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Re-Design of a Skin Graft Culturing System

A Major Qualifying Project Report

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Abstract

The Pins Lab has developed a tissue engineered skin system to address the problem of non-healing skin wounds. The goal of this project was to redesign the device in which the grafts are cultured and to improve the image analysis of these grafts. Our redesigned device greatly reduced the handling time, and potentially reduced the risk of graft contamination. Our improved image analysis system allows for consistent, semi-automated analysis of histological sections of the skin grafts grown in Pins Lab.

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1. Introduction

Every year there are millions of cases of injuries to the skin that will not heal without intervention. The majority of these injuries are caused from trauma to the skin, ulcers and to a lesser extent burns. The gold standard is an autograft, which involves the transplant of a patient's own skin. The drawback to this method is that it creates a secondary injury site, and in cases with extensive skin loss there may not be enough remaining undamaged skin for transplantation. There is therefore a need for an off-the-shelf, tissue-engineered skin replacement.

The goal is to culture a skin graft in vitro that is as close to natural skin as possible. To facilitate in vitro culture, there must be a device to provide support to the graft. The current device used in Professor George Pins' lab is shown in Figure 1.

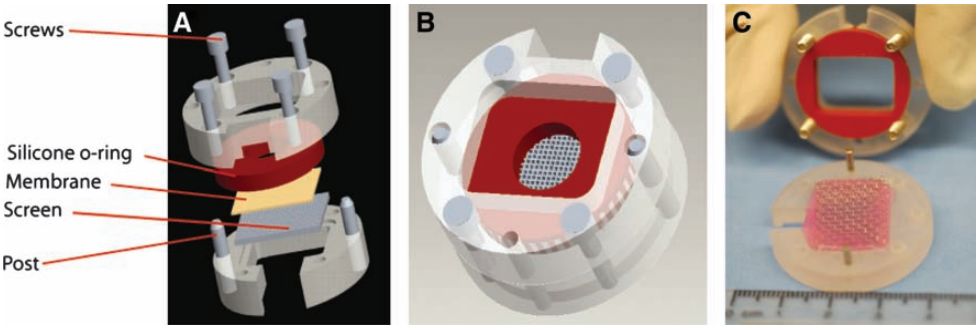

Figure 1: Schematic and Picture of the Pins Lab System (Bush, 2010)

This device was designed for use in Professor Pins' Lab, however, there are a number of problems associated with its use. First, it is difficult to disassemble. The screws seen in the first pane of Figure 1 must be completely unscrewed during disassembly, which is time consuming and difficult because of the sterile conditions under which the device must be disassembled. This method of securing the device is very inefficient because prolonged handling time leads to increased susceptibility to

contamination. The result of contamination would be the loss of a sample. The second major problem associated with this system is that the cells can begin to grow up the side of the silicone O-ring. This creates a problem upon removal of the graft because the epidermal layer of the graft could separate from the dermis. This results in a failed experiment because removing the epidermis from the graft renders it useless. In general, assembly and disassembly of the device are difficult, especially under sterile conditions.

To maximize efficiency, a new system must be devised. Our team has been tasked with the design and creation of this new system. We were given three specific objectives:

1. Create a system to streamline the skin graft production process which maximizes reproducibility and minimizes handling.
2. Develop techniques to image and evaluate cultured skin grafts non-destructively
3. Design a series of functional engineering, biochemical, and biological assays to characterize the keratinocytes on skin grafts.

We were given the additional statement that: "The ideal device would facilitate creation of the biomaterials scaffold, cell seeding and culture of two cell types (fibroblasts and keratinocytes) on the scaffolds, and imaging of the cultured skin grafts using microscopy."

To accomplish the goals of our project, we have followed the engineering design process as outlined in Engineering Design: A Project Based Introduction by Clive Dym and Patrick Little. The design process is detailed in Chapter 3: Project

Strategy. Details of the alternative designs follow that section. Next we discuss the methods with which our designs will be verified, followed by discussion. Then our final design is outlined along with the evidence validating its effectiveness.

2. Literature Review

2.1 Anatomy of Skin

The skin is a critical part of the body. It is the largest organ of the body and is essential for survival. The multiple distinct layers serve important functions that maintain the internal environment necessary for survival. An understanding of natural human skin is necessary for the creation of the tissue engineered skin grafts on which this project is focused.

2.1.1 Functions

One of the primary functions of skin is its ability to act as a barrier between the internal and external environments. Skin regulates the rate at which water leaves the body, while also keeping harmful bacteria and disease-causing agents from entering the body. Skin also serves as a barrier to protect internal organs from damage caused by light or other outside sources (Fox, 2011).

In addition to serving as a barrier, skin helps maintain homeostasis. Blood vessels within the skin can dilate to increase release of heat to the environment or constrict to reduce heat loss. Skin also functions in the release of sweat, another method by which heat is dispersed from the body, and a constant internal temperature is maintained (Fox, 2011).

The skin also serves an important sensory function. It contains a number of sensors that respond to touch, pressure, heat, and other stimuli. Sensing of the environment is critical for responding appropriately to a specific stimulus. For example, by sensing something such as potentially damaging pressure or heat, the body can respond and protect the internal environment from damage.

