- ISO 13485: Medical Devices

In addition to the ISO standards listed above, the team will also follow Good Laboratory Practices (GLPs) and Good Manufacturing Practices (GMPs) as these are regarded across the industry. GLPs are defined by the Food and Drug Administration (FDA) which sets the standards to be followed "for nonclinical laboratory studies that support or are intended to support applications for research and marketing permits for products regulated by the Food and Drug Administration" [54]. The GLPs review and define practices including: Organization and Personnel, Facilities, Equipment, Testing Facilities Operation, Test and Control Articles, Protocol and Conduct, Records and Reports, and Disqualification of Testing Facilities [55].

The FDA defines Current Good Manufacturing Practices (CGMPs) for pharmaceutical products. While this project is not based on drug development, it is important that CGMPs are known and regarded as the team will still be developing a product via cell culture for consumption. CGMPs are put in place and enforced to ensure the safety of products and ensure that it matches the claims and ingredients defined in manufacturing. These guidelines define the "minimum requirements for the methods, facilities, and controls used in manufacturing, processing, and packing of a drug product" [56]. The team will follow similar practices in order to ensure the safety and integrity of the design and product.

As of July 2018, the FDA announced that they should take control of regulations regarding meat products produced via cell culture [57]. While the FDA has relevant stake in this area, it is also important to take into account food standards defined by the USDA.

3.3.2 USDA Standards for Meat Production

The Food Safety Inspection Service (FSIS) is the primary party responsible for ensuring that the producers of the meat industry are producing safe products. Their responsibilities include the inspection of meat products during slaughter, inspection of meat carcasses for infectious agents, and ensuring that plants are following proper sanitation procedures [58]. The USDA also regulates packaging, including labeling for meat grade, safe handling, and inspection marks [59]. Since our project is not dealing with any livestock directly, these regulations are not relevant, however they are important to understand.

Looking into standards that are applicable from the USDA, they have multiple documents outlining requirements of beef production [60]. Some of these meat production standards that are relevant to our project include:

- Pathogen Intervention - The production process must include at least 2 pathogen intervention steps (315)
- Microbial Testing - All boneless beef products must be tested for certain indicator microorganisms (318.7)
- Fat Percentage - The limits for percent fat in a boneless beef product should be within 12.0-18.0% (331)

3.4 The Revised Client Statement

Bioreactors are a powerful tool for upscaling biological processes and increasing the ability to monitor important environmental and growth factors. For this project, the team recognizes that a bioreactor design could help accomplish many of the teams design requirements. Due to this, the following revised client statement was adapted from our initial client statement to include some of the key points discussed above.

Current processes for culturing meat are inefficient and time consuming. Seeding bovine cells on plant scaffolds has created a unique opportunity to combine natural scaffold techniques with potential biological upscaling techniques. The team's revised goal is to design a bioreactor model for culturing meat on plant-based scaffolds, while increasing the overall efficiency and scalability of the entire process. To do this, the bioreactor must be able to monitor important environmental and tissue growth parameters via several instrumentation techniques.

3.5 Management Approach to our Project

We started our project in August of 2019, doing background research and project planning for the first term of our year (August to the middle of October). The team also made preliminary designs and concepts along with brainstorming for Chapter 4. Finally, the team began planning experiments and tests for terms B (mid-October to mid-December) and C (January to early March). In B-term, the team learned basic laboratory techniques to prepare for detailed testing and conducted a control experiment of seeding primary bovine muscle cells on a plant scaffold using cloning wells. Chapter 4 was written and Chapter 5 was drafted. In C-term, the team conducted a variety of experiments to test methods for seeding and growing cells on plant scaffolds in a scalable model. These experiments were analyzed and successful methods that met project objectives were incorporated into the final design, which was conceptualized in this term.. Chapter 5 was also completed. In D-term, the team faced some obstacles due to the COVID-19 pandemic which resulted in restricted lab access and the remainder of the school year was moved to remote learning. The team overcame these obstacles and continued to meet remotely, conceptualized the final design further, completed the remaining chapters of the report (Chapters 6, 7, 8, and 1), as well as created a recorded final presentation for a virtual Project Presentation Day.

Chapter 4. Design Process

The following section will outline the needs analysis the team defined for the components of the bioreactor, a description of current and conceptual designs, and a final design selection which was determined by completing a Pugh Analysis.

4.1 Needs Analysis

In order to understand the overall needs of our bioreactor design, we looked at three different sectors of needs: seeding and growing, media delivery, and manufacturability. Seeding and growing refers to the capability of the design in adhering cells onto the plant scaffold and the growth of those cells within the device. Media delivery incorporates the methods in which media is delivered to the plant scaffolds and cells. Manufacturability defines the scalability of the design.

4.1.1 Seeding and Growing

In regards to the most important needs for the bioreactor model, the team evaluated the following objective criteria for seeding and growing. The objective criteria was weighted based on a scale of 1 (least important) to 5 (most important), as seen in Table 4.1.

Table 4.1: Objective Criteria for Seeding and Growing

Objective	Weight
Ease of Use	5
Confluency	4
User Interaction Time	4
Environmental Monitoring	5
Automation	3

The objective criteria weighted as the most important were ease of use and environmental monitoring. Ease of use is important as seeding and growing is an important step to culturing meat and the process must be user friendly and not complicated. Environmental monitoring is also critical as the product needs to be safe and monitoring contamination will help to ensure product quality.

The next level of objective criteria was confluency and user interaction time. Confluency is in reference to the amount of cells on the scaffold, ideally the scaffold should be 85-90%

confluent. User interaction time defines the amount of time the user is working with the device to seed and grow the cells.

Lastly, automation is considered as moderately important, which references the ability to automate the system to seed and grow the cells and monitor their condition, which can help to reduce user interaction time.

4.1.2 Media Delivery

In regards to the most important needs for the bioreactor model, the team evaluated the following objective criteria for media delivery. The objective criteria was weighted based on a scale of 1 (least important) to 5 (most important), as seen in Table 4.2:

Table 4.2: Objective Criteria for Media Delivery

Objective	Weight
Volume of Media Used	5
Optimizing Media Volume to Scaffold Area Ratio	5
Recycling Media	3
User Interaction Time	4
Ability to Automate	3

Volume of media used and optimizing media volume to scaffold area ratio were the highest ranked objective criteria for media delivery. Volume of media used is important as the project aims to reduce the amount of media, which also relates to optimizing the media volume to scaffold area ratio.

User interaction time is ranked with the second highest score as it aims to minimize the amount of time someone would have to replenish or supply the media to the cells to sustain them.

The last two objective criteria were given a weight of three, which were recycling media and ability to automate. Recycling media is the ability to reuse media and thereby reducing the amount of water and resources used in the system. Ability to automate refers to the automation of the system to supply media to the cells with minimal user interaction.

4.1.3 Manufacturability

In regards to the most important needs for the bioreactor model, the team evaluated the following objective criteria for manufacturing potential. The objective criteria was weighted based on a scale of 1 (least important) to 5 (most important), as seen in Table 4.3:

Table 4.3: Objective Criteria for Manufacturing Potential

Objective	Weight
Scalability	5
Reproducibility	4
User Interaction Time	3
Ease of Use	4
Amount of Training Required	3

Scalability was weighted as the most important objective criteria as it refers to the bioreactor's ability to be scaled up to manufacturing production to ultimately get the product to market.

Reproducibility and ease of use were ranked with the next highest weight of four. Reproducibility refers to the ability to repeat the manufacturing process and produce the same quality of product each time. Ease of use is in regards to the level of difficulty in which the manufacturing process would take and how easy the process is to carry out.

User interaction time and amount of training required were each weighted as a three. User interaction time refers to the amount of time an individual would have to interact with the bioreactor system to manufacture the cultured meat product. Amount of training required refers to the resources, tools, and knowledge necessary for a user to be able to safely and effectively carry out the manufacturing procedure.

4.2 Conceptual Designs, Prototypes, and Feasibility Studies

In order to create a functional final design, numerous concepts were developed to improve upon the currently used standard method of seeding bovine myocytes onto spinach leaf scaffolds. After hypothetically evaluating their advantages over current methods, some aspects of early prototypes were combined into an additional option to be considered.

4.2.1 Current Method

Currently, the method for seeding and growing cells on a plant scaffold is using cloning wells, as described in *Appendix A*. The Gaudette lab uses whole plant scaffolds or punches of leaves for cell adhesion. The cells in cloning wells on plant scaffolds are incubated for two days to allow for cell adherence on the scaffold. The cloning wells are then removed to allow for cells to grow and proliferate on the scaffold for a set amount of time, depending on the size of the scaffold.

Figure 4.1: Cloning wells used for cell seeding on scaffold [61]

This method has a few setbacks. The first being that there are many materials used, most of which are required to be sterilized. This makes the process time consuming and expensive. Second, the process requires an immense amount of user interaction time, therefore making it not a very scalable process. These challenges are the motivation behind looking into bioreactor designs that do not rely on cloning wells.

4.2.2 Inspiration for Bioreactor Designs

Although cell seeding and media delivery are two separate concepts, the team looked to combine these into one coherent design for this project. The idea behind this was to create a process that was more contained, space efficient, and hence more scalable.

With the idea of scalability and efficiency in mind, the team then searched for inspiration of what the bioreactor should accomplish on a more conceptual level. Since the team needed to mimic the ideal growing environment of mammalian cells, the team drew design inspiration from sketches and figures of the circulatory and respiratory systems of the body, like seen in *Figure 4.2* below.

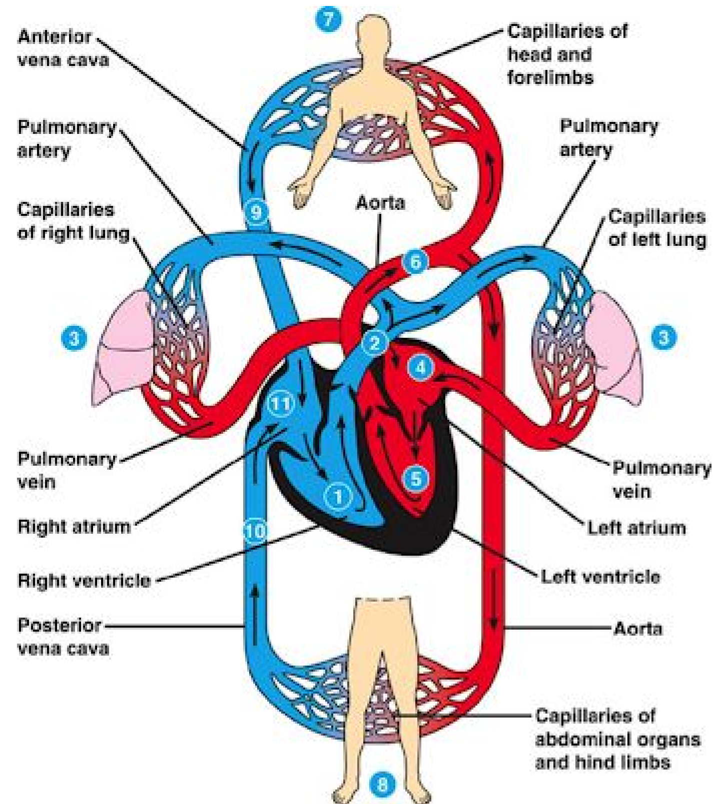


Figure 4.2: Diagram of circulatory and respiratory systems in body [62]

From this diagram, the team drew several important conclusions about what was needed for ideal media delivery. First, the bioreactor itself would require a pumping system to move media both in and out of the main cell growing compartment. Second, the bioreactor would likely need some sort of filtration system for cleaning/recycling media [62]. This inspired the team to make this simple diagram, which was the basic underlying design of all of the specific bioreactors seen later in this section.

