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DEPARTMENT OF BIOMEDICAL ENGINEERING
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PERFUSION DECELLULARIZATION-RECELLULARIZATION BIOREACTOR FOR LARYNGEAL TISSUE ENGINEERING

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ABSTRACT

Tissues and organs of the human body can be partially damaged or rendered completely dysfunctional when subjected to trauma or disease. In the case of tissues with minimal regeneration capabilities, the only option for regaining function is transplantation. This procedure is very expensive and there is a shortage of qualified donors. Even if a donor is found and the transplant performed, the immunosuppressants that must be taken by the recipient can lead to complications and the organ or tissue may be rejected. Recent tissue engineering research has shown promising evidence that tissues and even whole organs can be generated using the recipient's cells. This is done by utilizing a decellularization-recellularization process. The decellularization step lyses the cells of the donor organ, turning it into an acellular scaffold. This scaffold is then recellularized by exposing it to organ-specific cells. This process has been performed on whole organs and tissues. Of specific interest to this design project is whole organ regeneration of the larynx. Previously only partial laryngeal engineering has been successful. A key component in successfully engineering the larynx is proper bioreactor design. The bioreactor is the unit that will house the larynx throughout the decellularization and recellularization processes. Design of a quality bioreactor includes determining optimal orientation of the larynx and techniques for recellularization of the scaffold.

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PROBLEM STATEMENT

The purpose of this project is to design a sterile bioreactor for whole organ tissue engineering of the human larynx, as well as comparable large animal models such as the pig or dog larynx. The bioreactor must be capable of performing two different processes: perfusion-decellularization of the larynx to create an acellular scaffold, and perfusion-recellularization of the acellular scaffold using vocal fold fibroblasts and other cell sources.

BACKGROUND

Larynx Anatomy

The larynx is the organ that is located at the superior aspect of the airway channel, connecting the trachea and pharynx, ultimately leading to the oral cavity. Although the larynx is a relatively small organ, it has many important functions. The primary functions of the larynx include the production of voice (phonation), regulation of the flow of air into the lungs, and preventing the passage of food or other foreign matter into the airway during swallowing [1]. Other functions include coughing, the Valsalva maneuver, and providing sensory information [2]. Figure 1 shows three different views of the larynx and highlights its gross anatomical structure.

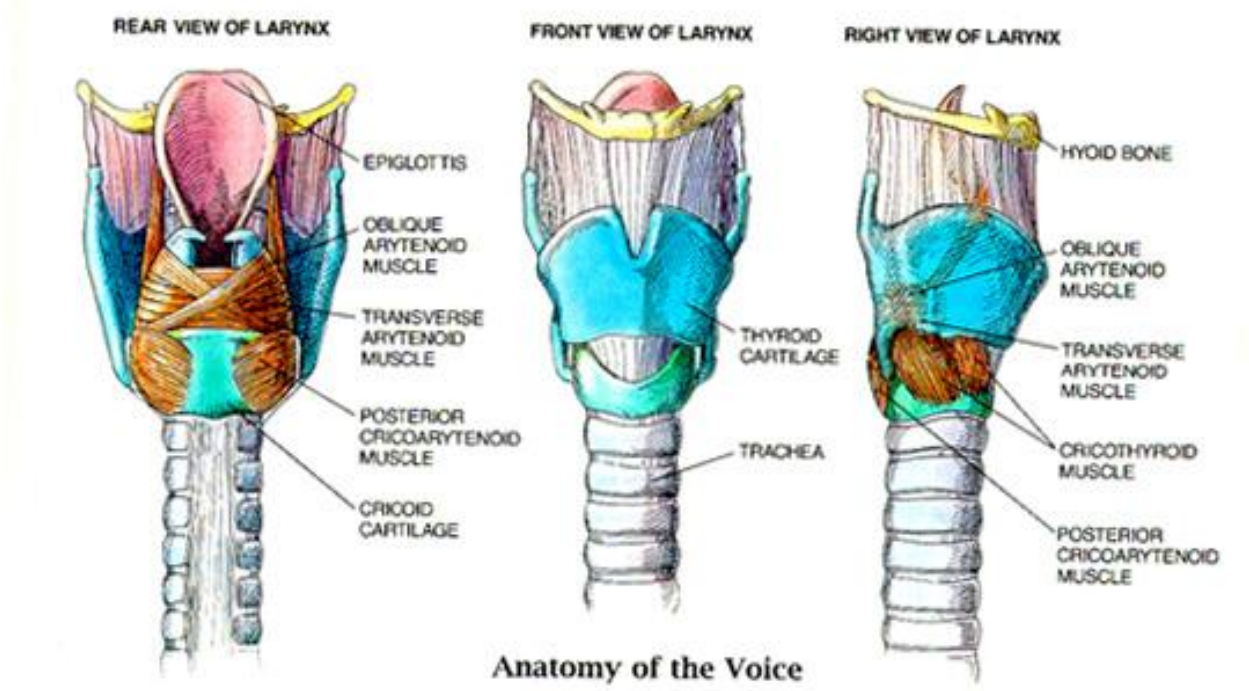

Figure 1: Major anatomical structures of the larynx. [http://www.edoctoronline.com/media/19/photos_040EAD64-F02E-4068-A04D-1B1C94AFDB10.jpg]

As shown in Figures 1 and 2, the larynx is composed of many different structures and tissue types. This allows for the many precise and intricate movements that are necessary to achieve its aforementioned functions. A brief overview of the gross anatomy of the larynx follows, highlighting pertinent information for the understanding of this report and design project.

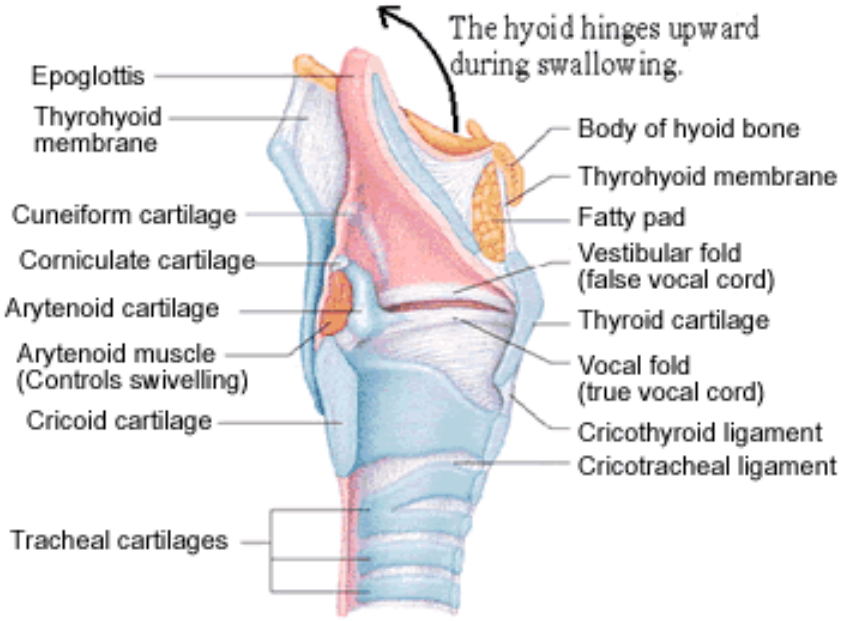

Figure 2: Additional laryngeal anatomy [http://www.edoctoronline.com/media/19/photos_167BFD91-F36A-473D-A56E-ACBAB131E39C.gif]

Structural support for the larynx is provided by six cartilages: three large unpaired cartilages and three smaller, paired cartilages. Briefly, the thyroid, cricoid, and arytenoid (paired) cartilages serve as attachment points for many muscles in the larynx. The epiglottis is a leaf-shaped cartilage that protects the vocal folds and airway during swallowing, as it folds over the opening to the vocal folds. The corniculate (paired) cartilages are smaller in size and extend the arytenoid posteriorly and medially. Finally, the cuneiform (paired) cartilages are also much smaller and are responsible for the small whitish projections above the arytenoid cartilages (see Figure 2) [2].

The vocal folds, which are concerned with phonation, are composed of two strong vocal ligaments and the vocalis muscle [2]. This structure is covered by four layers of tissue with distinguishable structures: the epithelium and superficial, intermediate, and deep lamina propria [3]. Two classes of muscles attach to the larynx and are defined by their points of attachment. The extrinsic muscles pass between the larynx and its surrounding structures; the intrinsic muscles are confined entirely within the larynx. Both the extrinsic and intrinsic muscles allow for the finely tuned pitch and frequency control of the voice as well as respiratory functions. Muscles are innervated by the superior laryngeal nerve and the recurrent branch of the vagus nerve [2].

To accelerate the decellularization and recellularization process, the vasculature is used to perfuse media and detergent into tissues [4]. The left and right common carotid arteries feed blood into the smaller thyroid artery, as shown in Figure 3.