2.1.2 Composition

The skin is composed of two major layers: the dermis and the epidermis. Figure 2 shows a cross section of the skin. The outermost layer is the epidermis, which is constantly replicating (Fox, 2011) and contains mainly keratinocytes. The keratinocytes proliferate in the basal layer of the epidermis and slowly migrate to the outer surface of the skin and become keratinized. These keratinocytes form a stratified squamous epithelium, with the outer keratinized layer forming the main protective barrier from the outside environment. Also in the epidermis are melanocytes, which help protect against UV radiation and give the skin color, and Langherhan's cells, which serve as a primary immune response (Boyce, 2005).

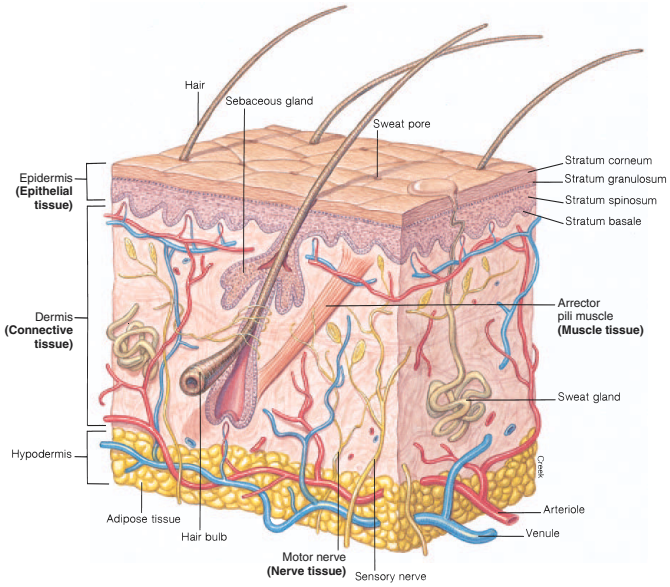

Figure 2: A cross section of skin (Fox, 2011)

The dermis is composed mainly of extracellular matrix that serves as a support for the epidermis as well as contributing to its elasticity. In addition to the extracellular matrix the dermis is composed of fibroblasts, endothelial cells, smooth muscle cells, and mast cells (Boyce, 2005). The dermis also contains the blood

vessels that nourish both the dermis and the epidermis. Finally, hair follicles and sweat glands originate in the dermis and extend up through the epidermis (Fox, 2011).

At the dermal-epidermal junction is the basal lamina. The basal lamina is responsible for keeping cells localized within the proper locations, as well as serving as the source of proliferation for the epidermal layer (Vracko, 1974). In addition to these functions, the basal lamina has a specific topography that guides the orientation and proliferation of keratinocytes (Pins, 2000). The basal lamina is particularly important in wound healing, because in an injury where the basal lamina is undamaged, complete healing will occur. In contrast, in an injury such as a third-degree burn where the basal lamina is damaged, scarring and incomplete healing will occur (Vracko, 1974). The basal lamina is of particular importance to this project because Professor Pins' lab has developed methods to mimic the topography of the native basal lamina (Pins, 2000).

2.2 Clinical Application

Professor Pins and his lab have developed a more advanced version of the bi-layered tissue engineered skin graft that incorporates aspects of several previous systems and novel μDERM technology. This new technology could prove very valuable to the medical field. Every year there are approximately 35 million injuries where the skin cannot heal properly without medical intervention (Clark, 2007). Over 163,000 split-thickness and full-thickness skin graft are performed annually on Medicare recipients alone and according to Wright Medical Group, and over 215,000 skin grafts are performed annually in the United States (Burd, 2005). Skin grafts are

commonly used in the treatment of diabetic ulcers. These ulcers do not heal on their own without therapeutic intervention. In the United States approximately 21 million people have diabetes (Clark, 2007). Approximately 2 million people with diabetes have persistent chronic ulcers and approximately 82,000 of these people may need amputations (Clark, 2007). In 1995, amputations associated with diabetic ulcers cost Medicare approximately $1.5 billion (Clark, 2007). In addition to diabetic ulcers, many people also suffer from venous ulcers and pressure ulcers. Of the 2 million people with persistent chronic ulcers, over 600,000 people suffer from venous ulcers and approximately 1.4 million people are affected by pressure ulcers (Clark, 2007).

Another common cause of significant skin loss is thermal injury, which accounts for approximately 12,000 skin grafts annually(The Burn Foundation). As shown in Figure 3, burn injuries can be broken into three categories: first-degree (burns that damage the epidermis only), second-degree (burns that damage the epidermis and into the dermis), and third degree (burns that extend through the entire dermis). There are also fourth degree burns, in which the burn extends through skin subcutaneous tissue and into the underlying bone and muscle. When burns reach the full thickness of the skin (third or fourth degree) and are more than 4 cm in diameter, the wound is usually unable to heal on its own and skin grafts are required (MacNeil, 2007). It is very important that the wound be closed as soon as possible because if a burn cannot heal on its own, complications can arise in the body, including infection, multiple organ dysfunction syndrome, acute respiratory distress syndrome, and sepsis (NIH, 2011).