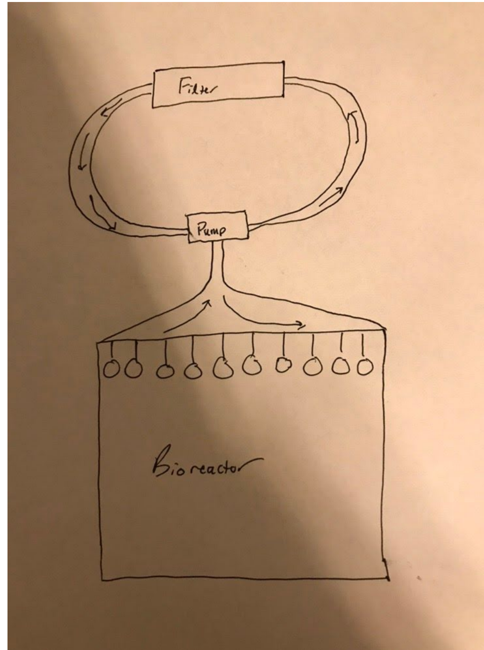


Figure 4.3: Simple design concept inspired by human circulatory and respiratory systems

With this simple design concept in mind, the team then began brainstorming designs that could recreate the system in *Figures 4.2 and 4.3* while fulfilling the criteria outlined in Section 4.1. The following sections describe some of the conceptual designs the team came up with throughout the brainstorming phase of our project based on these thoughts.

4.2.3 Hydroponic Mist Bioreactor

The first conceptual design the team considered was a mist type bioreactor. This design would incorporate the cell seeding technique of cloning wells (the current method), however seeded scaffolds would then be sustained via intermittently spraying media. An early schematic of how this reactor would look can be seen below in *Figure 4.4.*

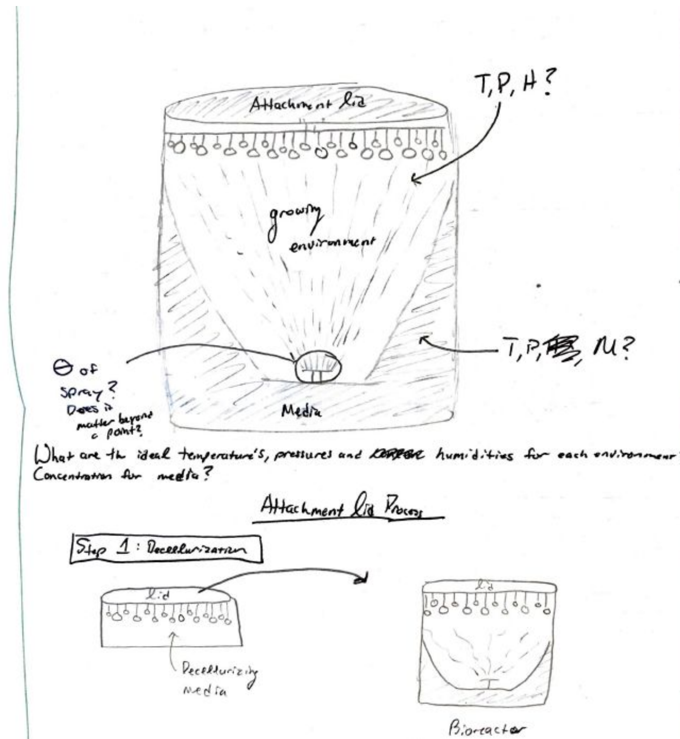


Figure 4.4: Early Mist Bioreactor Design

The misting design is the original inspiration for a closed system, and several design aspects from this schematic inspired future concepts. *Figure 4.5* below is another conceptual version of the misting bioreactor.

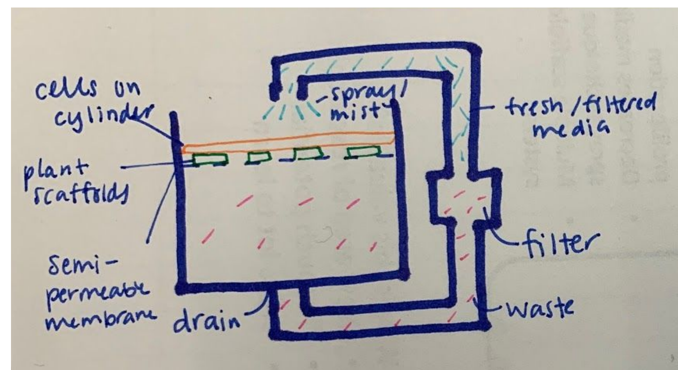


Figure 4.5: An updated design of the misting bioreactor, including an external filtering system

This updated misting design introduced the concept of actively filtering media, to both reduce media usage and user interface. Some concepts from this design would be included in our final design.

4.2.4 Spinning Bioreactor

The next major concept the team considered was the spinning “washing machine” bioreactor. This was the first design to incorporate both the cell seeding and media delivery phases into one contained system, unlike the misting bioreactor. An early concept of this design can be seen below in *Figure 4.6.*

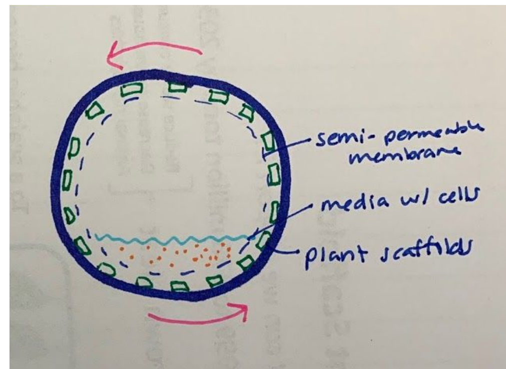


Figure 4.6. An early concept of the spinning bioreactor design

This design would also include some sort of active filtering system, to change the media at ideal intervals. In addition, multiple layers of scaffolds could be used in this design, as seen in *Figure 4.7* below.

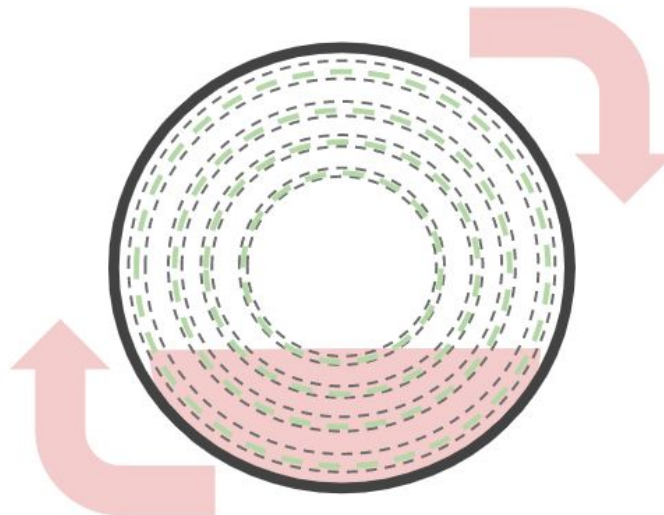


Figure 4.7: An updated concept of the spinning bioreactor, seen with multiple layers of scaffold

To achieve this design the scaffolds would be placed in between two biocompatible mesh pieces, and then folded into subsequently smaller circles. *Figure 4.8* below shows how this process would work.

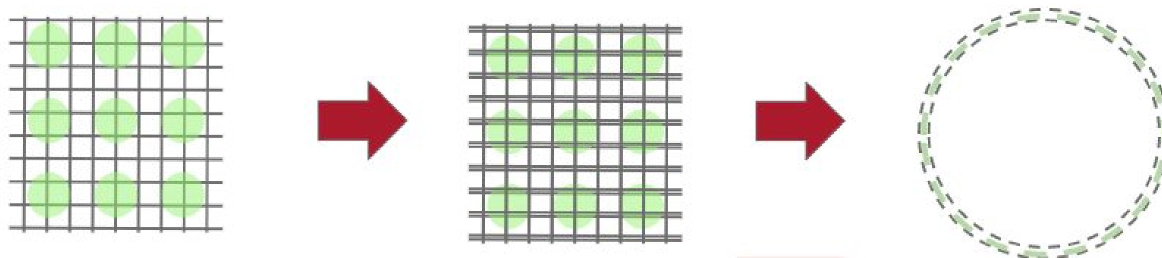


Figure 4.8: Plant scaffolds (green) placed between mesh pieces and folded into a circular shape

This method of folding and packing the scaffolds would maximize the amount of volume used in the 3D space of the bioreactor itself.

4.2.5 Combination Designs and Upgrading Past Designs

This section discusses some of the designs that either combine the types of bioreactor discussed in early sections, or improve on current methods done in the Gaudette Lab. Not all of these will be included in the final design selection, but they could inspire improvements to methods for future projects.

First, the team discussed adding a filtration system to the current cloning well method to improve efficiency and decrease user interface time. This concept would essentially be an improved control, as the overall design and methods are not completely redesigned like the mist or spinning bioreactors. This would add some automation to the media delivery process, and since the scaffolds are seeded in the same cloning well, the system itself would act as a small scale bioreactor. For this reason, the team chose to include the updated cloning well design in the final design selection.

In addition to the two main bioreactor designs, the team also considered combining the two ideas into one bioreactor design. This would involve the spinning bioreactor for cell seeding and agitation, followed by misting media to maximize efficiency of media delivery. Unfortunately, in order to maximize the amount of volume used in the spinning bioreactor design, the space available for incorporating the mist design would likely be minimal. Because of this, the reactor would likely need to incorporate two separate chambers for seeding and misting, which would negate the spatial efficiency accomplished by the standalone spinning bioreactor design. For this reason, the team decided to not include this design in the final design selection.

Finally, the team considered using a cannulation method for media delivery as another alternative design. This method would first include cell seeding via cloning well, and then transferred to a hanging apparatus similar to *Figure 4.4*. The stems of the scaffolds would then be punctured by needle, and media delivered through the petiole of the stem into the leaf

vasculature system. Although this method does take advantage of the leaf's intricate vasculature network, there are several factors that made the team hesitant to move forward with it. First, cannulation does not work on every leaf, as imperfections in the leaf itself can cause imperfect needle puncture. Due to this, leaf selection would have to be done beforehand to ensure quality leaves, and then the leaves themselves would have to be cannulated by hand. This would drastically increase the amount of user interface. In addition, leaves not selected would go to waste. Because of these reasons, the team decided not to move forward with the cannulation design.

4.3 Final Design Selection

In this section, the selection process of identifying the best overall bioreactor design is outlined based on the analysis of each design in each critical subsection previously described in Section 4.1.

4.3.1 Seeding and Growing Selection

Shown in Table 4.4 is a Pugh analysis of the primary bioreactor designs. Based on the requirements described in Section 4.1.1, the three designs were compared against the current standard of culturing cells onto decellularized scaffolds and weighted according to their identified importance.

Table 4.4: Pugh Analysis of Seeding and Growing Criteria

Evaluation Criteria	**Assigned Weight**	**Control - Manual Cloning Wells**	**Washing Machine**	**Mist Bioreactor**	**Automatic Filtered Cloning Wells**
Ease of Use	5	0	-1	-1	0
Confluency	4	0	+1	+1	0
User Interaction Time	4	0	+1	+1	+1
Environmental Monitoring	5	0	+1	+1	+1
Automation	3	0	+1	+1	+1
Score		0	11	11	12

Based on the conclusions reached with this analysis, all three designs are very similar, with the automated filtered cloning well design slightly edging out the other two options based on the criteria set forth in Section 4.1.1.

4.3.2 Media Delivery Selection

Shown in Table 4.5 is a Pugh analysis of the primary bioreactor designs. Based on the requirements described in Section 4.1.2, the three designs were compared against the gold standard of culturing cells onto decellularized scaffolds and weighted according to their identified importance.

Table 4.5: Pugh Analysis of Media Delivery Criteria

Evaluation Criteria	**Assigned Weight**	**Control - Manual Cloning Wells**	**Washing Machine**	**Mist Bioreactor**	**Automatic Filtered Cloning Wells**
Volume of Media Used	5	0	+1	+1	+1
Optimizing Media Volume to Scaffold Area Ratio	5	0	+1	0	0
Recycling Media	3	0	0	+1	+1
User Interaction Time	4	0	+1	+1	+1
Ability to Automate	3	0	+1	+1	+1
Score		0	17	15	15

Based on the conclusions reached with this analysis, as with the Pugh analysis completed for Section 4.1.1 criteria, all three designs are mostly similar to each other, with the washing machine design slightly edging out the others based on the criteria set forth in Section 4.1.2.

4.3.3 Manufacturing Potential Selection

Shown in Table 4.6 is a Pugh analysis of the primary bioreactor designs. Based on the requirements described in Section 4.1.3, the three designs were compared against the gold standard of culturing cells onto decellularized scaffolds and weighted according to their identified importance.