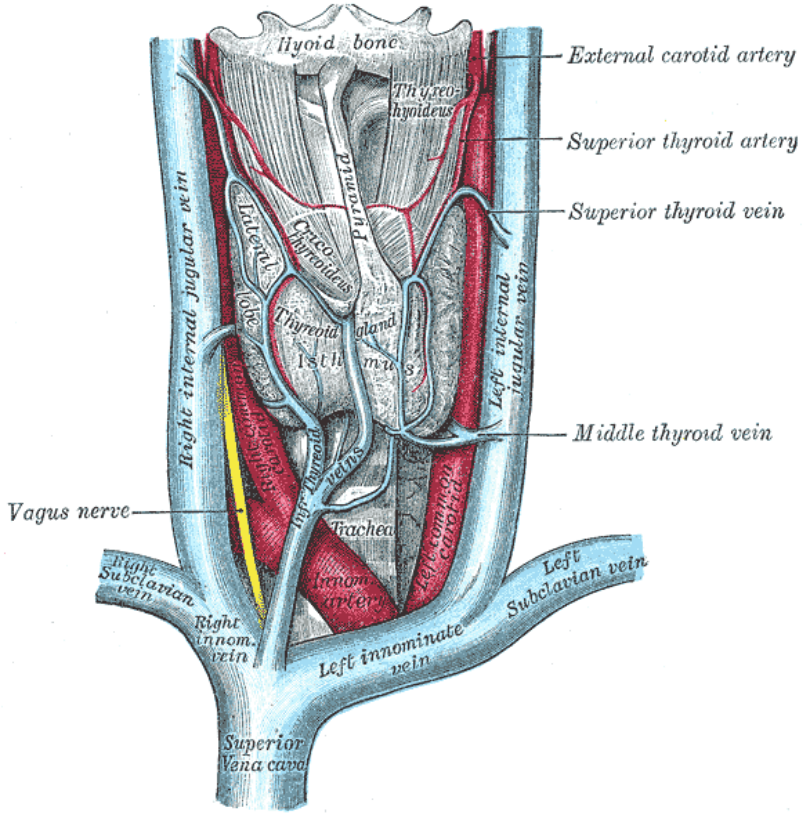

Figure 3: Blood supply to the larynx
[http://www.fpnotebook.com/_media/entLarynxAnteriorVesselsGrayBB1174.gif]

Tissue Engineering

Stem cells are remarkable for their ability to differentiate into one of many other cell types in the body. The major hallmarks of stem cells are their lack of specialization and their ability to differentiate into more specialized cell types, such as neurons, muscle cells, red blood cells, and others. This process is shown in Figure 4. For this reason, stem cells tend to function as an internal repair system, dividing and differentiating to replace damaged tissue. This is especially useful in organs such as the stomach, in which cells are regularly damaged and destroyed, and stem cells can easily replace the damaged cells to maintain function [5]. Stem cells are of great interest in tissue engineering for their unique ability and versatility. With their ability to differentiate into any other cell type, both embryonic and adult stem cells could be used to replace damaged cells in a body part and to help cure diseases.

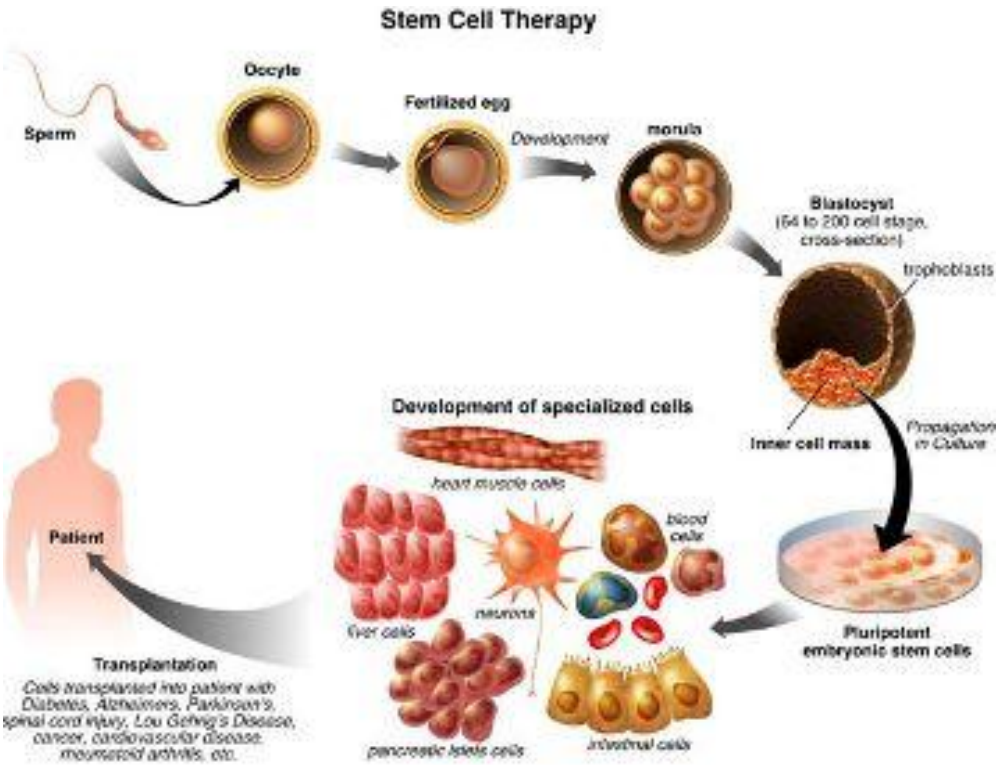

Figure 4: Differentiation of stem cells into specialized cells such as neurons and blood cells [http://nanobiotechnews.com/wp-content/uploads/2011/07/stem_cells.jpg]

The use of a decellularized matrix to repair damaged tissues is quickly becoming very popular in tissue engineering. By taking a donated tissue, using perfusion techniques to lyse cells within the tissue, then repopulating it with healthy cells (either stem cells or specialized cells of the body part in question), it is possible to restore lost function. An example of this is shown in Figure 5, which shows a rat kidney both before and after the decellularization process. This process is especially popular in whole organ tissue engineering, being used on donor organs such as the heart, lungs, and liver in order to provide an environment in which healthy cells can thrive [6].

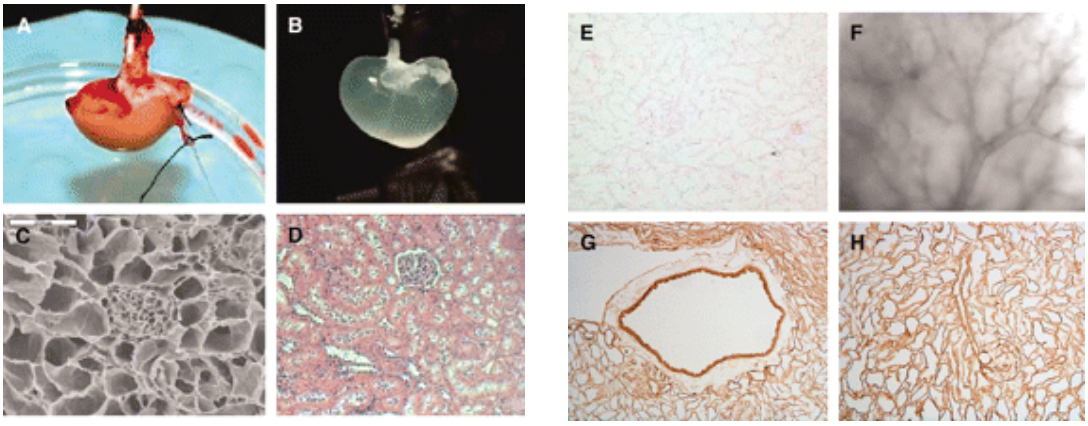

Figure 5: Rat kidney both before and after decellularization. [http://jasn.asnjournals.org/content/20/11/2338/F1.expansion.html]

Naturally, such a process is difficult to perform *in vivo*; thus, an *ex vivo* environment must be created to simulate the conditions of the physiological environment. This is the purpose of a bioreactor, or a device in which biological processes develop in a controlled environment. Bioreactors were traditionally used in industrial processing and waste management until recently, when the popularity of perfusion-decellularization tissue engineering led to a demand for devices such as bioreactors to create an optimal environment in which cells could grow and develop outside the body [7]. There are many different types of bioreactors, as shown in Figure 6, which support different physiological conditions, cell types, organs, and other factors. Regardless of the type, bioreactors are a very useful tool for controlling the environment in which cells grow and proliferate. And when used in conjunction with decellularized matrices and stem cells, there is a lot of potential to engineer healthy, functioning tissues and organs.