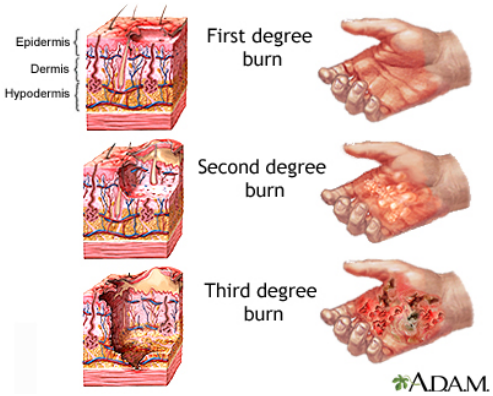

Figure 3: A Comparison of the three degrees of burns (http://www.burn-recovery.org/images/burn-classification.jpg)

The "gold standard" for the treatment for non-healing skin wounds is to use split thickness grafts that contain all the epidermis but only part of the dermis. These grafts are taken from healthy areas on the patient to cover the damaged areas. They provide a protective layer on the wound to prevent infection and a means to heal the area. If sufficient epidermal cells remain around the damaged tissue, these cells can migrate and will allow the growth of new epidermis at the injury site. However, autografts require a secondary wound site and may not be available for large scale wounds or in patients with compromised wound healing.

Skin grafting technology and burn treatment techniques have made great strides in the last thirty years. In the 1970's, burn coverage of more than 20-30% of the body was normally fatal, but with the improved medical care and skin grafting technology it is possible to survive a burn with up to 80-90% coverage (NIH, 2011). This improvement in treatments has cut down fire related deaths down from approximately 9,000 in 1975 to only around 3,500 in 2010 (ABA, 2011).

The cost to treat both venous and pressure ulcers each year is about 8 billion dollars. Also, as the population of the United States ages, instances of diabetes will continue to rise, leading to an increased incidence of diabetic ulcers. With

improvements in skin grafting technology, the medical care system will save millions of dollars and improve the treatment of the many people afflicted by these conditions (Clark, 2007).

2.3 Laboratory Application

In addition to the clinical uses of tissue engineered skin, there are many non-clinical research applications. Another primary application for tissue engineered skin is as 3D models (MacNeil, 2007) that are valued in many aspects of research and experimentation on the skin. These grafts help researchers by reducing animal experimentation and providing reliable data on the various properties of skin such as cell-cell and cell-extracellular-matrix interactions, skin barrier penetration, wound healing, angiogenesis, skin contraction, and regulation of pigmentation. They can also be used for research into various skin diseases like melanoma or psoriasis (MacNeil, 2007).

These tissue engineered skin models provide an excellent alternative to animal testing. They are being used to test the thousands of chemical additives in human skin products. Humanitarian protests have made the need for an alternative to animal models very important, however, because these models lack immune or circulatory systems, animal models are still needed to test immune and circulatory responses to the products. Many companies such as L'Oreal and Skin Ethic have been developing skin models to conduct dermatotoxicity tests. These models are still not perfect; in addition to the already mentioned lack of immune and circulatory systems, most of these models contain only keratinocytes and lack other cells such as fibroblasts. This decade will likely see an increase in 3D models, where

physiological interactions between cells that cannot occur in a monolayer culture will be possible (MacNeil, 2007).

2.4 Skin Substitutes

The goal of skin substitutes is to provide a replacement for wounds that will not heal on their own. Systems for cultivating skin substitutes are dependent on the type of skin substitute being cultivated. These skin substitutes differ in composition, materials, and function. Currently, the gold-standard approach is to use a patient's own skin as a source by harvesting skin directly from the patient and implanting it in the wound, allowing for revascularization and subsequent skin regrowth (Groeber, 2011). This is known as an autologous graft, or "autograft". This form of skin substitute has very little risk and does not cause an immune reaction. However, this approach is not always possible, such as in cases where a patient has had severe burn damage where there may not be enough undamaged skin to harvest and apply to burn sites (Shevchenko, 2009).

In cases where an autograft cannot be performed, appropriate and effective alternatives are required. Therefore, research into developing skin substitutes that are equal in quality to autologous grafts is extremely important. Shevchenko lists the properties of an ideal skin substitute as such (Shevchenko, 2009):

1. The biomaterial in the substitute must not be toxic or otherwise harmful;
2. The substitute should be biodegradable and reparable;
3. The substitute must be mechanically and biologically similar to the skin it replaces;
4. The substitute must allow for regrowth of the tissue it replaces;

5. The substitute must protect the wound and provide relief to the patient;
6. The substitute must be cost effective and readily available.

The basic functions of a skin substitute are: mechanical and biological protection; promoting delivery of growth factors, cytokines, and dermal matrix; providing a structure for cell incorporation into the wound (Shevchenko, 2009). Skin substitutes can replace the dermal layer or epidermal layer of the skin, and many replace both. Dermal-epidermal layer substitutes, or "composite" substitutes, most accurately imitate the structure of natural skin. Figure 4 illustrates the division of a composite substitute into its dermal and epidermal components.