Table 4.6: Pugh Analysis of Manufacturing Potential Criteria

Evaluation Criteria	**Assigned Weight**	**Control - Manual Cloning Wells**	**Washing Machine**	**Mist Bioreactor**	**Automatic Filtered Cloning Wells**
Scalability	5	0	+1	0	0
Reproducibility	4	0	+1	-1	0
User Interaction Time	3	0	+1	+1	+1
Ease of Use	4	0	+1	+1	+1
Amount of Training Required	3	0	0	0	0
Score		0	16	3	7

From the conclusions drawn from this pugh analysis, the washing machine design significantly outcompetes the other two design options based on the criteria set forth in Section 4.1.3.

4.3.4 Pugh Analysis Results

The overall scores for each pugh analysis can be seen below in Table 4.7.

Table 4.7: Total Scores for Bioreactor Designs

Design	**Control**	**Washing Machine**	**Mist Bioreactor**	**Automatic Filtered Cloning Wells**
Seeding and Growing Score	0	11	11	12
Media Delivery Score	0	17	15	15
Manufacturing Potential Score	0	16	3	7
Total Score	0	44	29	34

After completing pugh analysis on all three bioreactor designs for the criteria defined under the three major needs sections, the washing machine design offers the most promising bioreactor design, with a total score of 44, 10 higher than the automatic filtered cloning wells design and 15 higher than the mist bioreactor design.

Chapter 5. Design Verification

5.1 Standard Laboratory Testing Procedures

There were several procedures required to move forward with design verification and sample analysis that remained unchanged during the team's experimentation. Those procedures are as follows.

5.1.1 Passaging of Bovine Satellite Cells

Cells were maintained and passaged in order to be used for experiments throughout the experimental process. This allowed for the ability to know the amount of bovine myocyte cells were used for each seeding experiment. This procedure can be found in *Appendix B*.

5.1.2 Bulk Decellularization of Spinach Leaves

Bulk decellularization involves removing the non-structural components of multiple spinach leaves at the same time. Approximately fourteen spinach leaves are treated and decellularized over the course of a week. Some example images can be seen below in *Figure 5.1*. This procedure is outlined in detailed steps and can be found in *Appendix C*.

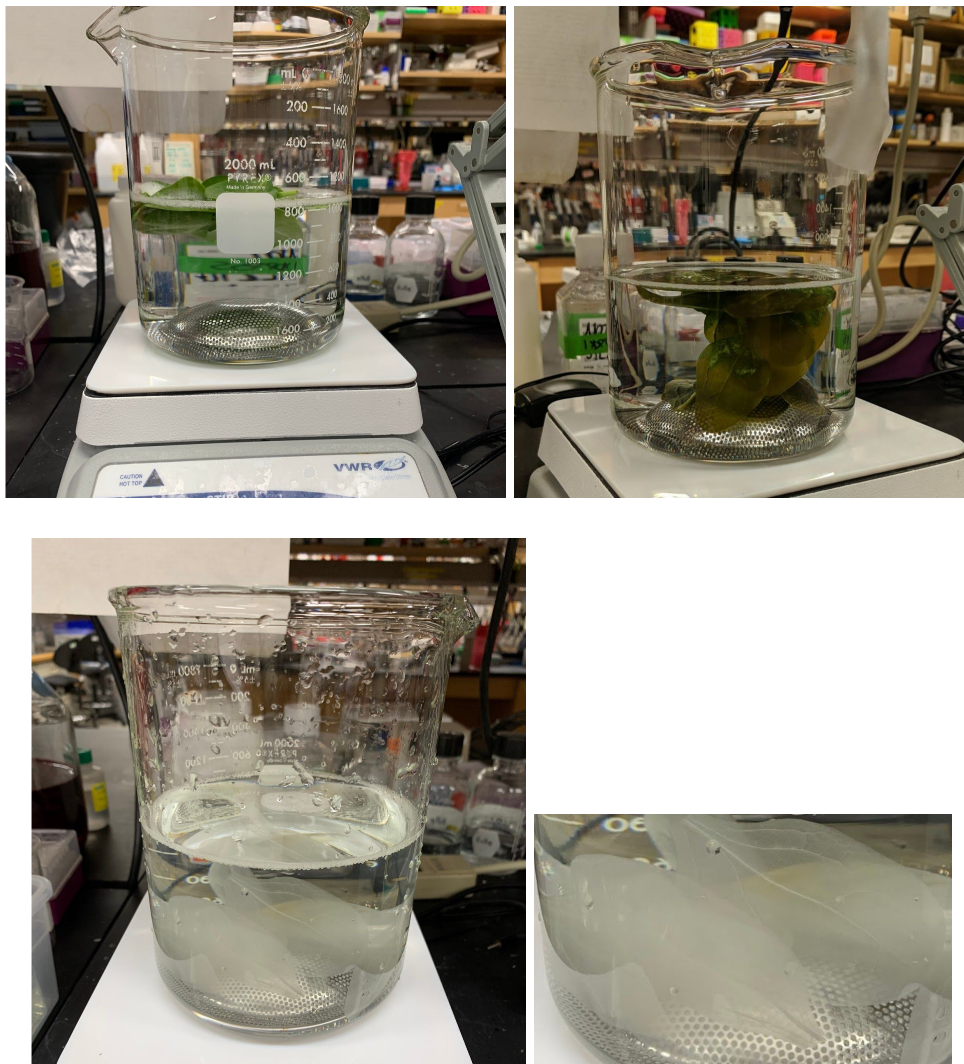


Figure 5.1: Bulk decellularization of spinach leaf scaffolds. Top left image shows the leaves on day 1 of decellularization. The top right shows the leaves on day 2 of decellularization and the bottom left image shows the leaves on the final (7th) day of decellularization. The bottom right image shows a closer view of the bottom left image.

5.1.3 Rehydration and Sterilization of Spinach Leaves

Plant scaffolds are lyophilized after being decellularized. This process requires them to then be rehydrated before cells can be seeded. This procedure outlines the details in order to rehydrate and sterilize leaf scaffolds to prepare them for experiments. This procedure is detailed in *Appendix D*.

5.1.4 Cell Seeding on Spinach Leaves using Cloning Wells

The standard laboratory practice is to seed bovine myocytes onto a plant scaffold using cloning wells, which are essentially sterile plastic cylinders that ensure that cells stay on the

scaffolds while they attach. This was used as a comparison for all seeding experiments since as mentioned, this is the current standard. The details of this procedure can be found in *Appendix A*.

5.1.5 Fluorescent Staining and Imaging of Spinach Leaves

In order to qualify the results from each experimental setup, the seeded leaf punches were stained with Hoechst and Phalloiden stains in order to observe the cell nuclei and actin filaments of the samples. These stains allow for observation of these cell features under a fluorescent microscope. This procedure is in *Appendix E*.

5.1.6 Live/Dead Assay

In order to analyze the viability of cells seeded and adhered to the plant scaffold, a live/dead assay was performed and scaffolds were imaged for analysis. The purpose of this protocol was to observe the amount of living and dead cells on the scaffold. This procedure is in *Appendix F.*

5.1.7 Cyquant Analysis

A cyquant analysis was performed to quantify the amount of cells on the plant scaffold. This procedure is in *Appendix G*.

5.2 Seeding Experiments

5.2.1 Control

Our first experiment conducted was meant to serve as a control experiment with which we could compare all of our seeded leaf scaffolds to. This allows us to determine the effectiveness of the experiments we conduct by comparing the relative confluence, degree of cellular adherence, and cell health and determine which, if any, of our designs are as effective at seeding and growth on spinach leaf scaffolds as the cloning well method. As cloning wells are the current gold standard for seeding cells on scaffolds in the lab, similar results between our experiments and the baseline control would demonstrate comparability of our design to the standard.

In this experiment, 0.5 inch diameter punches were obtained from lyophilized spinach leaves decellularized using the bulk method (as detailed in *Appendix C*). The leaf punches were then rehydrated and sterilized. Next, 200,000 cells were seeded on each leaf scaffold, each in their respective wells in a 6-well plate. The cells were allowed to adhere and grow for two days in an incubator, with environmental conditions of 37°C and 5% CO_2. After two days of growth, the seeded punches were stained with Hoechst and Phalloiden and imaged with fluorescence. One sample from this test can be seen in *Figure 5.2*.

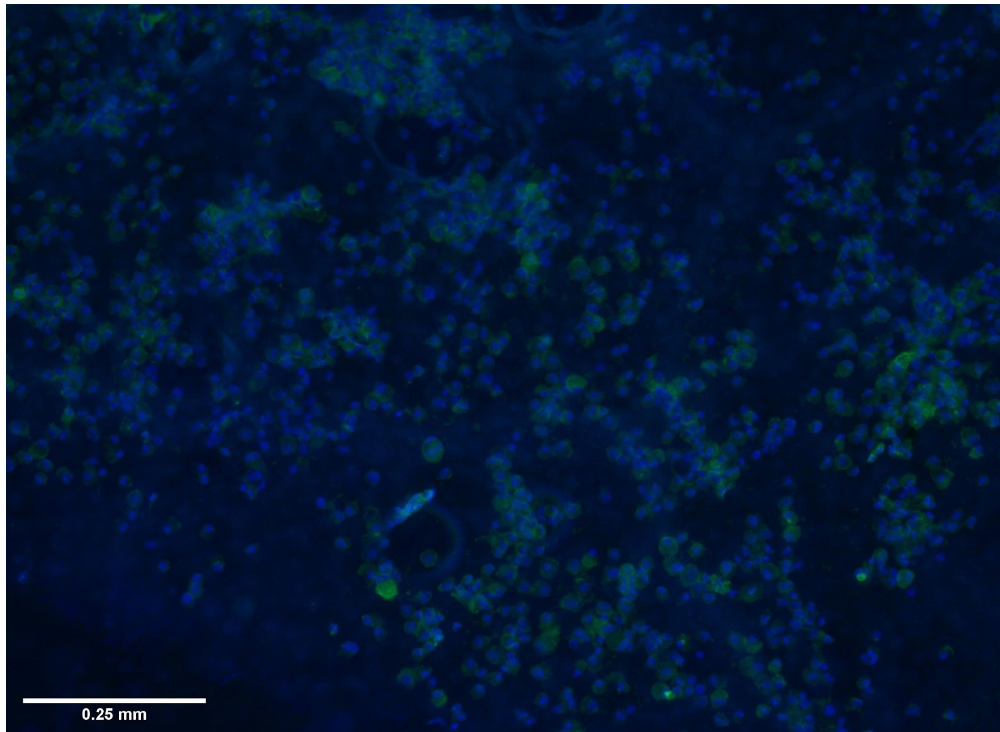


Figure 5.2: Control test results from Hoechst and Phalloidin staining

5.2.2 Design Concept 1: PDMS Ridges

The purpose of this design concept was to test the idea that cell agitation would encourage the cells to attach to the decellularized spinach leaf scaffolds. A mold was made in order to create a Polydimethylsiloxane (PDMS) piece that would create ridges along the inside circumference of the tube, as seen in *Figure 5.3*. The idea was that the ridges would agitate the cells as they floated around.

The PDMS piece was made by mixing the curing agent and polymer, in order to make the PDMS, at a 10:1 ratio. The PDMS was poured into a 3D printed mold, allowed to sit overnight, and the next day placed in an oven at 65°C for 15-30 minutes to set the pieces. These pieces were cut to size to be able to fit around the circumference of the 50mL conical tube.

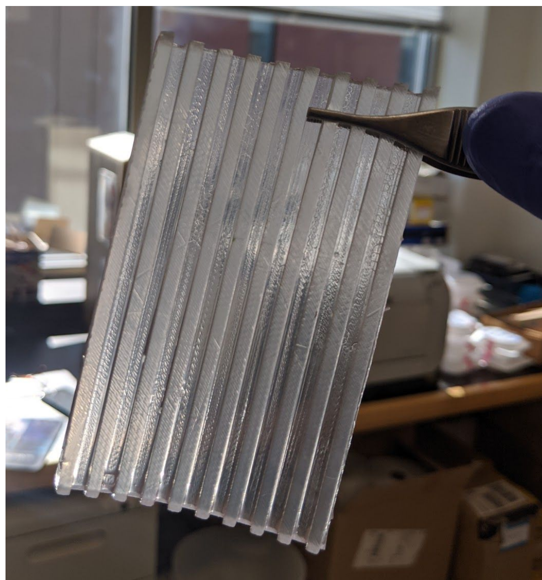


Figure 5.3: PDMS piece used for cell agitation

This test setup consisted of three 50mL conical tubes, each lined with a PDMS along the tube. The PDMS pieces were placed into the tubes using sterile gloves in order to ensure sterility of the setup. Each tube contained 15mL of complete media, five decellularized and sterilized leaf scaffolds, and 150,000 cells. The scaffolds were each about one centimeter in diameter, and were decellularized, rehydrated, and sterilized. The setup began by placing the PDMS pieces in each tube, followed by adding 15mL of media to each tube, then a sample containing 150,000 cells, and finally the sterile leaf punches, which were transferred using sterile forceps. All setup was completed inside a biosafety cabinet in order to ensure the overall sterility of the test.