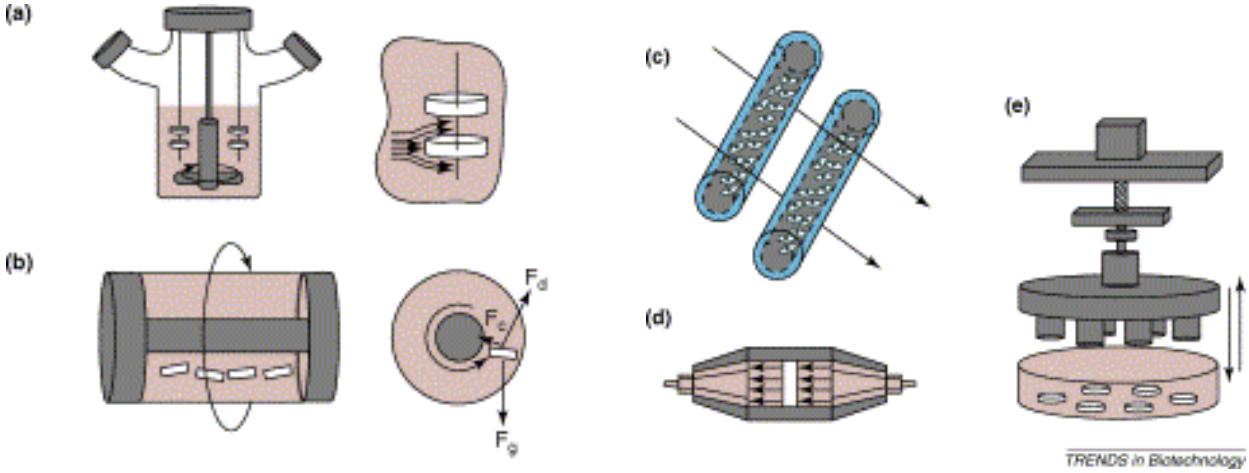

Figure 6: An example of different bioreactor types, including a rotating bioreactor (b) and a direct perfusion bioreactor (d) [http://ars.els-cdn.com/content/image/1-s2.0-S0167779908000346-gr2.jpg]

Significance

As with other organs, the larynx may be rendered dysfunctional from disease or trauma and current therapeutic techniques are ineffective. Each year almost 136,000 patients are diagnosed with laryngocarcinoma (laryngeal cancer) and require partial or complete laryngectomy, or removal of the laryngeal tissue [4]. Retaining function of the larynx following these procedures may not be possible, leaving patients mute and/or with respiratory problems. The only plausible treatment at this point is allographic transplantation.

A final treatment strategy for the kidney, pancreas, heart, lung, liver and intestine is transplant. In 2009 alone, 29,346 organ transplants were conducted in the United States [8]; however, due to anatomical complexities leading to difficulties in surgical technique, only two full-larynx transplants have ever been recorded [9,10]. Researchers have suggested that eliminating the need for immunosuppressants would increase the rate of laryngeal transplantation [4]. A recellularized laryngeal scaffold would not require the same level of immunosuppression as normal transplantation because they are grown from patient-specific cells. Thus, this project would be a big step in providing implantable larynges that would require minimal immunosuppression to patients.

PREVIOUS WORK AND CURRENT DEVICES

The concept of decellularizing and recellularizing an organ is not novel. Several research teams have successfully completed the task in other organs, including the lung [11], heart [12], liver [13], and kidney [14]. Researchers and clinicians have taken this a step further with the trachea. In 2009, the first successful full organ transplantation was completed using this decellularization-recellularization technique. Clinicians matched the geometry of the patient with a donor using MRI and CT scans to obtain a perfect match. After decellularization of the donor scaffold, the tissue was slowly rotated in a double-chamber bioreactor (Figure 7) that separated the inner lumen and outside of the trachea. This separation allowed for the different conditions of cellular development, promoting successful growth for the luminal epithelial cells and the cells on the outer surface [15]. The simple, tubular geometry of the trachea simplified many aspects of bioreactor design and definitely contributed to its published success. While bioreactors are commercially available, none exist that support whole organ laryngeal generation.

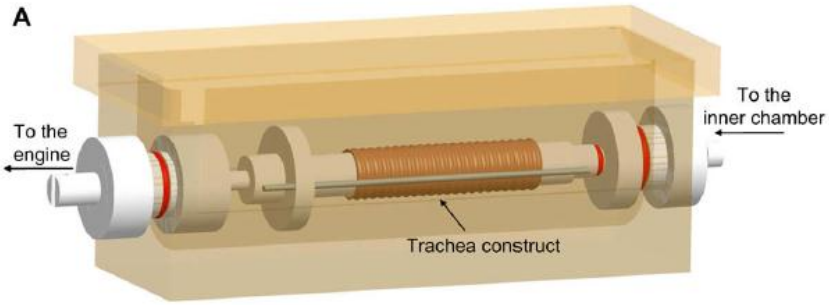

Figure 7: Example of currently available tracheal bioreactor [Asnaghi et al, 2009]

CLIENT DESCRIPTION

Dr. Nathan V. Welham (PhD, CCC-SLP) is an Assistant Professor in the Division of Otolaryngology-Head and Neck Surgery at the UW School of Medicine and Public Health. Clinically, Dr. Welham is certified in speech-language pathology and the evaluation and treatment of patients with disorders of the voice, resonance, swallowing and airway disorders. He practices as the Voice and Swallowing Clinic and the Pediatric Voice and Swallowing Clinics at UW Health. In addition an established clinical career, Dr. Welham has had extensive research experience [16]. Dr. Welham has 31 citations listed on PubMed and most recently published on proteome analyses, vocal fold scarring and treatment, and surgical treatment of sulcus vocalis. He has also worked with and developed animal models to study vocal fold scarring [17].

CLIENT REQUIREMENTS

In accordance to the wishes and specifications of the client Dr. Welham the laryngeal bioreactor will be made to meet the following criteria. It is essential that any portion of the bioreactor that comes in contact with the larynx or cell culture media be sterilizable. Autoclavable components

are ideal, though where this is not possible, replaceable components, to be changed after each use, are acceptable as well.

The bioreactor will be required to function continuously for days at a time within an incubator environment, which maintains temperatures of approximately 37ºC with high humidity levels. This continuous function must be upheld throughout the entire decellularization and recellularization processes, which combined are estimated to take no more than 4 weeks.

In order to streamline this process as much as possible, a single unit will be used to house the larynx for both decellularization and recellularization, with an intermediate wash cycle. It is necessary that this unit provide easy access to the larynx to allow for cell seeding, cell media replacement and any additional adjustments that need to be made. This will be achieved while maintaining dimensions that can easily fit within a Forma Series II Water-Jacketed CO_2 incubator from Thermo Scientific.

It is also required that the bioreactor maintain separate environments for the lumen of the larynx and the exterior. This separation needs to be maintained so that different cell media can be delivered to the larynx interior and exterior. In addition, the interior must be exposed to air in order to aid in appropriate cell development.

A Product Design Specification list, found in Appendix A, was composed to summarize the minimum requirements necessary to construct a functional bioreactor. Since whole organ engineering of the human larynx has never been performed, the criteria in this list are based on relevant literature and previous tissue engineering work.

DESIGN ALTERNATIVE: SPRAY

The first bioreactor design consists of a 10x10x20cm box with a removable lid. The removable lid will allow easy access to the inside of the bioreactor which will help during initial placement of the larynx onto the support tube and during exterior media changes. As previously mentioned, there will be a support tube inside the bioreactor that is also removable. The support tube will be taken out of the bioreactor so that it can be attached to the excess trachea below the larynx. The two will be sealed together using a cable tie and then placed back into the bottom of the bioreactor. To stabilize the larynx, sutures will be run through the superior cornu of the thyroid cartilage and fixed to the bioreactor walls. This will aid in supporting the larynx during the decellularization-recellularization process.

Once the larynx is attached inside the bioreactor, a balloon will be inserted through the epiglottis side of the larynx and positioned below the vocal cords. Once below the vocal cords, the balloon will be inflated to create two separate culture environments: one below the balloon on the luminal side of larynx and another environment above the balloon which is exposed to the exterior media that surrounds the larynx.

The media applied to the inner environment will be sprayed through a nozzle tip that is located on top of the support tube. The nozzle is attached to a tube that runs the inner length of the support tube and is connected to a pump. When the pump is turned on, media will be sprayed

through the nozzle tip at various time intervals. By atomizing the media, the cells will be exposed to both air and media which is a design requirement. After the inner media is sprayed onto the cells, it will trickle down the wall of the lumen and collect in a void below the support tube. By collecting the media at the bottom, the other end of the pump can be attached to the void and the media can then be recycled.

The exterior media will be added by removing the lid of the bioreactor and filling it until the larynx is completely submersed in media. Likewise, to remove the exterior media the lid will be removed and the media will be aspirated out of the bioreactor. In the wall of the bioreactor near its base will be a tube that draws exterior media into to it (Figure 8). This exterior media will then be perfused through the left and right carotid arteries by pumps to simulate blood flow through the larynx and provide oxygen and nutrients to the cells located within the extracellular matrix (ECM). Once the media passes through the internal vasculature, it will spill out the bottom of the larynx and into the exterior media. The exterior media can then be pulled back in by the pumps and the cycle repeats.