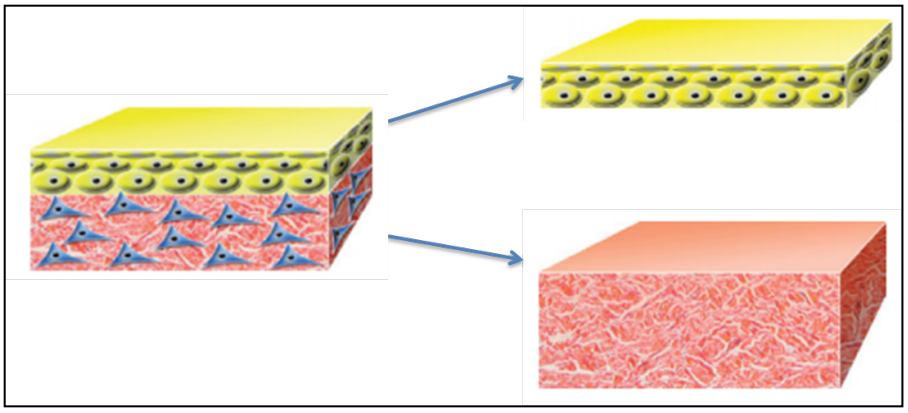

Figure 4: Division of a dermal-epidermal graft (left) into an epidermal layer (top) and dermal layer (bottom) (Shevchenko, 2009).

2.4.1 Skin Substitute Types and Materials

Design of skin substitutes is geared towards mimicking the structure and function of the skin as an organ. Grafts are primarily meant to resemble the histological form of natural skin (Shevchenko, 2009). Basic skin substitutes are comprised of skin cells seeded onto an extracellular matrix. Commonly, these substitutes also contain growth factors and cytokines as a method of promoting wound healing (Groeber, 2011). Additionally, the science of skin substitutes is

relatively new, and research continues to investigate novel material combinations and cultivation methods.

Skin substitutes can most basically be divided into several groups based on the source of extracellular matrix and cultivated cells as shown in Table 1.

Table 1: Types and Materials of Skin Substitutes (Shevchenko, 2009)

Type	Basis	Example	Advantages	Disadvantages
Autologous	Self-derived cells and scaffold	Meshed skin from thigh	Will not cause immune reaction	Limited supply
Allogeneic	Tissue from a similar donor	Cadaverous tissue	Balances availability with similarity to human tissue	Will eventually be rejected
Xenogeneic	Foreign scaffold stripped of cells	Acellular bovine dermis	Abundant	Requires processing
Biosynthetic	Polymers such as collagen and glucosamino-glycan	PET polymer-based dermal matrix	Potentially mass-produced and customizable	New and underdeveloped technology

Allogeneic skin grafts solve the issue of donor skin scarcity but introduce the problem of the replacement skin not being attuned to the patient's immune system. This may result in the rejection of the graft after a certain period of time, in which case the graft must be covered with, or replaced with, autologous tissue (Groeber, 2011). Resources for cadaver skin are scarce and use is complicated by the possibility of disease transmission, which makes allogeneic grafts a less ideal alternative to autologous grafts. A diagram of the composition of Karoskin, a commercially available allograft sold by Karocell Tissue Engineering, is shown in

Figure 5. The epidermal and dermal layers contain allogeneic human keratinocytes and fibroblasts, respectively (Shevchenko, 2009).

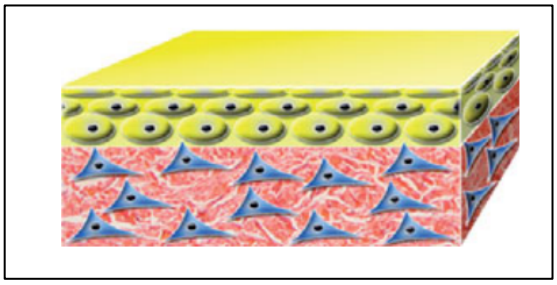

Figure 5: Karoskin allogeneic graft diagram, with populated epidermis in yellow, and dermis in red. (Shevchenko, 2009).

Substitutes of xenogeneic origin are commonly distributed in the form of bovine or porcine dermis (Shevchenko, 2009). This dermal tissue has been decellularized (including the matrix-forming fibroblasts) to a purely structural cell matrix in order for human cells to repopulate and incorporate into the dermal layer. This form is common in dermal replacements, where cow or pig dermis has all cells removed and is permanently fixed to the wound site (Shevchenko, 2009). Figure 6 shows an example of a porcine dermal matrix, the OASIS Wound Matrix by Cook Biotech, in which intestinal submucosa is decellularized and applied permanently to the wound site, after which the patient's cells populate the extracellular matrix.

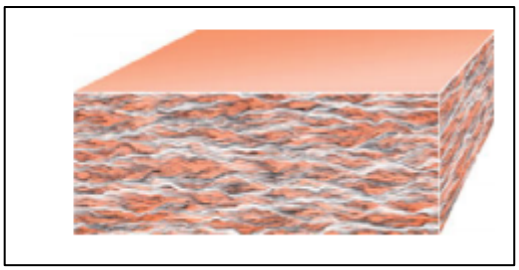

Figure 6: Xenogeneic porcine dermal substitute diagram. (Shevchenko, 2009).