The conical tubes were placed on an elevated rotator in an incubator for three days at 37°C and 5% CO_2. After 24 hours and 48 hours, the tubes were removed from the elevated rotator, and the caps were removed for five minutes, in order to allow for air exchange inside the tubes. They were then replaced into the incubator, and continued to rotate. On the third day, the scaffolds were stained with the Hoechst and Phalloidin and imaged to analyze effectiveness of this method for cell seeding on the scaffolds.

The initial results from this test were compared to a control where the scaffolds were seeded using a cloning cylinder (*Appendix A*), the current standard. In a comparison of confluency and repeatability, the control showed more promising results. *Figure 5.4* shows one of the samples from this experiment, after Hoechst and Phalloidin staining.

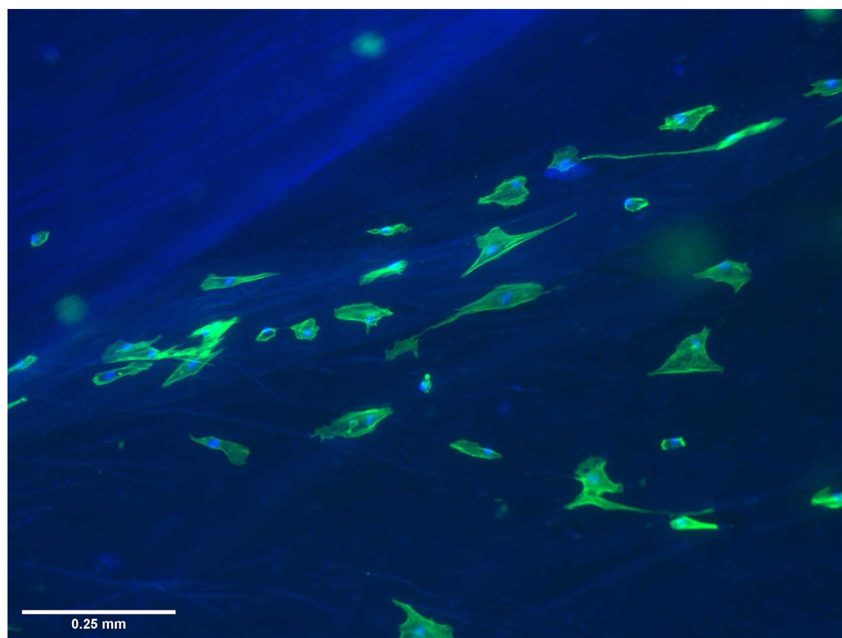


Figure 5.4: PDMS agitation test results from Hoechst and Phalloidin staining

Comparing these results along with the overall experience of this design, it did not prove to be a design that was worth pursuing further. The setup for this design was time consuming and not user friendly, this in conjunction with the comparison of cell confluency gave reason to not move forward with this design concept.

5.2.3 Design Concept 2: PDMS Lattice

This design investigated the seeding of cells on decellularized spinach scaffolds that were fixed to the edge of the conical tube via a PDMS lattice structure rather than letting them float freely as tested in the previous design (PDMS ridges). The PDMS lattice structure was made by first making a thin layer of PDMS with a 30:1 ratio (base elastomer to curing agent) to make a more rigid solution. The solution was poured in a thin layer in a weigh boat and left to set overnight. Once set, the layers of PDMS were removed from the weigh boats and placed in the oven at 65°C for 30 minutes to set the pieces further. Small circular punches were taken from the PDMS to create a lattice design.

This test was carried out with two 50mL conical tubes, with two rehydrated and sterilized decellularized leaf scaffolds per tube, each scaffold was punched to the same size diameter (about one centimeter). Inside the biosafety cabinet, each scaffold was placed on the wall of the inside of the conical tube using sterile forceps. Then, the PDMS lattice structure was inserted, using sterile forceps, lining the inner circumference of the conical tube and fixating the scaffolds against the edge. 57,500 cells were suspended in 10mL of media which was added to each conical tube. The two conical tubes were then placed on the elevated rotator in the incubator. After 24 hours, the tubes were removed and placed in the biosafety cabinet. The caps were

opened to allow the cells to breathe for five minutes before being placed back on the rotator for another 24 hours.

Figure 5.5: Note about 4mL of media was lost in one tube over the two day incubation period

After 48 hours, the scaffolds were stained with the Hoechst and Phalloidin and imaged to analyze effectiveness of this method for cell seeding on the scaffolds. Most scaffolds were damaged during the staining process so there were limited scaffolds to image.

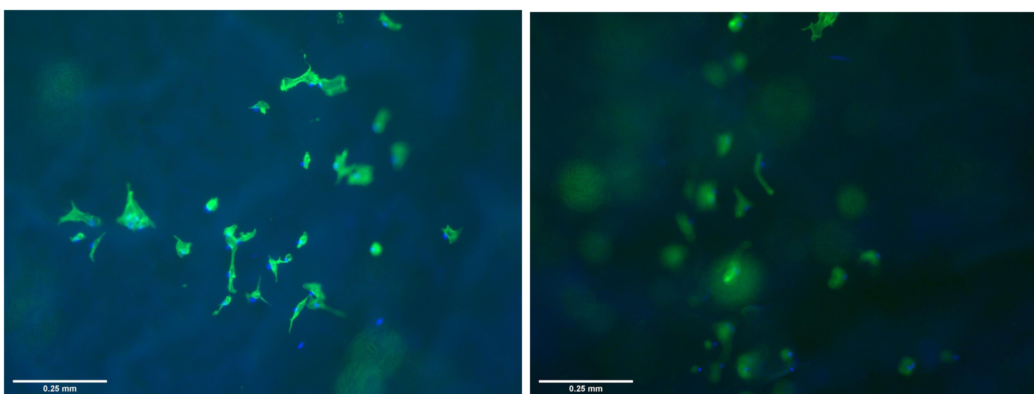


Figure 5.6: PDMS lattice test results from Hoechst and Phalloidin staining

This test was not very user-friendly during setup, and the achieved results were comparable to the experimental setup with the PDMS ridges, but neither of these experiments yielded results comparable to the control.

5.2.4 Design Concept 3: Unrestricted Scaffolds without PDMS

In order to understand the feasibility of unrestricted scaffolds as a design concept, this experiment was conducted. It was hypothesized that without agitation or fixation, the cells would

float to the bottom of the conical tube, and the attachment to the decellularized leaf scaffolds would be minimal.

This test setup consisted of three 50mL conical tubes. The PDMS pieces were placed into the tubes using sterile gloves in order to ensure sterility of the setup. Each tube contained 15mL of complete media, five decellularized and sterilized leaf scaffolds, and 150,000 cells. The scaffolds were each 1cm in diameter, and were decellularized, rehydrated, and sterilized. The setup began by adding 15mL of media to each tube, then a sample containing 150,000 cells, and finally the sterile leaf punches, which were transferred using sterile forceps. All setup was completed inside a biosafety cabinet in order to ensure the overall sterility of the test.

The conical tubes were placed on an elevated rotator in an incubator for three days at 37°C and 5% CO_2. After 24 hours and 48 hours, the tubes were removed from the elevated rotator, and the caps were removed for five minutes, in order to allow for air exchange inside the tubes. They were then replaced into the incubator, and continued to rotate. On the third day, the scaffolds were stained with the Hoechst and Phalloidin and imaged to analyze effectiveness of this method for cell seeding on the scaffolds.

The initial results from this test were compared to a control where the scaffolds were seeded using a cloning cylinder, the current standard. In a comparison of confluency and repeatability, the control showed more promising results. *Figure 5.7* shows one sample from this experiment, after the Hoecsht and Phalloidin staining.

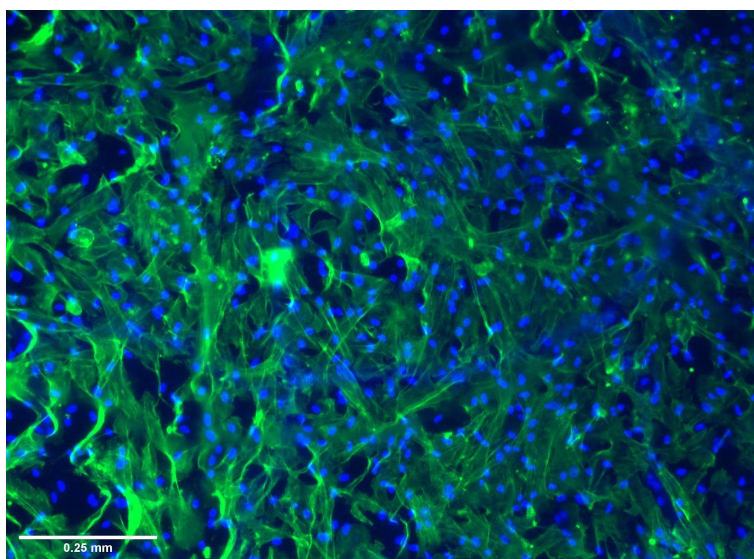


Figure 5.7: Unrestricted scaffold test after Hoechst and Phalloidin staining

Comparing the results of cell confluency to the control, the cells seem to be much more confluent and the attachment is also promising, as indicated by the amount of actin (the green) present. This design concept is also promising as it requires minimal setup outside of adding the

cells to the tubes, and placing them in the incubator. This fact is promising for the idea of creating a scalable design.

5.2.5 Selected Design

In order to choose a final design, we decided to compare these design concepts to our design specifications. The factor comparison of each design can be seen in Table 5.1. The team ranked each design concept on a scale of 1-5 (5 being the best) in each category that relates to the main design specifications.

Table 5.1: Weighting for Design Concepts based on design specifications

Evaluation Criteria	PDMS Ridges	PDMS Lattice	Unrestricted Scaffolds
Amount of Media	4	4	4
Ease of Use	3	1	5
Scalability	4	2	5
Totals:	9	7	14

Based on the weighting in Table 5.1, the team decided to move forward with the unrestricted scaffold design concept. This was based on it being the easiest user experience, as well as the design that is most promising in terms of scalability, which is one of the most important factors for the design of this bioreactor.

5.3 Scaling Design for Growing

5.3.1 Media Volume to Scaffold Ratio Tests

In order to reduce the amount of media in our prototype and relate back to an objective to reduce the amount of water used to produce meat, the team investigated an optimal ratio for media volume and scaffolds used when seeding and growing cells. The ratios tested in this experiment were:

- Two milliliters of media per one scaffold (2:1 ratio)
- One milliliter of media per one scaffold (1:1 ratio)
- One half milliliter of media per one scaffold (0.5:1 ratio)

This test was carried out with a total of nine 50 mL conical tubes, three per ratio. In the first three conical tubes, three scaffolds were added in six milliliters of media with 100k cells per tube (2:1 ratio). In the second three conical tubes, three scaffolds were added in three milliliters of media with 100k cells per tube (1:1 ratio). In the last of the three conical tubes, three scaffolds were

added in one and a half milliliters of media with 100k cells per tube (0.5:1 ratio). The nine tubes were then transferred out of the biosafety cabinet and onto the elevated rotator in the incubator. After 24 hours, the tubes were removed and placed in the biosafety cabinet. The caps were opened to allow the cells to breathe for five minutes before being placed back on the rotator for another 24 hours.

For each ratio, there were a total of three 50mL conical tubes with scaffolds: one tube was used for imaging, the second was used for a cyquant analysis, and the third was used for a live dead analysis. The results of the cyquant analysis were inconclusive due to lack of experience in completing the procedure.