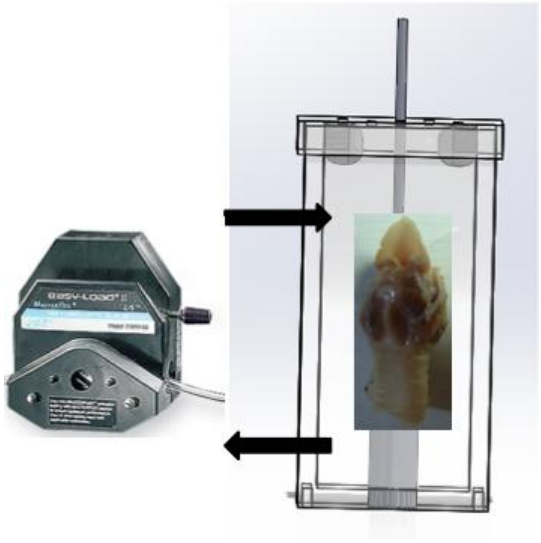

Figure 8: External media will be pumped in through the bottom and perfused back out through the left and right internal jugular veins (not shown) on the top of the larynx.

DESIGN ALTERNATIVE: FILL-REFILL

Like the first bioreactor design, the second bioreactor will be a 10x10x20cm box with a removable lid. A balloon will be inserted below the vocal cords and inflated to create two separate environments like in the first bioreactor design. The exterior media will be added in a similar way as it was in the first design; however, the inner media will not be delivered by spraying the cells on the inside of the lumen.

Instead, media will be pumped in and out of the lumen so at any given time, the cells are either exposed to media or to air. To perform this action, a reversible pump will be used. The pump will line the support tube and push media out until the lumen is full of media. After a given amount of media has been expelled from the tube, the tube will be sealed so that media cannot flow back down. This seal will trap the media on the inside of the lumen and expose all the cells

that line the lumen to this interior media. To expose the cells to air, the tube will be opened and media will be allowed to flow down. To expose the cells to media, the pump will push more media in and the cycle will continue. This design is advantageous over the first design because all cells are more equally exposed to media, and it is more controllable than the spraying technique of the first design.

DESIGN ALTERNATIVE: ROTATION

The third design, shown in Figure 9, more closely emulates the tracheal bioreactor currently available. Thus, this design is similar to that of the tracheal bioreactor, but modified to fit the size and shape of the larynx. The larynx is mounted on the inside of the chamber and media is perfused through the vasculature via pumps, just like with the previous two designs. The main difference between this design and the previous two, other than the fact that it is horizontally oriented, is that this is a rotational bioreactor. The larynx exterior is completely surrounded by media, while the inner lumen is half full with media and half full with air. The bioreactor will be designed to rotate slowly, guaranteeing full exposure of the inner lumen to media and to air. Unlike a traditional continuous unidirectional rotation, this bioreactor will rotate a set distance in one direction before changing directions and rotating in the opposite direction. This will ensure that the larynx is fully exposed to media, without the additional complications that a full rotation would bring, such as tangling the perfusion tubing and vasculature.

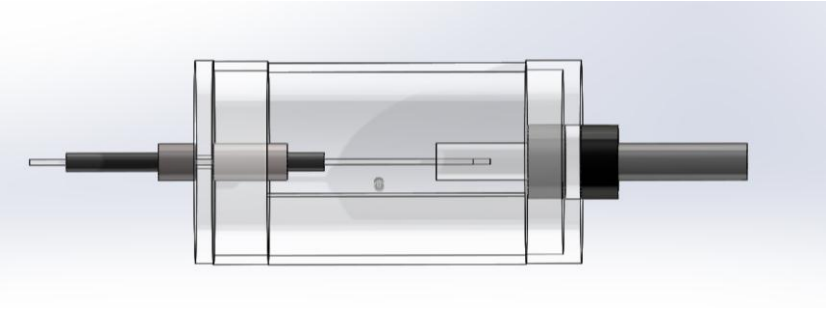

Figure 9: The third design alternative, which utilizes rotational movement and is horizontally oriented.

DESIGN MATRIX AND EVALUATION

	Weight	Design 1 Spray	Design 2 Fill-Refill	Design 3 Rotation
Cost	0.1	9	9	4
Decellularization-recellularization	0.3	7	7	8
Physiological accuracy	0.2	6	8	7
Adaptability	0.15	9	9	9
Maintenance	0.25	8	8	5
Total	**1**	**0.75**	**0.80**	**0.68**

Table 1: Summary of Design Matrix

The three design alternatives were evaluated and compared in the categories of cost; how conducive the bioreactor is to the decellularization-recellularization process; physiological accuracy of cellular development; adaptability of the infrastructure; and media maintenance of the bioreactor. Cost was given a low weight since the most expensive component of the designs will be the perfusion pumps, a feature in all three designs. Since perfusion through the vasculature is a client requirement, pumps are included in all designs. The rotation design features additional mechanical motors and therefore will be more costly than the other designs.

Since the main objective of the bioreactor is to decellularize a larynx into an acellular scaffold followed by recellularization into a functional organ, this criterion was given the highest weight. The decellularization process will be the same in all designs and therefore the major difference between the alternatives is in the recellularization process. While all designs provide a favorable environment for recellularization, the rotation design received a slightly higher rating because this technique has proven successful in similar recellularization processes.

Physiological accuracy was defined as the similarity between the bioreactor environment and the *in vivo* laryngeal environment. This includes orientation of the larynx as well as the air and media exposure techniques utilized in each design. The fill-refill design will best achieve the *in vivo* environment since the larynx will be secured in the anatomical position. The technique of having the inner lumen entirely exposed to either air or media is a more realistic representation of the *in vivo* conditions.

Adaptability encompasses two areas: the ability to secure larynges of varying sizes and alterations to be made to the bioreactor. All designs feature securing methods that allow for different sized larynges. Since the recellularization process is largely unknown at this point, the bioreactor must be able to handle experimental variations on media composition and levels in addition to minor mechanical alterations to optimize the recellularization process. All three alternatives were designed with this capability and therefore all received the same score.

The final important criterion, receiving the second highest weight, is maintenance of the bioreactor. This was characterized by the ease of maintaining the different medial levels and compositions in the external, internal, and perfusing environments. While the maintenance of the external and perfusing environments will be similar in all three designs, the internal environment will be the most difficult to access and the media composition will be most frequently altered. The similarities in the spray and fill-refill technique allow for the easiest access to the internal environment, thus enabling the media to be easily replaced.

FINAL DESIGN

Compiling the results of the alternatives in the design matrix, the fill-refill design is the clear choice for the final design. This design received high rankings in all matrix categories. The lowest score it received was in the decellularization-recellularization category; however this is only due to the unknown nature of what defines a successful recellularization process for the larynx. What separates the fill-refill design from the other two alternatives is the physiological accuracy. The air exposure technique allows for the inner lumen to be entirely exposed to media,

as it would be in the embryonic development, or to air, as it would be in the postnatal environment.

The bioreactor consists of an outer box with volume reducing inserts. A top view of this design is shown in Figure 10. The functional dimensions of the outer box are 15x20x25cm (7,500 cm^3). With the inserts in place, the inner volume is reduced to 10x10x21cm (2,100 cm^3). When the bioreactor is empty and the inserts are removed, the user can easily use one or both hands in the bioreactor. Being able to fit hands inside the bioreactor is important for placing the trachea, sealing the trachea, and seeding cells onto the decellularized scaffold. Once the larynx is in place, both inserts can be positioned prior to filling the bioreactor. Since media can cost up to $400 for 500mL, the inserts significantly reduce the overall operational cost of the bioreactor.

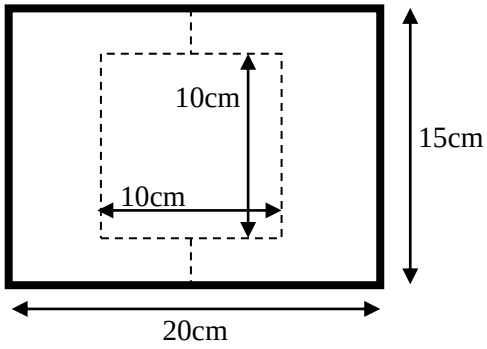

Figure 10: Top view of bioreactor to show dimensions. There are two volume reducing inserts with outlines denoted by dashed lines.

In order to recellularize the entire larynx, excess trachea on the inferior side as well as excess pharynx on the superior side must be excised with the larynx. These additional structures provide area for attachment to the bioreactor without compromising the laryngeal cells. A fixed cylinder 1.27cm in diameter is located at the base of the bioreactor. A second cylinder can be slid onto the fixed cylinder in order to account for different sized tracheas. The bioreactor can be used for tracheas with inner diameters ranging from 1.27cm to 2.54cm. The trachea can then be positioned on the cylinder and sealed with suture or a zip tie. A cuffed silicone endotracheal tube is used to seal the pharynx. The sealed trachea and pharynx separate the inner lumen from the external environment.

Two pumps help supply the larynx with media. One pump attaches to the arteries and perfuses media through the vasculature. After circulating through the vasculature, media will exit through the veins and contribute to the external media. The second pump fills and drains the inner lumen environment through the same tube. This tube is attached beneath the base of the bioreactor and pumps media through the hollowed center of the fixed cylinder.