As an alternative to natural tissue, biosynthetic substitutes can be created to replace skin layers. These biosynthetic substitutes come in cellular and acellular

form, and are ideally biodegradable (Shevchenko, 2009). In these biosynthetic substitutes, scaffolds and cell matrices are commonly made out of materials such as silicone, collagen, and polymers such as polyglycolic acid, glycosaminoglycan, etc. (Shevchenko, 2009). After cultivation, these substitutes are applied to the wound site and are populated by the patient's cells. PolyActive, created by HC Implants in the Netherlands, shown in Figure 7, is comprised of a dermal-epidermal matrix made from polyethylene oxide terephthalate seeded with cells harvested from the patient and cultured within.

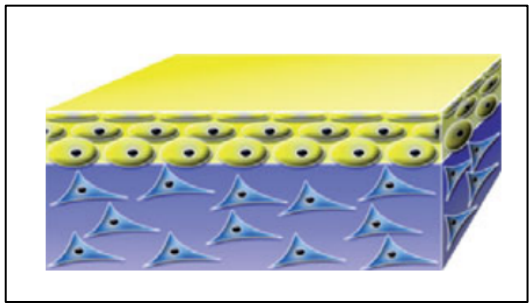

Figure 7: Diagram of synthetic dermal-epidermal substitute with autologous cells. (Shevchenko, 2009).

Some grafts are a combination of xenogeneic and biosynthetic material. Apligraf, manufactured by Organogenesis, is one such example of a mix between biosynthetic and xenogeneic material. In Figure 8, the extracellular matrix is derived from bovine collagen, while the cells are cultivated from an allogeneic source.

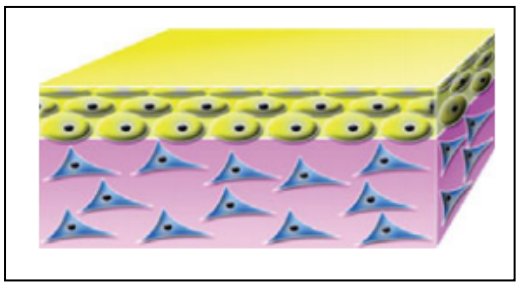

Figure 8: Diagram of xenogeneic-biosynthetic skin replacement. (Shevchenko, 2009).

2.4.2 Impact on Device Design

Tissue engineered skin culturing involves submersion of a graft in culture medium, and seeding of cells to both sides of a collagen gel. Therefore, this device must facilitate access to both sides of the collagen gel for the purpose of seeding. Ideally, it will be able to form a seal on both sides of the graft to make the seeding process easier. To facilitate this process the device must be able to be flipped over and still have media come into contact with the graft. The process must also be as easy as possible in order to prevent contamination, and should be designed to require minimal handling by the user. Finally, the material chosen must not be cytotoxic to fibroblasts and keratinocytes.

Of particular importance is the ability to cultivate at the air-liquid interface, which is necessary for the formation of a stratified squamous epithelium and the outer keratinized layer of skin. The device must therefore allow for this, either through providing this interface itself, or by allowing the graft to be removed non-destructively. The process must also be simple in order to prevent contamination, and should be designed to require minimal handling by the user.

2.5 Tissue Engineered Skin Systems

There are two major types of tissue engineered skin systems. The first are small systems designed to create small samples for a laboratory environment. The second are mostly autonomous systems designed for mass production. This project is focused on smaller systems for use in a laboratory.

2.5.1 The History of Tissue Engineered Skin Substitutes

One of the first systems to replace skin artificially involved Dow Corning Silastic medical grade silicone. In this two-step process, first a collagen and chondroitin-6-sulfate dermal analogue was implanted and covered by a layer of silicone. After several weeks when the dermis had begun to be vascularized, the Silastic was removed and it was replaced with a meshed epidermal autograft. This product was later commercialized as the Integra Dermal Regeneration Template or DRT. These experiments, originally performed in the early 1980s, relied on an artificial epidermis initially because of technological limitations in the artificial skin compositions at that time. Burke points out that the first necessary improvement was to create a bilayer artificial skin construct in which the permanent dermal and epidermal components were implanted at the same time (Burke, 1981).

One of the first examples of full-thickness tissue engineered skin was produced in the lab of Steven T. Boyce in the late 1980s. Prior to this point the only cultured skin substitutes were composed of a layer of keratinocytes that were grafted onto de-epidermized dermis autografts or artificial dermal equivalents. One drawback to this technique is that it requires two separate graft implantations, first the dermal portion, then the epidermal portion. In addition, this technique is still subject to the limited availability of autograft material, and difficulty of both materials integrating into the damaged area. Boyce hypothesized that by culturing human keratinocytes onto a collagen-GAG dermal substitute before implantation, the graft would perform better than epidermis-only grafts or dermis and epidermis grafted separately. This system combines the adhesion and formation of vasculature

supported by the dermal substitute as well as the wound closure and protection from the keratinocyte layer (Boyce, 1988).

US Patent 5711172, granted in 1998, describes a later iteration of Boyce's process to produce a dermal-epidermal tissue engineered skin composite. The dermal component is created by freezing a combination of collagen and GAGs between two sheets of Teflon supported by a frame. This membrane is then laminated with a combination of collagen, GAGs, and dimethyl sulfoxide. This layer acts like the basal lamina in natural skin and prevents epidermal cells from migrating into the membrane. Other bioactive molecules could be included in the dermal component, however it is acellular. Keratinocytes are then cultivated separately and cultured onto the surface-laminated dermal component created in the previous steps. The cells then grow in submerged culture into an epithelial layer attached to the dermal component. In studies conducted by Boyce and his colleagues, it was found that limited stratification of the epidermis occurred, as well as limited incorporation with the surrounding tissue. These substitutes were found to be better than no wound covering based on percent of the original wound healed, but not as effective as autografts or xenografts (Boyce, 1998). The results of this study are shown in Figure 9.