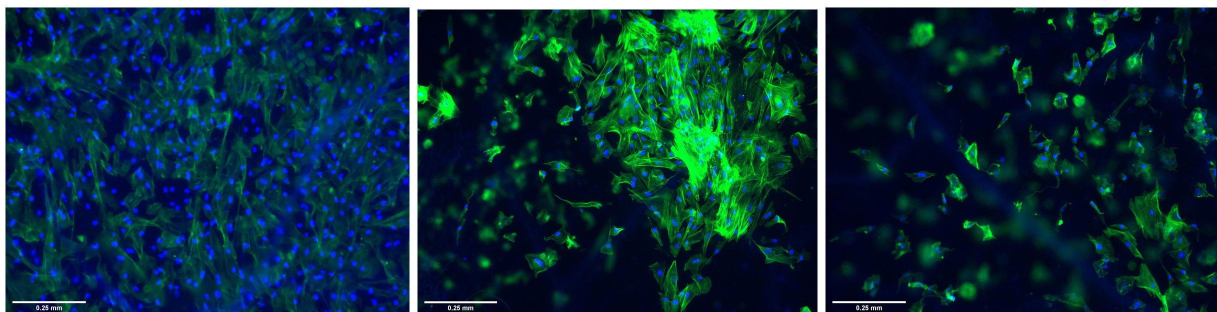
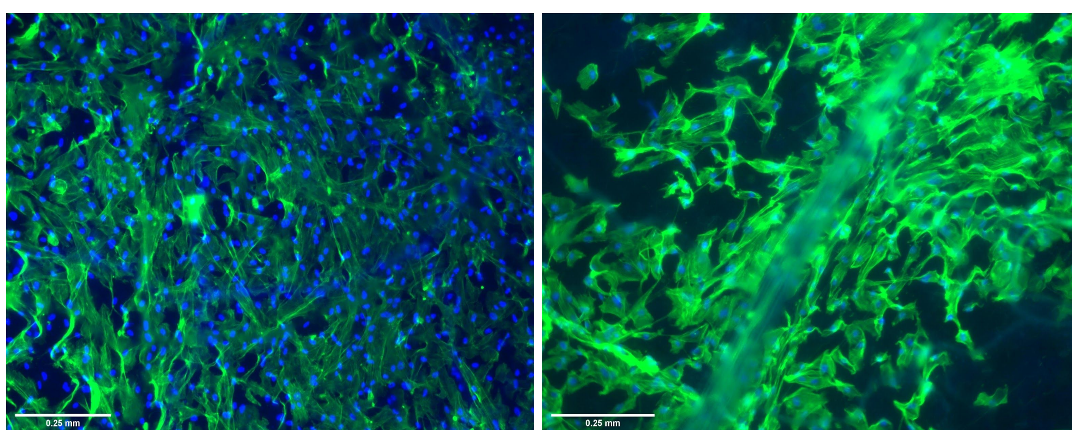


Figure 5.8: (from left to right) results from 2:1, 1:1, and 0.5:1 ratios

Figures 5.8 shows the imaging results from this experiment. All ratios proved to have cell attachment on the scaffold, however the 2:1 ratio was clearly the best with the greatest amount of adherence and nuclei on the scaffold.

This experiment also proved another breakthrough concept; that cells were seeded on both sides of the scaffold for the 2:1 ratio, as shown in *Figure 5.9*.



Figures 5.9: (left to right) results from side one and side two of a scaffold in the 2:1 ratio test

These results were very promising as they showed that cells can adhere to both sides of the plant scaffold, which was not possible using the current standard of adhering cells via the

cloning well method. This demonstrated that the process of adhering and growing cells suspended in media via the rotator was a more efficient and scalable process as it optimizes resources (like media) for the production of lab-grown meat.

5.3.2 Adding Scaffolds Test

This test was performed to demonstrate the potential for cells that have been seeded onto one scaffold to migrate to a secondary scaffold, ideally after the first scaffold has reached confluence but also if the seeded cells became detached from the scaffolds for any other reason.

For this experiment, 14 leaf punches were first prepared in the typical method used in the lab. Four were left as the standard 0.5 inch diameter circular scaffold punches. Four were cut into identical triangles, four others were cut into identical squares, and the remaining two were one inch diameter punches that were then cut into a rectangular shape. The purpose of having different shaped punches was that it allowed for differentiation between the punches added to the samples on different days.

Next, 3.5 mL of media was placed into two 50mL conical tubes. Two standard leaf punches were then added to the media in each tube via sterile forceps. Then, 500,000 cells in 0.5 mL of media was added to each tube via pipet, for a total of four mL, or the standard two mL media to one leaf punch ratio. The tubes were then placed onto the rotating stand and left to incubate for two days. After the incubation period, the media was removed via pipet and eight milliliters of media was added to each tube. Next, two triangular scaffolds were added to each tube via sterile forceps, for a total of four scaffolds in eight milliliters of media. The tubes were once again placed on the rotator stand and allowed to incubate for two days. After the second incubation period, the previous step was again repeated with the square scaffolds, resulting in six scaffolds in 12 mL of media. After one additional day, the final rectangular shaped scaffold was added with two additional milliliters of media, for a total of seven scaffolds in 14 mL of media. For the entirety of this experiment, the cells were allowed to breathe between incubation periods during the addition of scaffolds and media changes, otherwise they were housed in the enclosed and sealed conical tube.

On day six of the experiment, one day after adding the final scaffold, the leaves were removed from media, stained with Phalloidin and Hoechst, and imaged using fluorescence microscopy.

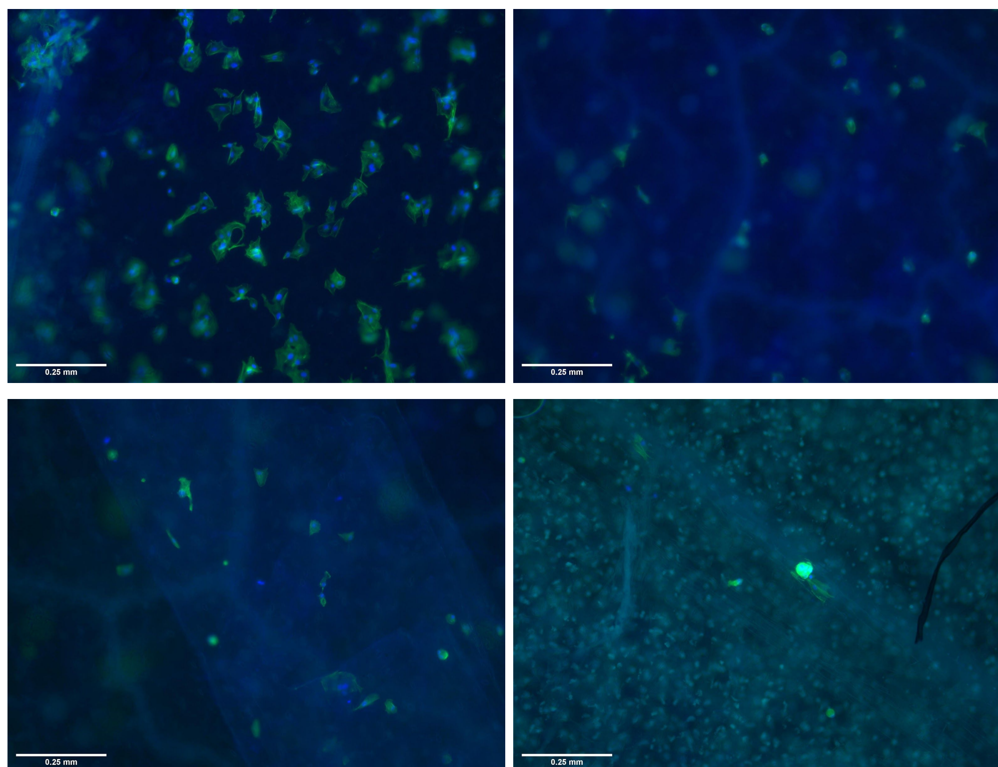


Figure 5.10: Day zero (top left), day two (top right), day four (bottom left), day five (bottom right)

These results show that even though no more cells were added over the course of this experiment, cells appeared to attach to the scaffolds that were added after the initial ones. This demonstrates that cells did migrate from one scaffold to another, and gives promising results in terms of being able to scale up this process.

5.3.3 Extended Toxicity Test

To test the toxicity of the growth media at different time intervals, the team designed a week-long experiment aimed at investigating pH changes and the death of cells on scaffolds. The goal of this experiment was to find the critical time at which the media no longer supports the cells on the scaffolds, but rather restricts their access to nutrients and causes their eventual death.

The test consisted of 6mL tubes, each filled with two spinach scaffolds and four milliliters of growth media. Three of these tubes would be samples, and the other three would be controls. The controls had media replaced every two days, whereas the samples never had media replaced.

Every two days, a live-dead assay was done on both a control and sample tube. One of the scaffolds in the control tube was covered in 70% ethanol for 30 minutes before the assay preparation, in order to have a dead control. The other scaffold was used as a live control. Images in *Figure 5.11* below show characteristic images from days two, four, and six.

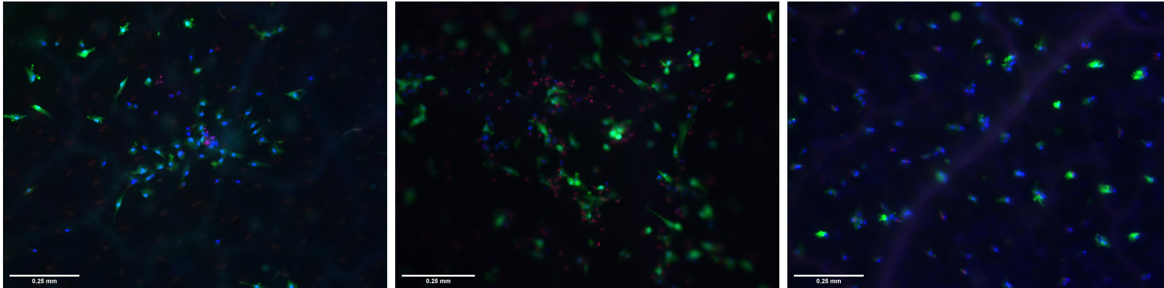


Figure 5.11: Day 2 (left), day 4 (middle), and day 6 (right) live-dead assays

In addition, the media from each of the sample tubes was kept for pH analysis via absorbance. However, this part of the experiment was not completed due to time constraints and the restriction of lab-work in D-Term.

Looking at these images we can conclude that there was minimal cell death in the day two sample (indicated by where the red dots or the dead assay line up with the blue dots or the cell nuclei). Day four shows more dead cells on the sample, but not as many as expected since this was the sample after four days of not changing the media, which is twice as long as the lab standard. Day six shows significantly more cell death, indicating that six days is far too long to go without a media change in cell culture of the bovine myocytes.

Chapter 6. Final Design and Validation

6.1 Final Design

The final design focused on three main criteria: decreasing the amount of media per test, the ease of use for the user, and the ability to monitor critical properties while being away from the device. The computer aided design (CAD) model, along with the real model can be seen below in *Figure 6.1*.

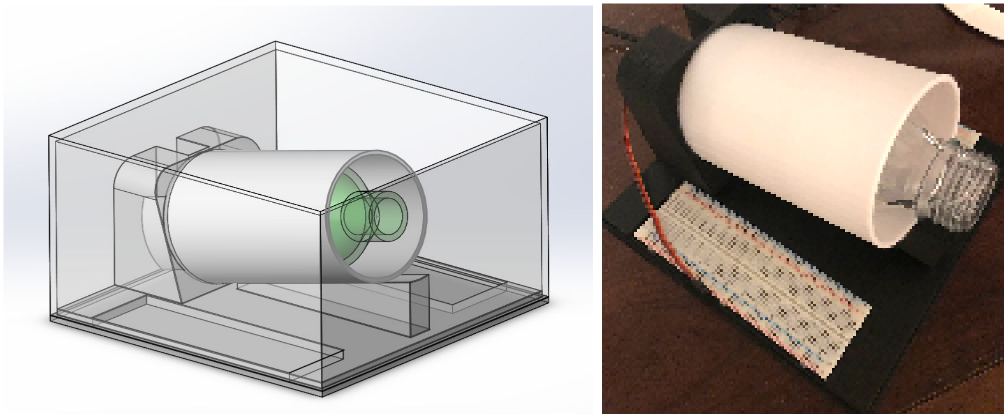


Figure 6.1: Final Design of Bioreactor in CAD vs 3D printed

This design is made up of multiple hardware and software components, which will be discussed in the following sections.