MATERIALS

Bioreactor

Polycarbonate was chosen as the primary material for the housing of the bioreactor. A list of all of the materials, use, manufacturer and their cost can be found in Table 2. Many properties make polycarbonate an ideal choice for bioreactor design, as it is a clear material and allows the user to see what he or she is working on. Additionally, it allows for observation of the decellularization and recellularization processes and gives the user the opportunity to intervene if either process is not going as planned. Most importantly, polycarbonate is able to withstand the heat and pressure of the autoclave process. Taking preventative measures and limiting the autoclave cycles to 20

minutes at 121°C will extend the life of the polycarbonate and ensure that it retains mechanical integrity [18].

Although it was not considered as a design parameter, polycarbonate is fairly easy to work with and can be machined with normal equipment. It is joined with a quick (1-2 minutes to working strength) liquid solvent that provides strong, liquid-tight bonds.

The only other material used in the bioreactor was grade 316 stainless steel for fittings between the pumping system and the bioreactor. This grade of stainless steel minimizes the chance of material leaching when exposed to the decellularization chemicals and will not rust during autoclaving. Additionally, clinical grade trachea tubes were used to create the seal between the two media types. These are disposal and do not present any contamination concerns.

Pumps

Two pumps from Langer Instruments were chosen to provide the perfusion pumping in this design. These pumps provide interchangeable tubing that can be replaced after each use and does not present any risk for contamination, as no fluid is passed through the pump head itself. Instead, the fluid is peristaltically pushed through the tubes using a series of rollers within the pump head. The Langer Instrument pumps offer the same functionality as other leading competitors but at nearly half the price.

Item	Use	Manufacturer	Cost	Total ($)
0.5" thick 12 x 12 in polycarbonate sheet	Bioreactor base	Grainger	1 x $28.40	28.4
0.236 " thick 12 x 12 in polycarbonate sheet	Bioreactor sides; inner box; cover	Grainger & Midland Plastics	6 x $11.84 *	71.04
0.5" diameter 1 ft polycarbonate rod stock	Trachea support	Grainger	1 x $3.61	3.61
1 " diameter 1 ft polycarbonate rod stock	Trachea support	Grainger	2 x $11.75	23.5
Very fast set solvent cement for acrylics	Bioreactor cement	SciGrip from Midland Plastics	1 x $8.00	8
Stainless steel 0.25 " 90 elbow NPT thread	Fill-refill lumen	McMaster-Carr	1 x $7.83	7.83
Stainless steel 0.25" x 0.25 " barbed hose fitting	Fill-refill lumen	McMaster-Carr	1 x $13.39	13.39
Stainless steel 0.25 " x 0.125 " barbed hose fitting	Fill-refill lumen	McMaster-Carr	1 x $18.39	18.39
Low flow rate peristaltic pump (model BT100-2J)	Vascular perfusion	Langer Instruments	1 x $350	350
Ultra low flow rate pump head (model DG-2)	Vascular perfusion	Langer Instruments	1 x $140	140

3 Stop tubing for DG pumping (model Tubing-3s)	Vascular perfusion	Langer Instruments	1 x $39 **	39
Low flow rate peristaltic pump (model BT100-1F)	Fill-refill lumen	Langer Instruments	1 x $598	598
Medium flow rate pump head (model YX1515x)	Fill-refill lumen	Langer Instruments	1 x $168	168
1/16" thickness tubing for YZ1515x	Fill-refill lumen	Langer Instruments	1 x $34 **	34
Trachea tubes, various sizes	Compartment-alization	Dr. Welham	Free	0
Arduino Uno Microcontroller	Automation	Arduino	1 x $29.11	29.11
0-5V Input Control Module	Automation	Langer Instruments	1 x $56.20	56.20
				1588.47

Table 2: List of materials for the bioreactor housing, pumps, tubing and accessory equipment.
* Prices based on published Grainger price
** Prices reflect a 6 pack of tubing

FABRICATION

As previously mentioned, the polycarbonate material was machined to custom sizes and shapes using regular metal/plastic tooling (Figure 11). Machined edges were required to provide a smooth bonding surface for the cement. The fixed vertical cylinder at the base of the bioreactor and trachea inserts were turned to custom diameters on a lathe and fit to coincide with one another.

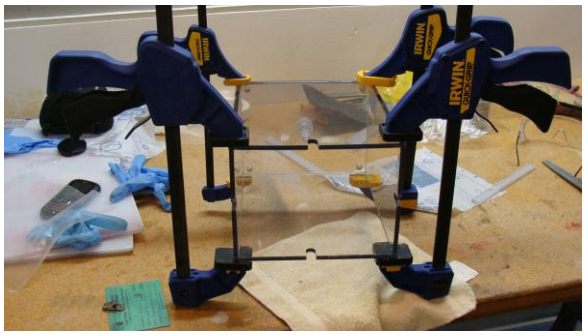

Figure 11: Cementing the outer box of the bioreactor.

PUMP TESTING

Testing

After constructing the bioreactor, the outer box as well as the inserts were tested and confirmed liquid tight. It was not necessary for the inserts to form a liquid tight seal with each other or the outer box.

Testing of the two pumps was also conducted (Figure 12). The time to fill both the pump tubing

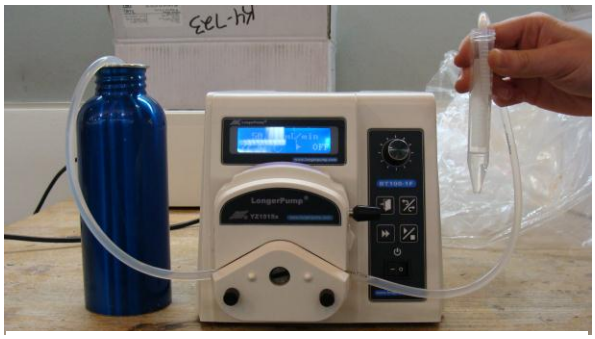

Figure 12: Pump testing set up.

and a volume of 25mL with water was recorded at varying speeds for each pump. Since the tubing associated with each pump will likely be replaced at least once during the decellularization-recellularization cycle, the pump tubing was emptied prior to each test. Additionally, during the fill-refill process of the inner lumen, the used media drained from the inner lumen will need to be entirely expelled from the pump tubing prior to refilling it with fresh media.

Fill time is important to consider in both the decellularization and recellularization processes. Once the larynx is excised, it must be transported to the bioreactor, secured in place, and submersed in decellularization solution. If this process takes a long time, cellular decomposition may begin prior to the addition of decellularization solution and may result in a compromised acellular scaffold. During the recellularization process, the air exposure time includes the time it takes to refill an emptied chamber and it is therefore important to know this fill time.

Results

The speed is set in revolutions per minute (rpm) for the pump supplying the vasculature. The maximum speed for this pump is 100rpm. Fill time in seconds was recorded at speeds of 10 to 100rpm in increments of 10rpm. This data is summarized in Table 3. Using this data, a plot was generated (Figure 13) to convert rpm to mL/min since this provides more useful information.

Speed (rpm)	10	20	30	40	50	60	70	80	90	100
Fill Time (seconds)	773.1	387.0	257.6	193.2	153.9	128.5	108.8	96.30	84.50	77.47

Table 3: Testing results for the vasculature pump

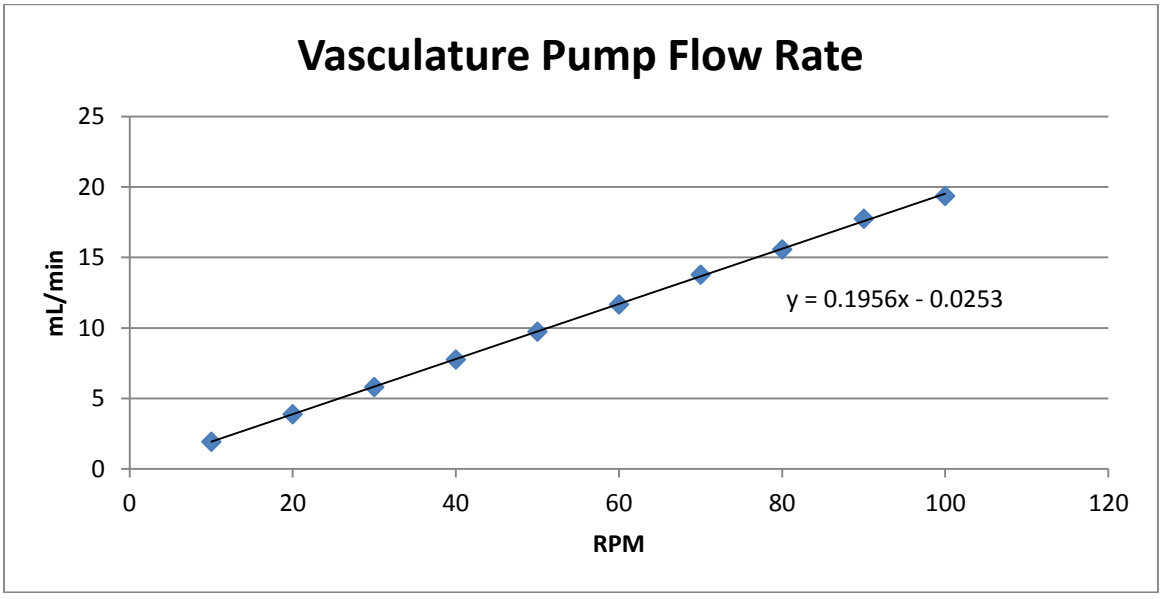

Figure 13: RPM to mL/min conversion for the vasculature pump.