TREATMENT	% ORIGINAL WOUND SIZE
1) Autograft	37.65
2) Human Xenograft	39.53
3) Composite Graft	16.23
4) No Graft	13.95

Figure 9: Results of healing with Boyce graft compared with no graft. Autograft and Xenograft on a mouse at day 41(Boyce, 1998).

As early as 1980, research began on culturing epithelial cells in air/ liquid interface culture (Lillie, 1980). These early experiments involved keratinocytes derived from rats and seeded onto collagen "rafts". These constructs were placed on stainless steel screens and medium was added so that the basal layer of the collagen raft was contacting the medium, but the epithelial cells were exposed to the air. This procedure leads to the formation of stratified epithelium that more closely mimics natural epidermis than cells grown in submerged culture (Bernstam, 1986).

2.5.2 Modern Tissue Engineered Skin Culture Systems

One of the most important commercially available tissue engineered skin systems is Apligraf. This system takes into account the findings of Boyce and others, and was designed by Organogenesis. US Patent 6730510, issued in 2004, covers this system and a schematic is shown in Figure 10.

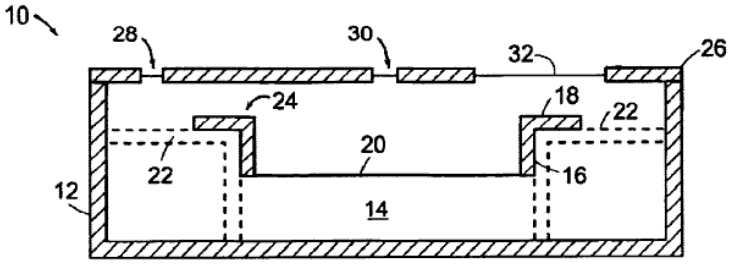

Figure 10: Organogensis Skin Culture System (20) suspended mesh in the middle, (14) medium up to bottom of graft, or higher to submerge, (28, 30, 32) ports at the top to allow the introduction and removal of materials. (US Patent 6730510)

This is a completely closed system that incorporates the seeding of cells as well as air/liquid interface culture within one device. This air/liquid interface culture that is critical for the growth of stratified epithelium. The culture device and a comparison between Apligraf and native human skin are shown in Figure 11.

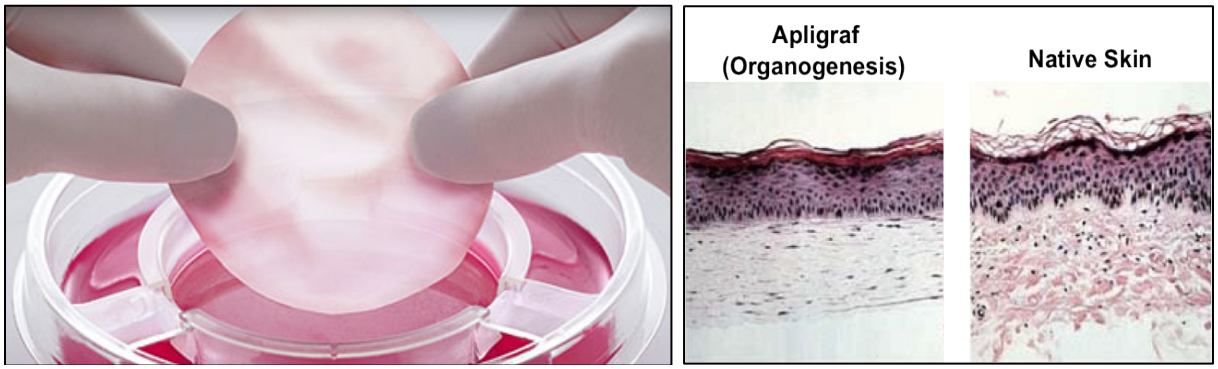

Figure 11: (A) Organogenesis Apligraf and Culture system. (B) Comparison between Apligraf and Native Human Skin (Organogenesis, 2012)

The Apligraf culturing process begins with the creation of a dermal matrix within the device. After the collagen membrane is formed, human dermal fibroblasts are seeded. These fibroblasts serve to contract the collagen to form the dermal analogue. Keratinocytes are then seeded onto this to form the epidermal layer. At approximately day 10 the level of medium in the device is lowered so that the graft sits at the air-liquid interface. This process is illustrated in Figure 12.