6.1.1 Hardware

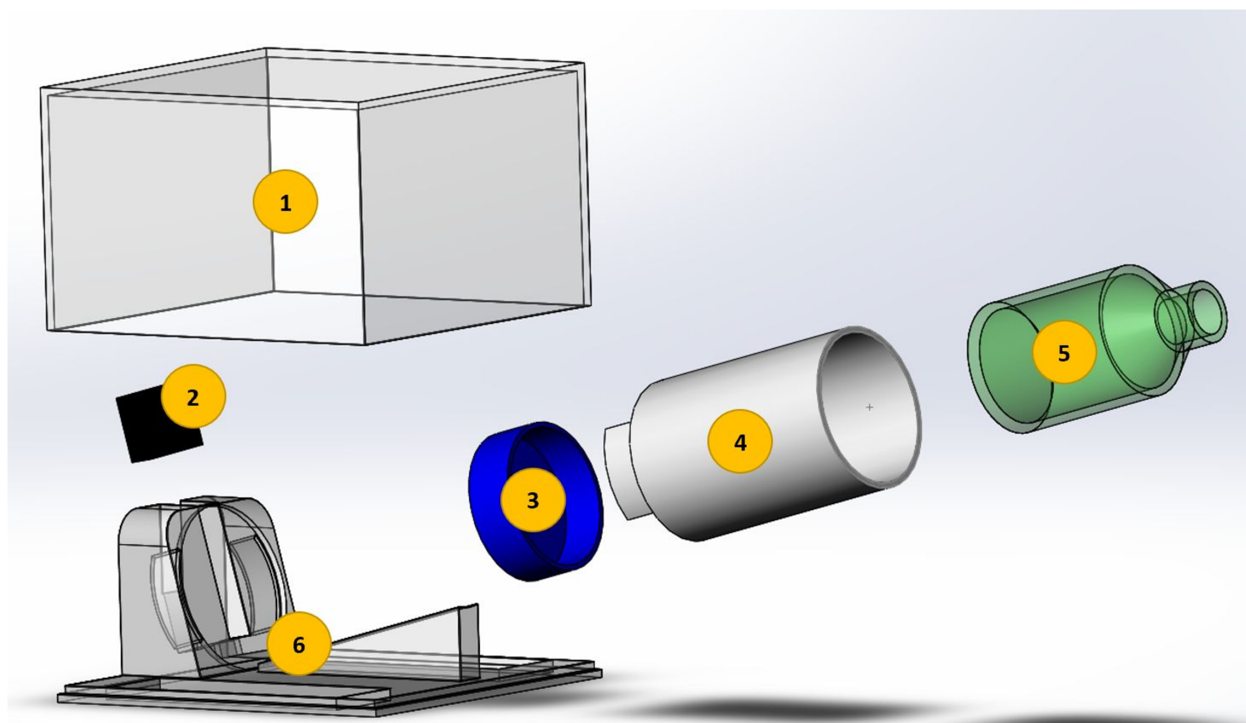


Figure 6.2: Exploded view of final prototype CAD model indicating each part

The final design consists of six non-electronic components: the stand, the outer guiding tube, the inner motor to bottle attachment, a 500mL glass bottle, a cover, and a servo motor. All of these parts are displayed in *Figure 6.2*. The first and most crucial of these components is the stand (labelled 1 in *Figure 6.2*), which keeps the other components (both mechanical and electrical) in place during rotation. Next is the outer guide tube (part 4 in *Figure 6.2*), which attaches to the stand to keep the bottle in place while rotating. Next is the continuous servo motor (part 2), which fits into the back side of the stand. Next is the motor to bottle connector (part 3). This connects to the motor via a matching gear slot on one side, and then slides over the 500mL bottle on another. The 500 mL glass bottle (part 5) fits tightly into the outer guide tube to ensure rotation. Finally, the cover (part 1) encloses the entire system, allowing for a stackable design.

6.1.2 Electronics and Instrumentation

The goal of the electronics on our prototype was to assist the user in gathering valuable information about the cell culture environment. Our final design includes the following electronics to achieve this goal. First is the Adafruit BME680 chip, which is the main sensing device on our prototype. This sensor gathers humidity, temperature, and pressure data for our bioreactor [63]. The device is also rated to measure volatile organic carbon (VOC) gas content, however this feature was not utilized. Next is the SGP30 sensor, which is an effective CO_2

sensor [64]. Again this device also has VOC monitoring, but was not used for this project. Both sensors can be seen below.

Next, a Huzzah ESP32 microcontroller was used to both gather and send sensor information, while also controlling the servo motor. The ESP32 is capable of both WiFi and Bluetooth, however WiFi was chosen for this specific application to assist in making the local visualization web page via Python [65]. See *Software* in the next section for more details.

All of these components were part of the final design to ensure the growing conditions inside the bioreactor stay fit for the bovine myocytes during their entire proliferation phase. These sensors along with the monitoring system to be described below act as a quality control system so that the system operator can constantly ensure that the culture environment is ideal for growth, producing the highest quality meat product at the end.

6.1.3 Software

After gathering sensor data on the ESP32, the data is then sent via WiFi to a personal Python server, which then visualizes it on a local webpage. This webpage can then be accessed by anyone in the local domain with the name "*bioreactor.lan:port_#",* where the port number is specified in the server code (and can be customized). This page allows for the live monitoring of the sensor outputs, and allows the operator to be constantly aware of the state of the bioreactor.

Figure 6.3: Bioreactor monitoring and controlling webpage

6.1.4 Device Procedure

This device provides the user with a simple procedure to grow lab grown meat. We have discussed the layout and design of the device (see *Figure 6.2*), and all of these components work

together in order to grow lab-grown meat. The procedure for utilizing this device at an independent location would be similar to the process outlined in the following steps, which mimic how these procedures were completed in the lab:

1. Day 0 - Add 2 decellularized scaffolds, 2 mL of media and 500,000 bovine myocytes into the 500 mL bottle; place the filter top on the bottle and place the glass bottle into the bioreactor, which is located in an incubator.
2. Day 2 - Remove 500 mL bottle from bioreactor, place in a biosafety cabinet; remove the media, add 4 new decellularized scaffolds and 4 mL of fresh media to the bottle, replace the filter top and replace the bottle into the bioreactor in the incubator.
3. Day 2 through 12 - Repeat process in step 2, adding double the amount of decellularized scaffolds and media as the previous day (i.e. on day 4, add 8 decellularized scaffolds and 8 mL of fresh media).
4. Day 14 - At this point, there are approximately 250 decellularized scaffolds with attached satellite cells; replace the growth media that has been used to this point with differentiation media and incubate the bioreactor for 14 additional days.
5. Harvest the cells from bioreactor, and use for production of a lab-grown meat product.

This is a rough outline of the process, but shows how simple this bioreactor design is to operate in order to create lab grown meat products. It is important to note that each time there are materials added to the system, it is just scaffolds and media; not cells. This is what makes our design unique. Further automation of media replacements would make this an even simpler design in the future.

6.2 Evaluation of Design Criteria

In Chapter 3, we outlined design criteria that guided our design process, in order to create a final product that was a viable solution to the proposed problem. In the following sections, we will evaluate our final design against these design constraints that were set.

6.2.1 Evaluation of Constraints

6.2.1.1 Ensure Health of Cells

One of the primary constraints of the bioreactor design is that it must ensure that the cells in culture remain healthy and viable, ensuring the product would remain safe to consume under current FDA standards. By using a filter cap on the incubation chamber, a direct supply of oxygen is available for free exchange by the cells. By designing the bioreactor to fit within an existing incubator space, the carbon dioxide and temperature requirements for adequate cell growth are met and the design requirement for pH maintenance and waste removal is ensured by simple and efficient media changes within each incubation space.

6.2.1.2 Scaffold Adequacy

The myocytes were grown on scaffolds in order to promote the growth of cultured muscle cells into a more structured meat product. Decellularized spinach leaves were chosen as the scaffold, as they fulfill the constraints noted in Chapter 3.2, meaning they were able to be decellularized by a bulk decellularization process, do not inhibit nutrient transfer to cells, are scalable in terms of the bioreactor design, be completely edible according to FDA standards, and be a viable scaffold for various bioreactor designs. . The spinach leaves used were decellularized using a bulk decell process that allows them to remain edible, the scaffolds offer a proven surface for cellular growth, and they can be easily manipulated to fulfill the needs of any bioreactor or available incubation space.

6.2.1.3 Live Monitoring

Another key constraint of this design was the ability to monitor the condition of the cells and media to ensure the health and continued growth of the cells. Our bioreactor design incorporates CO_2, relative pressure, humidity, and temperature sensors into its design. While pH is not included, the team deemed it not as important as first thought, as media changes will be regularly conducted, negating the need for pH monitoring. Because of this fact, the team also felt that live monitoring of sterility of the media and growth environment was unnecessary as the cells will still be handled regularly in a sterile environment, allowing the user to manually assess the sterility. The team also designed a webpage that allows visualization of the sensor data in real time and access to tools to see trends within the data, enabling real time feedback about the environmental condition within the bioreactor.

6.2.1.4 Cost Effective

The final constraint to this design was the overall cost of the final bioreactor. By opting for a design that fit inside conventional cell incubators, the team negated the requirement for expensive heat regulation capabilities and allowed the design to be entirely 3D printed from plastic and assembled using readily available components, drastically lowering the cost per unit. The bioreactor system is also a stackable unit, allowing for upwards of 16 units to be placed inside a standard cell incubator. Finally, due to the ability to seed both sides of a spinach leaf scaffold at once, less materials are required to grow the same quantity of cells, further lowering operating costs.

6.2.2 Evaluation of Objectives

6.2.2.1 Media Usage

The final design effectively reduces the amount of media to seed and grow cells on the plant scaffold. Media ratio tests were conducted to prove that smaller ratios of media could be used, however two mL of media to one scaffold was the most effective. This ratio still uses less media than the standard practice currently used in the lab as the bioreactor design seeds cells on not just one, but both sides of the plant scaffold.

6.2.2.2 Ease of Use

This design was easier for the user to interact with as it combined the seeding and growing process of cells on the plant scaffold in one step. Previously, cells would be seeded on one side of individual scaffolds using cloning cylinders, which then had to be removed after 24-48 hours. This design allows the user to simply place scaffolds in the bioreactor design with cells suspended in media, fulfilling the objective of producing an easy to use design.

6.2.2.3 Scalability

By producing a bioreactor design with a volume of 500mL, this process is scalable as 250 scaffolds can be put in each bioreactor to seed and grow cells at a two milliliter to one scaffold ratio, however this can be optimized even further on a larger scale. A total of 91 bioreactors of this size and six incubators are needed to produce one hamburger. The main concepts of this design could also be utilized in other ways to scale this process in other ways. Those main design concepts being the angled rotation of a vessel that suspends cells, scaffolds and media for seeding and growing of cells.

6.2.3 Evaluation of Standards

Because the target industry for this system is for meat production but it is also a lab-based bioreactor, it is important to look back on the standards we discussed in the early chapters, both for bioreactor design and also for meat production. This is a new field of research and there are still decisions being made related to how lab-grown meat products will be regulated, but at this point discussing standards that pertain to the design of this bioreactor both in terms of its design and the product that it is creating (i.e the meat).

6.2.3.1 Standards for Bioreactor Design

ISO 11737-2:2009 dictates how medical devices need to be sterile and remain sterile while in use. This bioreactor model could be fully automated to be a self sustaining system, therefore limiting human interaction with the device and the final product. The parts of the device can be sterilized by ETO gas, and by closing off the system, there will be very limited opportunities for the inside of the device to become unsterile.

6.2.3.2 USDA Standards for Meat Production

Two important USDA standards for meat production that were discussed in an earlier chapter include pathogen intervention and microbial testing. Microbial testing would be a step in the production process once the meat product has been removed from the bioreactor, but the inclusion of a media sensor is one way to ensure that no product is being produced with microbes in it. As a media absorbance sensor tests for the pH of the media in the system, this could also take advantage of the color change that results in cell culture media when contamination is present. In terms of pathogen intervention, any meat production process needs to include at least 2 pathogen intervention steps. As mentioned, this bioreactor design has the capability to be fully automated, thus limiting human interaction. It would simply require an initial setup of putting in the pieces it needs to produce meat. After the initial cells, scaffolds, and media are placed, there could be a procedure in place that initially sterilizes those parts in the bioreactor. This added pathogen prevention step would sterilize the process after the only form of human interaction occurs.