For the inner lumen pump, the speed is set in milliliters per minute (mL/min). The maximum speed for this pump is 63.63mL/min. Fill time in seconds was recorded at this maximum speed and then decreased by increments of 10.00mL/min. The lowest speed that fill time was recorded for was 13.63mL/min. This data is summarized in Table 4.

Speed (mL/min)	63.63	53.63	43.63	33.63	23.63	13.63
Fill Time (seconds)	30.49	36.54	44.98	58.32	82.99	143.5

Table 4: Testing results for the inner lumen pump

MODIFICATIONS

After testing the bioreactor with excised tissue, a few minor modifications had to be made. First, the client wished to connect the superior aspect of the lumen compartment to the pumping system. This modification is supposed to accelerate the decellularization process. Second, a draining hole was drilled into the bottom of the main housing after learning that pipetting the used decellularization fluid was time consuming and unnecessary. Finally, luer lock connections were purchased to connect the vasculature tubing to the cannula.

AUTOMATION

In order to improve the ease of use of the bioreactor, a microcontroller was added to automate the inner lumen pump. The bioreactor was designed, per the client's requirements, to create a separate inner lumen environment in order to expose these cells to both air and media during recellularization. In the early stages of recellularization testing, it will be necessary to test a range of media flow rates as well as media and air exposure time durations. Testing may also show that optimal recellularization conditions feature varying flow rates and duration times within a single trial. While it is possible to achieve all of these different states using the control buttons on the pump, the addition of a microcontroller improves the consistency of varying conditions during recellularization testing.

Materials

Three main components were necessary to automate the inner lumen pump: a microcontroller, an external control interface, and a low pass filter. An Arduino Uno microcontroller, shown in Figure 14, was selected for the microcontroller based on its relatively low cost and ease of use. To connect the microcontroller to the inner lumen pump, an external control interface from Langer Instruments was necessary. The control interface selected was

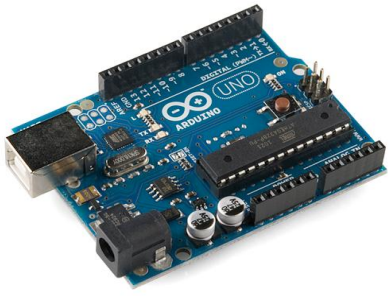

Figure 14: An Arduino Uno microcontroller [www.sparkfun.com]

the 0-5V Input External Control Module, based on the operating voltage of the Arduino Uno. Lastly, a passive low pass filter was implemented with the microcontroller to achieve in analog output voltage of 0-5V.

Design

The external control interface features five terminals to connect to the microcontroller. Terminal 1 controls the speed of the pump and requires an analog input of 0V to 5V. Terminal 2 controls whether the pump is running or not; when connected to 0V the pump will run and when connected to 5V the pump will stop. Terminal 3 controls the direction input; 0V input corresponds to a clockwise rotation and 5V input corresponds to a counter-clockwise rotation. Terminal 4 is the ground for pump speed control. Terminal 5 is the ground for the pump state (on or off) as well as for the pump direction [19]. A summary of the terminals are shown in Figure 15.

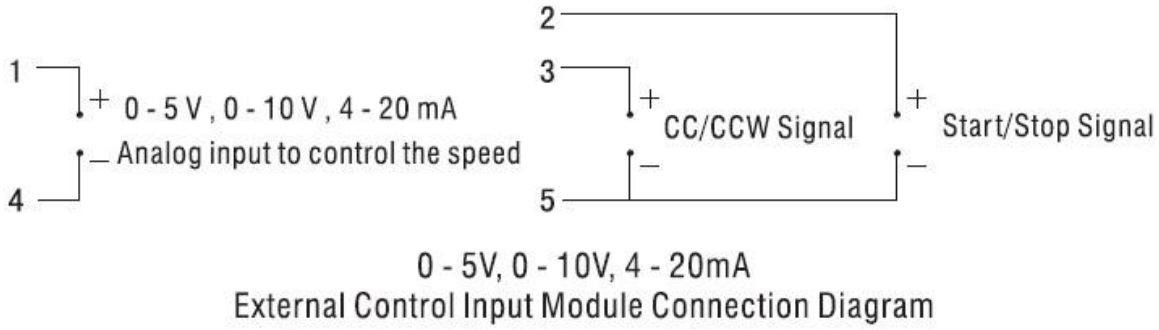

Figure 15: Summary of the five terminals on the external control interface [http://langerinstruments.com/manuals/BT100-1F.pdf]

The microcontroller was connected to the external control interface to control the pump state, speed, and direction. An example of working Arduino code to control the pump can be found in Appendix B. Since pump state and direction require only 0V or 5V, both were directly connected to digital output pins on the Arduino microcontroller. In order to access a range of pump speeds, an analog input between 0V and 5V is required. The only possible analog outputs from the Arduino are 0V or 5V. By adding a passive low pass filter, it is possible to input a range of analog signals to the pump. The low pass filter receives a pulse width modulation (PWM) signal from the microcontroller and converts it to an analog signal, which is what the external control interface receives.

To ensure consistent recellularization conditions, a sufficiently smoothed output signal is required from the low pass filter [20]. The low pass filter used is shown in Figure 16. To achieve sufficient smoothing, a resistor value of 10kΩ and a capacitor value of 4.7μF were selected.

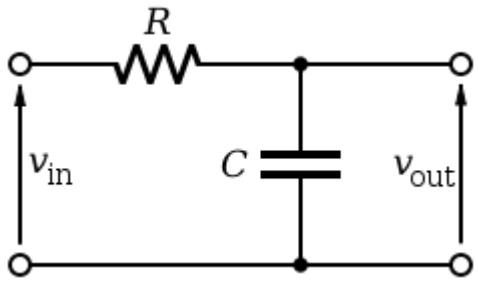

Figure 16: Passive low pass filter [http://en.wikipedia.org/wiki/Low-pass_filter]

Testing

A consequence of using the low pass filter is a reduction in the maximum voltage output and thus a reduction in the maximum flow rate. After connecting the microcontroller, external control interface, and low pass filter, a range of PWM outputs from the microcontroller were tested to determine the corresponding flow rates.

Results

The low pass filter sufficiently smoothes the PWM signal, however in there was still some variance observed. Table 5 summarizes the PWM value and corresponding flow rates in both RPM and mL/min. The high and low flow rates are included as well. Figure 17 shows a graph of PWM value and the resulting average flow rate.

PWM value	RPM		mL/min			
	LOW	HIGH	LOW	HIGH	Average	St. Dev.
0	0	0	0	0	0	0
25	4.8	4.9	3.097	3.162	3.1295	0.045962
50	9.8	9.9	6.324	6.388	6.356	0.045255
75	14.8	14.9	9.551	9.615	9.583	0.045255
100	19.8	19.9	12.77	12.84	12.805	0.049497
125	24.8	24.9	16.00	16.06	16.03	0.042426
150	29.8	29.9	19.23	19.29	19.26	0.042426
175	34.8	34.9	22.45	22.52	22.485	0.049497
200	39.8	39.9	25.68	25.74	25.71	0.042426
225	-	44.9	-	28.97	44.9	0
255	-	50.8	-	32.83	50.8	0