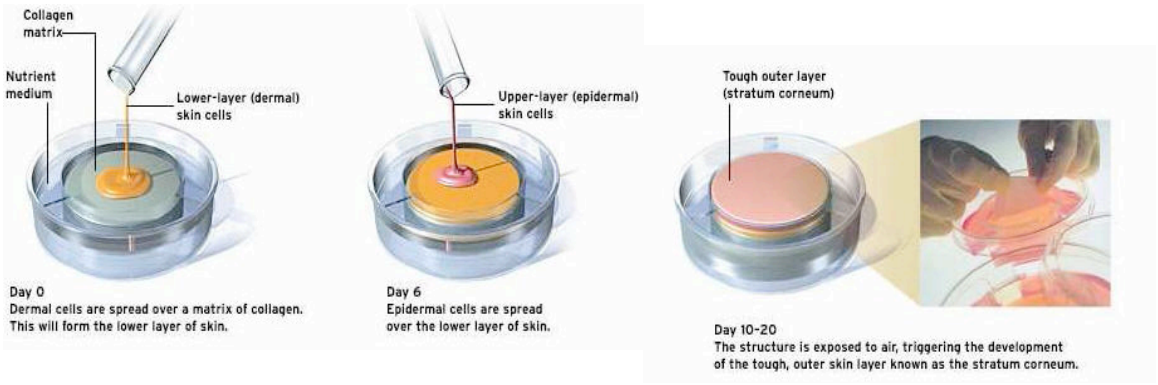

Figure 12: Illustration of the Apligraf creation process (http//bme240.eng.ucl/edu/students/07s/aedalati/pages/TE%20Ski10.jpg

An important advancement in the mass production of skin is the perfusion bioreactor system. Examples of perfusion bioreactor systems can be seen in Figure 13 and Figure 14.

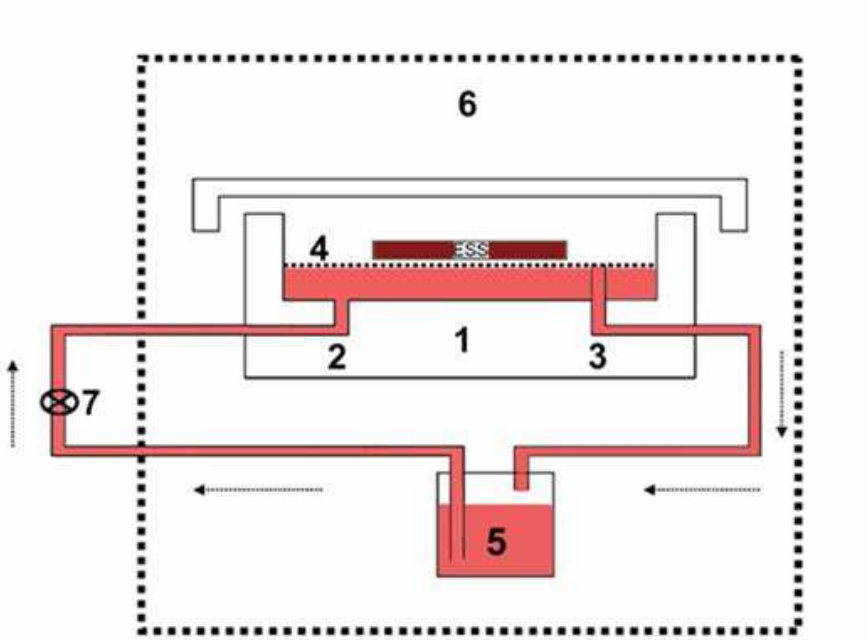

Figure 13: A Continuous Perfusion Bioreactor System- The engineered skin substitute sits on a cotton support at the air liquid interface (4). The pump (7) continually circulates the media, which ensures that the cells have proper nutrition. (Kalyanaram, 2008).

Figure 13 details a system created in the lab of Steven Boyce at the University of Cincinnati with Balaji Kalyanaraman. Kalyanaraman's research has shown that at flow rates of 5 ml/min there can be in increase in quality of the grafts produced compared to a static culture environment (Kalyanaraman, 2008). This type of culturing reduces the amount of work from the user, however the perfusion system uses more culture medium, and if used for small grafts it could be prohibitively expensive.

In Figure 14, a second similar system is shown schematically. This bioreactor forms a completely closed system. The authors indicate that this system could be useful in the production of multiple small grafts simultaneously, or one larger graft (Sun, 2005). While the system is designed to prevent contamination as much as possible, the perfusion system could potentially complicate this. In the case that

multiple grafts were being grown using the same perfusion system, the contamination of one would then lead to the contamination of all the others. Additionally, if different experimental conditions were being used for each graft, there would be cross contamination of these differences if all of the grafts were sharing the same medium.