6.3 Additional Considerations

6.3.1 Economics

Lab-grown meat has at least five years until it becomes a commercially sold product in grocery stores as it needs to be regulated and a manufacturing process must be identified. This project aimed to make a scalable design so lab-grown meat could be manufactured in a larger-scale for production. This bioreactor proves that a scalable method is possible and helps to bring lab-grown meat one step closer to grocery stores and restaurants.

6.3.2 Environmental Impact

The current landscape of the meat industry presents a multitude of environmental issues, as discussed in Chapter 2. Since our design decreases the amount of water and space needed to produce meat, there is the potential for a net positive environmental impact from our design. In addition, our design also aims to decrease the amount of cows necessary for growing meat,

which opens the door to a few more key environmentally friendly cases. First, less cows involved in meat production would mean less methane emissions, which would slow the onset of climate change. Second, less cows would mean less space needed for cattle farming, which could be repurposed to positively impact the environment. Finally, less cows means less food and water needed to raise the cows themselves.

6.3.3 Societal Influence

While some consumers may not consider cultured meat products to be "real meat" as it was not harvested directly from a living animal, the option to choose cultured meat products as an alternative to conventional meat products will cause a large societal change. The ability to still eat meat while drastically decreasing the water footprint of our food and decreasing the overall greenhouse gas emissions would be a major selling point, especially to environmentalists concerned about the impact of cattle farming on the planet. Similarly, current individuals who are vegetarian and vegan due to issues about the humane and ethical treatment of animals would be able to eat meat comfortably knowing that what they were eating was obtained in a slaughter-free manner. By harnessing the environmental impact as a major selling point, cultured meat products may be able to sway society into a form of meat eating that is both delicious and sustainable for the world.

6.3.4 Political Ramifications

There is potential for political ramifications if lab-grown meat were brought to the commercial market. With another meat alternative on the market, the agriculture economy could be impacted as there may be a reduced need for farmers. There are a variety of advocates for farmers and the disruption of their work may cause some uproars in the local communities, and potentially at the national level. Many countries have different perspectives and values regarding meat, so lab-grown meat could have a different impact on other cultures.

6.3.5 Ethical Concerns

As meat industry practices now generate many concerns in terms of animal rights, taking the animal out of meat production will ease many of these concerns. By switching to a lab-grown meat alternative, people will have the ability to enjoy the food that they like, while not having to worry about the way the animals were treated that they are now consuming. It will also change the way people view a vegetarian diet, as many individuals pursue a vegetarian diet in order to not eat animals.

6.3.6 Health and Safety Issues

This alternative meat solution will be produced using all edible products, which is the point of utilizing a plant scaffold. This will take out the chemicals that may be used in alternative cell culture to remove cells from plastic culture dishes for harvesting. By alleviating these concerns, we are creating a cleaner meat product than is currently on the marker, as what goes into meat now and how the cattle are grown aren't necessarily "healthy" for us.

6.3.7 Manufacturability

One of the three main design objectives for this project was the ability to scale the design up to a production level to both save time and resources for mass production of cultured meat products. As a result, tremendous consideration was placed into the design to maximize cell output and minimize user interaction time, as well as make the use of the bioreactor as efficient as possible. As such, the ability to 3D print the bioreactor and stack multiple such units in a standard cell incubator helps maximize the potential for replication and mass production.

Chapter 7. Discussion

7.1 Seeding and Growing Processes

Different seeding methods were tested throughout the design process to determine the easiest and most efficient process for seeding bovine muscle cells onto decellularized spinach leaf scaffolds. Ultimately, the team determined that seeding cells was most successful on unrestricted scaffolds, a process where scaffolds were added to cells suspended in media and allowed to rotate for 48 hours to allow for seeding of cells on the scaffolds. The success of these experiments was determined by staining scaffolds with Hoescht and Phalloidin to examine cell adherence onto the scaffold. Visibly, cells were adhered to most of the scaffold and cells were seeded at a lower density than traditional procedures. In previous lab practices, about 200,000 cells were used to seed onto one side of a scaffold. In this method, only about 50,000 cells were required for successful cell adherence on not just one, but both sides of the scaffold.

By allowing scaffolds to be unrestricted in a rotation with media, both sides of the scaffold were exposed to the seeding environment. Through imaging after staining scaffolds with Hoescht and Phalloidin, the team showed the success of cellular attachment on both sides of a scaffold, thereby doubling the productivity of the process and decreasing resources required for the seeding of cells on scaffolds.

When looking for ways to further enhance not just the seeding, but also the growing process of cells on scaffolds, the team investigated if cells would jump from one scaffold to another. This theory was tested by first seeding cells on unrestricted scaffolds via rotation. After two days, the media was then removed, along with any unattached cells left from the seeding process, and new scaffolds were added to the test tube with fresh, cell-free media. This process was repeated every other day for a week. After one week, scaffolds were stained with Hoescht and Phalloidin and imaged to examine cell adherence on added scaffolds. This experiment proved that cells did indeed jump from one scaffold to another and adhered onto the new scaffold. These results were revolutionary as they utilized less resources (ie. cells) for cell adherence and growth, while also making the process much more efficient.

7.2 Potential for Scalability

Throughout the design process, the idea of scaling up the final design to a production level helped steer our tests to maximizing efficiency for myocyte growth on the scaffolds. From our results, we have concluded that there is a definite potential for scalability and even increased efficiency when compared to both traditional and other cell culture meat production methods.

One of the easiest parts of our design that can be modified for scalability is the 2:1 mL of media to scaffold ratio that the team decided on using. The tests we conducted showed most optimal growth occurred in a 2:1 ratio, as compared to a 1:1 and a 0.5:1 ratio. However, all of these tests were conducted in very small scale, specifically in a 50 ml conical tube with a total of 4 ml of media and an appropriate number of scaffolds for each ratio. For our final design, upwards of 500 ml can be placed inside each bottle, which would result in 250 scaffolds for the 2:1 ratio. However, the team agrees that in such large scale conditions, the ml of media to scaffold ratio can be reduced to increase the number of scaffolds in each bioreactor unit. Simply by optimizing the media to scaffold ratio to suit the size of the bioreactor growth chamber, this design could both easily be made more efficient through more scaffolds, or be converted to a larger reactor model, a topic that will be discussed in more detail later in this section.

A second point of note for scalability in this prototype is the physical size of the unit itself. When first conceived and designed, the reactor unit was intended to contain its own heating element and environmental control system. However, as the project progressed the team decided to move away from bench top use and instead design the unit to be placed in a standard cell incubator. While the final prototype design is intended to be placed in an incubator, its overall shape still retains some of the original specifications and qualities the team had originally intended for the bench top use model, specifically the large, wide base on which the rotator sits, which was then adapted into the stackable box seen in the final design. Simply by redesigning the unit itself solely for use in an incubator instead of adapting the bench top design for incubator use, the team believes that the quantity of 500 ml reactor vessels that could be fit into a single incubator can be dramatically increased, potentially to the point to where a single hamburger can be grown in a standard double-stack, or two, incubator set-up commonly seen in labs.

Thirdly, and as mentioned previously, the team also believes that the bioreactor model can be scaled up to an even larger size. For example, beer is commercially brewed in extremely large vats that are not dissimilar to traditional cell proliferation bioreactors used for other types of cell growth. By adapting this technology into a reactor model of that scale, this technology could easily provide for the efficient growth of cells required to create 70 kg of meat.

Additionally, we also envision the possibility of developing stand-alone units that could be purchased directly by consumers. These units could be a small counter-top sized unit similar to a toaster oven or other kitchen appliance, or larger in size like a washing machine or refrigerator. Consumers would then be able to purchase scaffolds, media, and a culture starter, similar to a sourdough or yoghurt starter, and grow their own burgers in their own homes, small businesses, or restaurants. Furthermore, the burger example that has been prevalent in this paper is merely the beginning. Hot dogs, meatloaf, pork chops, boneless chicken, and even fish are just some of the products that this technology could be adapted to produce in the future.

7.3 Additional Features for Ease of Use

As previously mentioned in Section 7.1, many aspects of the rotating bioreactor design improved not only the process of seeding and growing, but also the overall ease of use of the device. The cell-jumping experiment showed that cells could continue growing on newly added scaffolds, which could eliminate the need for growing cells outside the bioreactor all together. However, other features were added to the prototype to monitor the growing environment outside the incubator to further decrease user interaction time.

To keep the user updated on the environment of the bioreactor, several sensors were added to final design. These included a temperature, humidity, pressure, and CO2 concentration sensor. These were then connected to a WiFi enabled microcontroller (ESP32 specifically), and the data were sent to a locally run Python server. The Python server was then used to host a local monitoring webpage, as seen in *Figure 6.3*.

Using a local webpage has several advantages over a dedicated application, with the first being that any device on the local network can access the page. This is key for monitoring the bioreactor while being away from the incubator, making it much easier for the user to see the current status of the bioreactor. In addition, the user can also control the speed of the bioreactor from the page by typing in a percentage on the left input box.

This page can only be accessed by users that know the host computer's local ip address, however extra security measures could be added (such as a login or pin to send commands to the bioreactor) in the future. This would be especially important if this page was to be port forwarded (meaning it could be accessed from any device on any network if the ip address is known, not just the local network).

Chapter 8. Conclusions and Recommendations

This project demonstrated the success of innovative and scalable procedures for the production of lab-grown meat using a bioreactor model. A new seeding method was tested and validated, demonstrating that scaffolds can be seeded in a free flowing system, with cells suspended in media in a system that is rotating at an elevated horizontal axis. Additionally, days after seeding new scaffolds were added to the system and it was shown through staining and imaging the scaffolds that cells would jump from one scaffold to another. These two novel discoveries allow for this procedure to be scaled to a larger size that is much more user friendly and limits the amount of resources used for the production of products. With further testing and validation, this bioreactor model could be scaled up to a larger size and manufacturing of lab grown meat. Through a training process, many people could have the ability to make their own lab-grown meat products with the right equipment and materials. The agriculture industry could be revolutionized as farmers could turn part of their barns into a clean room for the manufacturing of meat with bioreactors.

This project produced a proof of concept device that will require further testing and validation to bring this to market in the future. The team recommends that the bioreactor incorporate a media filtration system to further reduce the amount of water used by removing waste and maintaining viable media components for further production. Additionally, this system could be automated even further so that media filtration and replenishing as well as the addition of new scaffolds could be done with limited user interaction. This bioreactor design is a promising step towards the large-scale production of lab-grown meat products. Lab-grown meat is the key to having the ability to feed the world.

Meat production now starts in the lab and ends on your plate.
Are you ready to taste the future?

Chapter 9. References

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Chapter 10. Appendix

Appendix A: Seeding Myoblasts on Spinach Scaffolds using Cloning Wells

1. Remove plates from incubator and put back into biosafety cabinet
2. Using sterile forceps, transfer sterilized, decellularized spinach scaffolds into a well plate with only one scaffold per well
3. Place sterilized cloning wells onto leaves at this point using sterile forceps
4. Passage and count your cell supply
5. Pipette cell suspension into cloning wells on top of the leaves
    a. 200k cells/100uL per cloning well; can add more media if necessary
6. Deposit enough growth media to cover the leaf
7. Include a control plate of cells growing without a leaf scaffold
8. Incubate the plates
9. Check on the media daily and refeed every other day

Appendix B: Passaging Primary Bovine Muscle Cells

1. Place media, trypsin in water bath at 37C.
2. Remove T-75 flask and verify cell viability and confluence with scope. Place in bio-safety cabinet.
3. Remove cap and transfer media off cells with sterile Pasteur pipettes to a 50ml conical tube.
4. Add 5ml of trypsin to flask.
5. Put flask back in incubator and let sit for 5 min.
6. Remove flask and confirm cell detachment with scope. (Detached cells will float freely and appear round)
7. Add 5ml of 10% FBS in DMEM f-12 to T-75 flask. (This deactivates the trypsin)
8. Pipette contents of tube in 25ml pipette and place in the same 50ml conical tube.
9. Centrifuge the 50ml conical tube for 5min @ 1000rpm making sure to balance the centrifuge.
10. Being sure to spray down the 50ml conical tube, reintroduce it into the sterile field and aspirate off the supernatant being sure not to disturb the cell pellet. Resuspend the pellet
11. Resuspend the pellet in 15ml of media.
12. Transfer cell suspension to a sterile large non-TCP petri-dish.
13. Carefully move cell to 37C incubator and incubate for 30min.
14. Pipette contents of petri-dish in 25ml pipette and place in the same 50ml conical tube.
15. Centrifuge the 50ml conical tube for 5min @ 1000rpm making sure to balance the centrifuge.
16. Being sure to spray down the 50ml conical tube, reintroduce it into the sterile field and aspirate off the supernatant being sure not to disturb the cell pellet.
17. Resuspend the pellet in desired amount of media. (Varies between 0.5ml to 1ml based on pellet size)
18. Triturate the solution with a 1000ul pipette to ensure the solution is homogenous.
19. Remove 30ul of cell suspension and add it to the 30ul of trypan blue stain.
20. Load 10ul of the cell+trypan blue mixture in each side of the hemocytometer.
21. Count enough boxes to achieve a count of 100 cells of greater. Once you begin counting a box you must count the whole box.
22. Use this formula to determine the cell density.