Table 5: Summary of PWM values and resulting flow rates in RPM and mL/min

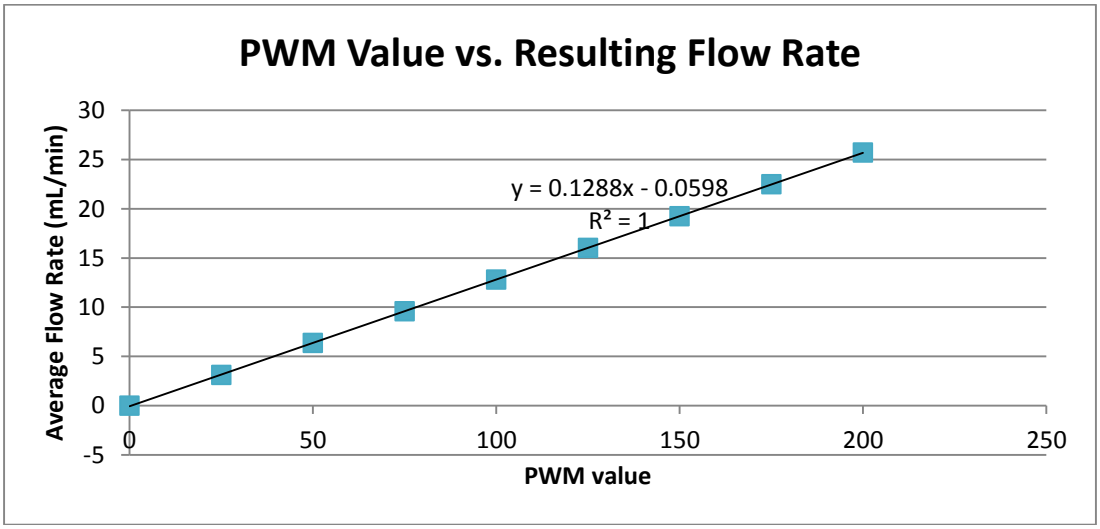

Figure 17: PWM value vs. Flow Rate

BIOREACTOR SETUP

The pumps should be taken out of the boxes and placed near the bioreactor. The 1/4" thickness tubing should be placed in the lumen peristaltic pump (model BT100-1F) head while the two 1/16" tubes should be used with the vasculature pump (model YX1515x). After the 1/4" tubing is placed into the pump head, take one of the loose ends and attach it to the barbed hose fitting located underneath the bioreactor. The other end of the 1/4" tubing will be inserted into a 500ml reservoir that contains reagents that will come into contact with the lumen of the larynx. Once the 1/16" tubing is in place take one of its free ends and attach it to a flask containing reagents that will run through the vasculature. Connect a luer lock to the other end and turn on the vasculature pump until the reagent flows out of the luer lock end which will prevent air from being forced into the blood vessels. Once reagents flow out of the luer lock end, it is ready to be attached to the cannulas that are connected to the arteries of the larynx.

The larynx needs to be sutured in several places before it can be placed in the bioreactor. The trachea needs to be sutured around the support cylinder which will prevent reagents of the lumen from escaping into the exterior environment. Next, the inflatable balloon of an endotracheal tube will be filled with air and inserted into the superior side of the larynx. Once in place just above the vocal cords, the surrounding tissues of the larynx and the esophagus will be sutured around the endotracheal tube to create a tight seal. To reduce the risk of perforating the blood vessels, flexible cannulas will be used and inserted into the left and right common carotid arteries. The arteries will then be sutured around the cannulas to hold them in place.

Once the cannulas are attached to the arteries, they can be connected to the luer locks of the vasculature tubing. After successful attachment, the larynx is ready to be inserted into the bioreactor. Holding the larynx near the epiglottis with one hand and at the trachea with the other, firmly attach the support cylinder to the bottom of the bioreactor. The volume reducing dividers can be placed into the bioreactor. Extra care should be taken so as not to loosen any of the larynx connections and to avoid all tubes exiting the bioreactor. The lid can then be placed on top of the bioreactor by having the endotracheal tube go through a hole in its center. To make the lumen pump a cyclic process, connect tubing to the superior end of the trachea tube and place the other end in the same 500mL reservoir used for the lumen pump tubing.

DECELLULARIZATION

Testing

To move this project forward, the first step is to successfully decellularize a larynx. Since the decellularization process is fairly well established in whole and partial organ decellularization, the focus for this project is to ensure full decellularization can be achieved and to determine how long this process will take using the laryngeal bioreactor. In the first iteration of decellularization testing, shown in Figure 18, 1% SDS detergent was perfused through the vasculature and circulated through the inner lumen. The rest of the bioreactor was filled with deionized (DI) water. This was done to analyze the extent of decellularization that would occur due to these two pumping systems. A second iteration was performed in which 1% SDS detergent was only perfused through the vasculature and the remaining volume was filled with DI water. After

decellularization, the larynx was washed in DI water and then sectioned into samples using a cryostat. The samples were stained with hematoxylin and eosin (H&E) which assays for nuclear content.

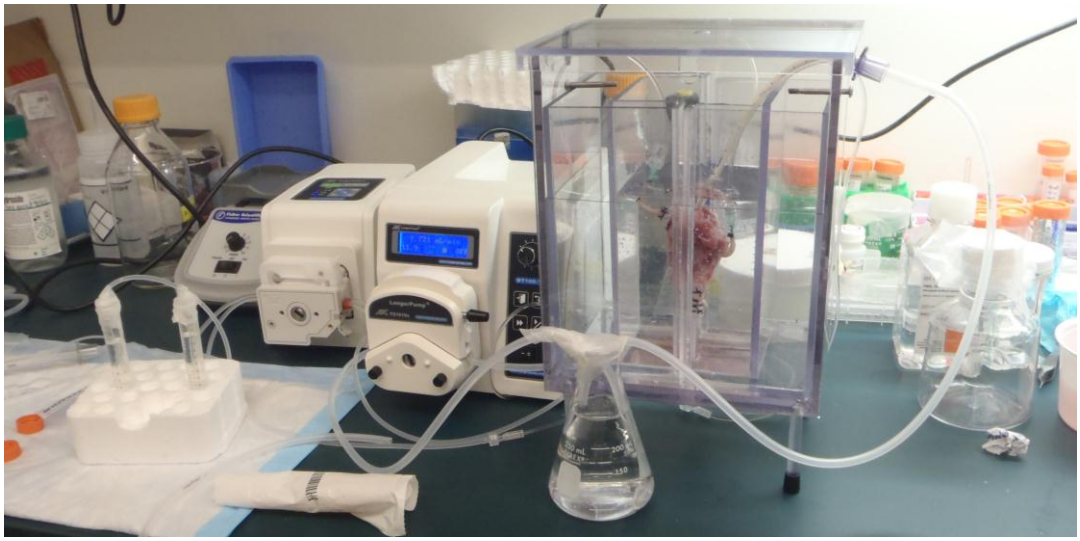

Figure 18: Setup for the first iteration of decellularization testing.

A third iteration was performed in which 1% SDS detergent was perfused through both the lumen and vasculature of the larynx. To help accelerate the decellularization process the laryngeal bioreactor was filled with 1% SDS instead of DI water used during the first and second trials. Also novel to the third trial was the use of antibiotics to help prevent tissue degradation. The larynx was bathed in 1% SDS + antibiotics for 4 days before being washed with DI water and antibiotics. After several wash cycles the larynx was sectioned into samples and stained with H&E. To compare results a control larynx was used during the third trial. This larynx went through the same procedure as the decellularized larynx but was not placed in the bioreactor. Instead, the control larynx was bathed in a static bath of DI water and antibiotics. The control larynx was taken out of the static bath and sectioned and stained during the same time as the decellularized larynx.

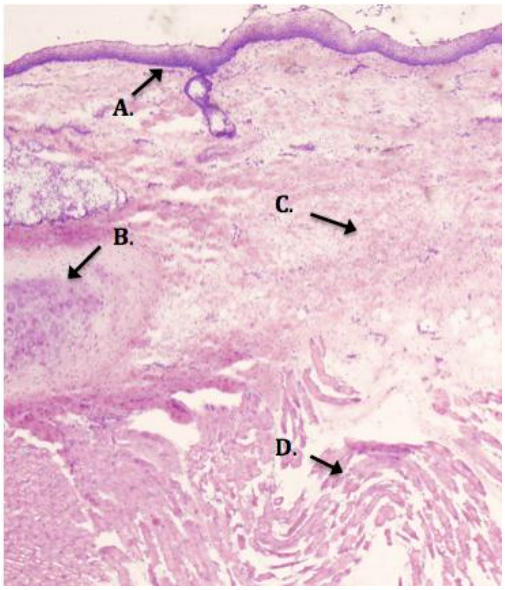

Figure 19: A non-decellularized larynx with intact epithelial cells (A), cartilage (B), lamina propria(C) and muscle tissue (D).

Preliminary Results

When assessing laryngeal decellularization, four tissues found within the larynx are observed in the H&E stains: the epithelial cells that line the lumen, lamina propria, muscle and cartilage (Fig. 19). Due to arterial damage, the first decellularization testing run was unsuccessful. Of the four tissues mentioned above, only the epithelial cells were removed from the laryngeal scaffold. In the second iteration, 1% SDS was perfused through the

vasculature for two days. The left vocal fold only had chondrocytes present in the cartilage matrix; however, the right vocal fold still had muscle cells and chondrocytes present. A small leak was observed during the perfusion of 1% SDS through the right common carotid artery which would account for a less decellularized larynx on the side of the right vocal folds. The H&E stains from the third iteration indicated an even decellularization of the lamina propria, epithelial and muscle cells on both the right and left side of the larynx. The only cell type still present in the laryngeal scaffold after decellularization was the chondrocytes. This was expected because the presence of a dense proteoglycan-collagen matrix insulates the chondrocytes and prevents the 1% SDS from penetrating the cartilage [21]. While there were no nuclei present in epithelia and lamina propria regions of the decellularized larynx, they were abundant in the control larynx that was bathed in DI water (Fig. 20).

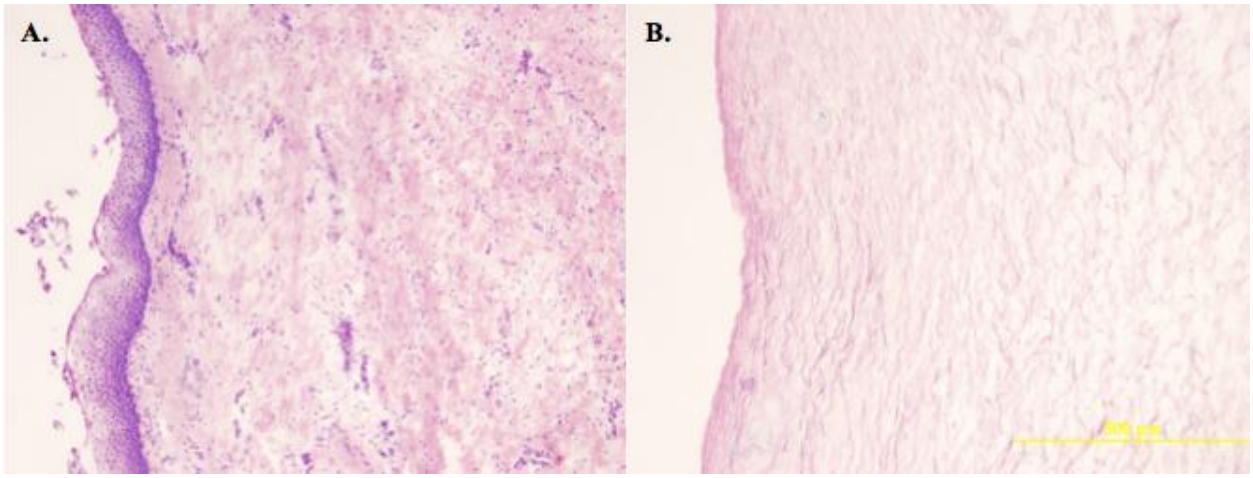

Figure 20: H&E stains from a non-decellularized larynx (A) show multiple nuclei stained purple. After four days of 1% SDS perfusion, the decellularized larynx (B) is absent of nuclei in both the epithelial and lamina propria regions of the larynx.

FUTURE WORK: RECELLULARIZATION TESTING

Following successful decellularization, the next immediate goal is the recellularization of the laryngeal scaffold. This will initially be pursued using only one cell type.

To test the utility of the perfusion system in introducing cells to the scaffold, fibroblasts will be perfused through the vasculature. H &E staining will then be used to stain the cytoplasm and nucleus of the attached cells; this is shown in Figure 5(D) on page 9. This will provide foundational information on how well and in what regions cells attach solely by means of the perfusion system. Multiple iterations of this will be performed to determine ideal conditions through variations in pump rate and cell concentration.

Once the above has been better determined, a greater variety of cell types will be introduced and tested. Since the larynx contains a number of tissue types, such as muscle and connective, it is required that the appropriate range of cells be present and localized to their respective regions. To fulfill this goal, cells will be introduced through direct seeding onto the scaffold, as well as

through perfusion. The direct seeding will ideally provide greater control as to the location of cells. Histological studies will be performed to determine cell location, survival, proliferation and type.

BUDGET

The cost of fully decellularizing and recellularizing the larynx must be taken into account. Decellularization of the larynx requires a sufficient amount of detergent (SDS) to lyse all the cells in the organ. SDS, however, is relatively cheap, and its cost is minimal in comparison to the cost of recellularization. The recellularization process requires the use of media to seed the larynx with cells. The cost of this media varies depending on the type. Dr. Welham has estimated that the media required to recellularize the larynx will cost anywhere from $100-400 for a 500 mL bottle. The interior of the bioreactor with the larynx inside has a volume of 1.5-2 L, depending on the size of the larynx. The media must be exchanged out once every seven days, and the recellularization process is expected to take roughly four weeks. Based on these estimations, it has been calculated that it will take between $1600 and $6400 to recellularize the larynx. Of course, this is just an estimate, as there are a number of variables to consider.

ETHICAL CONSIDERATIONS

Ethical considerations for this project lend themselves to future use of the bioreactor. The tissue engineered larynges could cause harm if not properly attended to in the decellularization-recellularization processes. If components are not properly sterilized throughout the entire process, contaminants could introduce unwanted pathogens to the donor recipient. This could stimulate a negative immune reaction to the engineered larynx.

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APPENDIX A: PRODUCT DESIGN SPECIFICATIONS

Perfusion decellularization-recellularization bioreactor for laryngeal tissue engineering

March 5th, 2013

Armand Grabowski, Taylor Milne, Brett Napiwocki, Sara Schmitz, Ben Smith

Problem Statement

The purpose of this project is to design a sterile bioreactor for whole organ tissue engineering of the human larynx, as well as comparable large animal models such as the pig or dog larynx. The bioreactor must be capable of performing two different processes: perfusion-decellularization of the larynx to create an acellular scaffold, and perfusion-recellularization of the acellular scaffold using vocal fold fibroblasts and other cell sources.

Client Requirements:

- The bioreactor must be sterile and have parts that can be replaced or autoclavable
- The bioreactor must easily be able to fit inside of the incubator
- The bioreactor should be capable of decellularizing and recellularizing the larynx
- The bioreactor must be able to function for days at a time

Design Requirements:

1. Physical and Operational Characteristics

a. *Performance Requirements:* The bioreactor needs to be capable of decellularizing a larynx to produce an acellular scaffold and subsequently provide an environment to recellularize the scaffold. This will be accomplished through perfusion pumps through the vasculature and trachea.

b. *Safety:* The device needs to be sterile if the larynx will be used in future transplantation. Additionally, the device should contain sufficient safeguards against user chemical exposure.

c. *Accuracy and Reliability:* The flow rate through the vasculature should not exceed physiological values and be able to maintain function throughout the entire decellularization-recellularization process.

d. *Life in Service:* For the decellularization process, the bioreactor should perform for a minimum of two days. For the recellularization process, the bioreactor needs to be able to perform for a minimum of 3 weeks. The bioreactor must also be reusable.

e. *Shelf Life:* The bioreactor should be capable of performing for 5 years.

f. *Operating Environment:* The bioreactor should be fit to operate in an incubator environment. Typical conditions include 37degrees C, 5% CO_2 and humidity. The device will also be exposed to various chemicals and liquids which are commonly found in a bioreactor environment.

g. *Ergonomics:* The bioreactor needs to movable and reasonable for one person to carry. It also must open to provide easy access to the tissue specimen.

h. *Size:* The reactor itself must be able to house a human or a large animal model larynx. The dimensions of the bioreactor must not exceed 50.8 x 54.1 x 68.1 cm.

i. *Weight:* The product must be handled easily by one person without excessive strain.

j. *Materials:* All materials used in the bioreactor must be sterile or autoclavable. None of the materials should degrade after exposure to detergents and other chemicals used during the decellularization and recellularization processes.

k. *Aesthetics, Appearance, and Finish:* The bioreactor must look like it fits in a laboratory setting.

2. Production Characteristics

a. *Quantity*: 1 deliverable

b. *Target Product Cost*: $1-3000

3. Miscellaneous

a. *Standards and Specifications*: N/A

b. *Customer/Patient related concerns*: N/A

c. *Competition*: There are companies that already make bioreactors but none that are specifically made for the larynx.

APPENDIX B: ARDUINO CODE FOR CONTROLLING THE INNER LUMEN PUMP

```
// 3 controls speed of pump - blue to board
// 11 starts 0 or stops 255  - purple
// 10 controls direction CCW 225 CW 0 - yellow
// 5 COM of speed - orange
// GND - COM of start stop and direction - green to board

int directionInput = 10;          // pin 10 controls CW/CCW direction
int startStop = 11;               // pin 11 controls pump start/stop
int speedcom = 5;                 // pin 5 com of speed
int speedrpm = 3;                 // pin 3 controls pump speed

void setup()
{
  pinMode(speedcom, OUTPUT);          // sets 3 as output
  pinMode(startStop, OUTPUT);         // sets 11 as output
  pinMode(directionInput, OUTPUT);    // sets 10 as output
  pinMode(speedrpm, OUTPUT);          // sets 5 as output

}

void loop()
{
  analogWrite(speedcom, 0);           // sets com of speed
  delay(1000);                        // waits 1 second
  analogWrite(directionInput, 0);     // sets direction CW
  delay(1000);                        // waits 1 second
  analogWrite(speedrpm, 75);          // sets speed approximately 10mL/min
  delay(1000);                        // waits 1 second
  analogWrite(startStop, 0);          // turns pump on
  delay(120000);                      // waits for 2 minutes

  analogWrite(startStop, 255);        // turns pump off
  delay(5000);                        // waits 5 seconds
  analogWrite(directionInput, 255);   // sets direction CCW
  analogWrite(startStop, 0);          // turns pump on
  delay(120000);                      // waits 2 minutes
  analogWrite(startStop, 255);        // turns pump off
  delay(5000);                        // waits 5 seconds

}
```