$$\frac{\#\ of\ cells\ counted}{\#\ of\ boxes\ counted} * 2 * 10,000 * \#\ of ml = \frac{cell\ count}{1\ ml}$$

23. Either seed 500,000 cells per T-75 flask, with 10-12ml of media, or use cells for other intended purpose. Recommended seeding density of ≈7000 cells per cm^2

Appendix C: Plant Decellularization using Bulk Decellularization

Materials:

- Plant material
- 1 L beaker
- Decellularization chemicals
  - SDS (Day 1)
    - For 2L of solution mix 200 mL of 10x SDS with 1800 mL of DIH20
      - 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
  - Triton X-100 or Tween 20 + Bleach (Day 2)
    - 0.1% Triton-X/Tween 20 with 10% bleach (or Cl tablets) in DI H2O
      - 48 mL of concentrated Clorox bleach and 20 mL of Triton-X100/Tween 20 Solution are added to 2L of DiH20 and mixed until in solution
  - DI H_2O (Day 3)
  - Tris Buffer (Day 4)
    - 10 mM Tris Buffer (605.7 mg in 500 mL of DiH2O)
    - Buffered to pH 9.0
- Stir bar
- Stir bar protector plate
- Stir plate
- Forceps

Procedure:

1. If dirty, wash the plant leaves with distilled water
2. Place a stir bar and a stir bar protector plate at the bottom of a 1 L beaker
3. Fill the 1 L beaker with SDS to the 800 mL mark
4. Put the plant leaves into the beaker
5. Depending on the amount of leaves and the type of stir plate, set the stir plate to an appropriate rpm. The rpm should be set so the leaves are moving around but are not being destroyed by the stir bar or the force of the flow.
6. Let the leaves soak in SDS for 24 hours
7. After 24 hours, replace the SDS with Triton X-100 or Tween 20 + Bleach
8. After 24 hours, replace the Triton X-100/Tween 20 + Bleach with DI H2O

9. After 24 hours, replace the DI H2O with Tris Buffer
10. After 24 hours in Tris Buffer, the decellularized leaves can be placed in the freezer for a few weeks until they are lyophilized
11. Remove leaves and freeze overnight in -20°C freezer. The leaves can stay in the freezer up to three weeks.
12. Lyophilize leaves for 24 hours
13. Store lyophilized leaf scaffold at room temperature until needed.
14. Please see the rehydration protocol for next steps

Troubleshooting:

- If the leaves are not decellularized enough, increase the rpm and the time in SDS.
- If the leaves are destroyed by the stirring mechanism, decrease the rpm and use less leaves.

Appendix D: Rehydration and Sterilization of Decellularized Spinach Leaves

Materials:

- Lyophilized decellularized spinach leaves
- Biosafety cabinet
- Petri dish(es)
- Non-TCP coated 6 well plates (or alternative)
- Orbital or linear shaker plate
- 10mM Tris buffer, pH 9.0
- DI water
- 70% ethanol
- Sterile 1X PBS
- Cell media
- Serological pipette gun and pipettes

Procedure:

1. Put lyophilized leaf pieces in Petri dish(es) or well plates
2. Rehydrate leaves in 10mM Tris buffer on shaker plate for 30 minutes
3. Aspirate Tris buffer from dish and rinse with DI water
4. Add new DI water to leaves and keep on shaker plate for 30 minutes
5. Aspirate DI water
6. Add 70% ethanol and wash for 30 minutes on shaker plate
7. Bring closed dish containing ethanol and leaves into the biosafety cabinet
8. Aspirate ethanol from dish and rinse with sterile 1X PBS.
9. Move leaf pieces into well plates with sterile forceps
10. Rinse leaves 2x with sterile PBS in well plates
11. If desired, can UV sterilize at this point in biosafety cabinet (but not necessary)
12. Aspirate remaining PBS and add cell media to leaf wells
13. Incubate leaves overnight at 37C

Appendix E: Phalloidin & Hoechst Staining of Bovine Myocyte Cells

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 sections/cells:

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

For fixed sections/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: Live/Dead for Plates and Suture 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

Appendix G: CyQUANT DNA Assay for Mammalian Cell-Seeded Plants

Materials:

- 1x PBS (Dulbecco's Phosphate buffered saline) (sterile and non-sterile)
- Sterile fine scissors and fine forceps
- 10, 200, and 1000 µL micropipettes
- Tips for 10, 200, and 1000 µL micropipettes
- 1.5mL microcentrifuge tubes
- 15mL conical tube
- Liquid Nitrogen
- Microcentrifuge tube rack
- Pipette Aid
- Serological Pipettes 10ml
- 50mL conical tube
- 96 well plate
- Daigger Vortex Genie 2® A. Daigger & Co., INC.
- Cyquant Cell Proliferation Assay Kit (Invitrogen)
  - Cell-lysis buffer (Component B)
    - Dilute 1 mL Lysis buffer to 19mL DPBS (1x Lysis buffer)
  - CyQUANT® GR dye (Component A) (light sensitive)
    - add 50 µL GR (fluorescent) to 20 mL 1x Lysis buffer
  - λ DNA standard
    - Dilute to 1.0 µg/mL by mixing 10 µL of the stock solution with 990 µL of CyQUANT® GR/cell-lysis buffer

A few hours or day before:

Leaf sample preparation:

1. Cut up each leaf sample with sterile fine scissors in very small pieces
2. Place each sample into one 1.5mL microcentrifuge tube and add 500 µL PBS to each
3. Flash freeze samples by dipping tube into liquid nitrogen
4. Repeat as many times as necessary until you can shatter leaf with fine forceps in tube
5. Spin down leaves by centrifuging at 2,000 rpm for 5 min
6. Remove 400 µL of PBS
7. Freeze at -80°C for at least 1 hour

Mammalian cell preparation (standard curve): (from T75 plate of control cells, adjust volumes if

smaller size plate):

1. In biosafety cabinet, aspirate off media using a Pasteur pipette.
2. Using a micropipette, add 10mL of sterile PBS.
3. Aspirate off PBS.
4. Add 5 mL of trypsin to plate and incubate for 5 minutes at 37C. Check under microscope to ensure floating cells.
5. Remove plate and add 5mL media. Draw up cell suspension into 15mL conical tube and spin down cells in centrifuge for 5min at 1000rpm.
6. Aspirate media and resuspend in media to count cells. See cell counting protocol.
7. Using a micropipette, add as much media as necessary to create a cell suspension of 12,000 cells/100ul (using/saving 1mL of suspension –final cell #: 120,000/1ml). (x 3 for triplicate need 300uL)
    a. Note: Can change cell concentrations if using/counting larger number of cells.
8. Centrifuge cell suspension down at 2,000 rpm for 5 minutes using the microcentrifuge.
9. Without disrupting the pellet, remove as much media as possible.
10. Add 500 µL non-sterile PBS.
11. Centrifuge at 2,000 rpm for 5 min using the centrifuge.
12. Remove 400 µL.
13. Freeze at -80°C overnight.

1 hour before assay:

Leaf sample preparation:

1. Remove samples in microcentrifuge tubes from -80C freezer.
2. Using a micropipette, add 500 µL of DPBS.
3. Centrifuge microcentrifuge tube at 2,000 rpm for 5 minutes.
4. Using a micropipette, remove 400 µL of DPBS.
5. Place microcentrifuge tube in microcentrifuge tube rack.
6. Place rack in -80°C freezer for at least 1 hour

Mammalian cell preparation (standard curve):

1. Make up standard dilution solutions in dark (see materials)
2. In a 96 well plate, place 100 µL of CyQUANT dye/lysis buffer into each well in Columns 1-4, Rows B-H.
3. Add additional 100 µL of CyQUANT buffer to each well of H1-H4.
4. Remove microcentrifuge tube from freezer.
    a. Note: Allow microcentrifuge tube and contents to reach room temperature. Do NOT place in water bath.
5. Following thaw, refreeze in -80°C freezer for at least 1 hour.

At time of assay: (in dark)

Plant standard curve preparation: (if no mammalian cells)

1. Make up standard dilution solutions in dark (see materials)
2. In a 96 well plate, place 100 µL of CyQUANT dye/lysis buffer into each well in Columns 1-4, Rows B-H.
3. Add additional 100 µL of CyQUANT buffer to each well of H1-H4.
4. Using a micropipette, take up the DNA Standard solution (1.0ug/mg DNA standard + GR/lysis buffer) and dispense 200 µL into each of the wells A1-A4.
5. Using a 1000 µL micropipette, take up 100 µL of CyQUANT buffer from the topmost well in a Column and dispense it into the well directly beneath it. Mix the solution in each well 6 times. Repeat this step through Row G.
    a. Each row will have ½ the concentration of the row before
    b. **Note:** Row H wells have NO standard curve (this is blank).
6. Using a micropipette, add 100 µL of CyQUANT buffer to each well from A1 to G4.
    a. **Note:** This will return the full amount to 200 µL.
    b. **Note:** Samples will saturate after 5 minutes of light exposure; work fast. Should be 200 µL in all wells of standard curve, if not add CyQUANT buffer until 200 µL is reached.

If using mammalian cell standard curve:

1. Thaw mammalian cell microcentrifuge tube from -80C freezer at room temperature.
2. Vortex lightly using Vortex Genie 2.
3. When thawing is complete, add 900 µL of CyQUANT buffer to Standard Curve microcentrifuge tube.
    a. **Note:** The total volume in the Standard Curve should be 1000 µL.
4. Lightly vortex using the Vortex Genie 2®.
5. Using a micropipette, take up the Standard Curve solution and dispense 200 µL into each of the wells A1-A4 of the 96 well plate.
6. Using a 1000 µL micropipette, take up 100 µL of CyQUANT buffer from the topmost well in a column and dispense it into the well directly beneath it. Mix the solution in each well 6 times. Repeat this step through Row G.
    a. **Note:** Row H wells have NO standard curve (this is blank).
7. Using a micropipette, add 100 µL of CyQUANT buffer to each well from A1 to G4.
    a. **Note:** This will return the full amount to 200 µL.

Sample preparation:

1. Remove rack from freezer.
    a. **Note:** Allow microcentrifuge tube and contents to reach room temperature. Do NOT place in water bath.

2. Using a micropipette, add 400 µL of CyQUANT lysis buffer.
3. Vortex lightly using the Vortex Genie 2®.
4. Using a micropipette, remove leaf material from sample.
5. Using a micropipette, take up of solution with cells and dispense 100 µL solutions into each of 4 wells.
6. Once all intended wells are complete, de-gass plate in vacuum to remove bubbles.
7. Place 96 well plate on plate reader.
8. Run plate reader. (480nm excitation, 520nm absorption)

Calculations

1. Place 96 well plate on plate reader. See protocol for Plate reader. Read operation related sections.
2. Copy obtained data into an empty Excel document. Calculate average, correlation coefficient, slope, and x-intercept of standard curve.

   Note: To calculate the correlation coefficient type in desired cell (the cell locations should
   correspond to cells for calculation)

   Ex: =CORREL(A3:A10,F3:F10)

   To calculate the average type in desired cell (the cell locations should correspond to cells for
   calculation)

   Ex: =AVERAGE(B3:BE3)

   To calculate the intercept type in the desired cell (the cell locations should correspond to cells for calculation)

   Ex: =INETERCEPT(F3:F10,A3,A10)

   To calculate the slope type in the desired cell (the cell locations should correspond to cells
   for calculation)

   Ex: =SLOPE(F3:F10,A3,A10)

3. Using average, multiple the average by 5 for 500µL.
4. Cell value for sample = (Average – y-intercept)/slope.