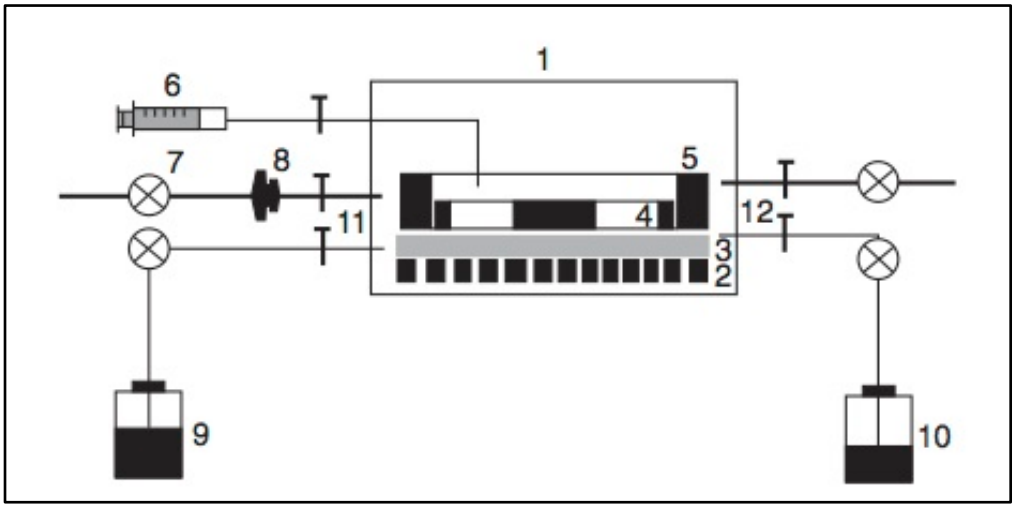

Figure 14: Pulsatile Perfusion Bioreactor System- Introduction of cells, medium, and filtered air are accomplished through silicone tubing at 6, 8, and 11, respectively. (Sun, 2005).

2.5.3 Modern Tissue Engineered Skin Culturing Process

The cultivation of a dual-layered tissue engineered biosynthetic substitute involves the creation of a collagen gel or sponge, followed by the application of fibroblasts, keratinocytes, or both, to the collagen scaffold. The general production cycle of a biosynthetic skin graft is shown in Figure 15, in which autologous or allogeneic cells harvested from a donor are combined with a natural or synthetic scaffold to produce a tissue engineered biosynthetic skin graft, and distributed to patients.

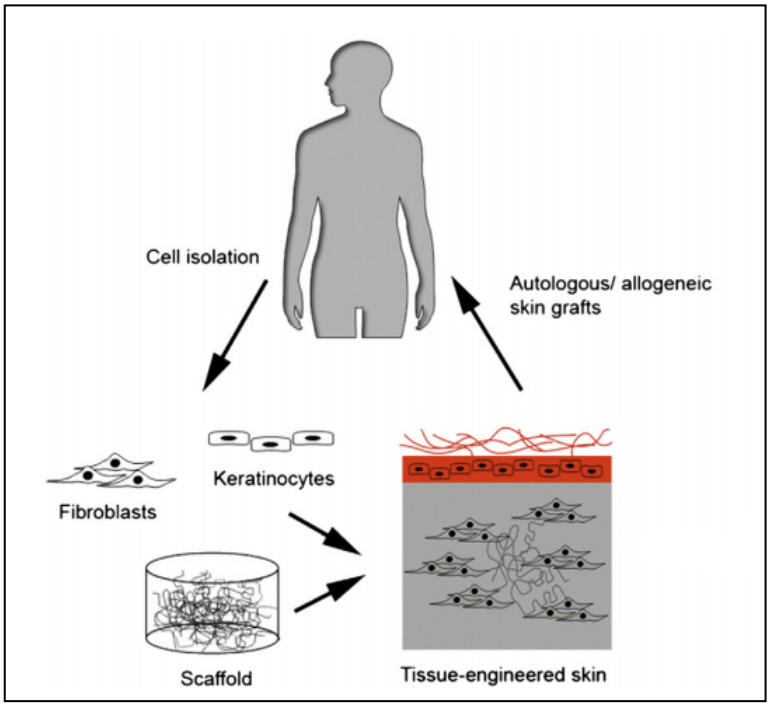

Figure 15: Chart describing the general process of producing a biosynthetic skin graft. (Groeber, 2011).

Creation of a Dermal Analogue

Scaffolds used as dermal analogues are commonly composed of collagen. This scaffold functions as a structural matrix upon which cells can grow. Kemp describes in further detail the process of creating a synthetic scaffold out of animal collagen as outlined in the patent for the Apligraf system (Kemp, 1996). A collagen gel is created mainly from a mixture of animal-derived collagen and sodium bicarbonate. It is allowed to chill in order to become a gel. Contraction agents, generally fibroblasts, are applied in order to contract the collagen gel and give the material the properties of dermal tissue (Kemp, 1996). This collagen gel may also incorporate fibrin to provide additional strength (Weinberg, 1989). The general method of applying fibroblasts involves first cultivating the fibroblasts in a medium, then seeding them onto one side of the scaffold. The scaffold is submerged, and cultured for

approximately three days (Krejci, 1991). These fibroblasts contract the collagen gel as they grow, forming a cell matrix that is similar to that of natural dermal tissue.

Cultivation of Epidermis

The opposite side of the collagen gel is seeded with keratinocytes generally derived from a human or animal donor. Keratinocytes are seeded and then cultured in submerged culture for approximately one week (Krejci, 1991). After full cultivation of keratinocytes, the seeded scaffold exhibits the properties of a synthetic dermal-epidermal skin graft.

Air-Liquid Interface

To ensure proper development of stratified squamous epithelium, grafts are cultured at the air-liquid interface. Exposure to air for one or two weeks allows cultured keratinocytes to form a keratinized layer. This hardened outer layer functions as a barrier, which is one of the major functions of skin that needs to be reproduced in TE skin substitute (Doucet, 1998). Figure 16 shows an example of the culturing cycle as it pertains to the air-liquid interface. Exposure to air through reduction of media level or raising of epithelial cells to the surface triggers the formation of a proper epithelium (Gangatirkar, 2007).

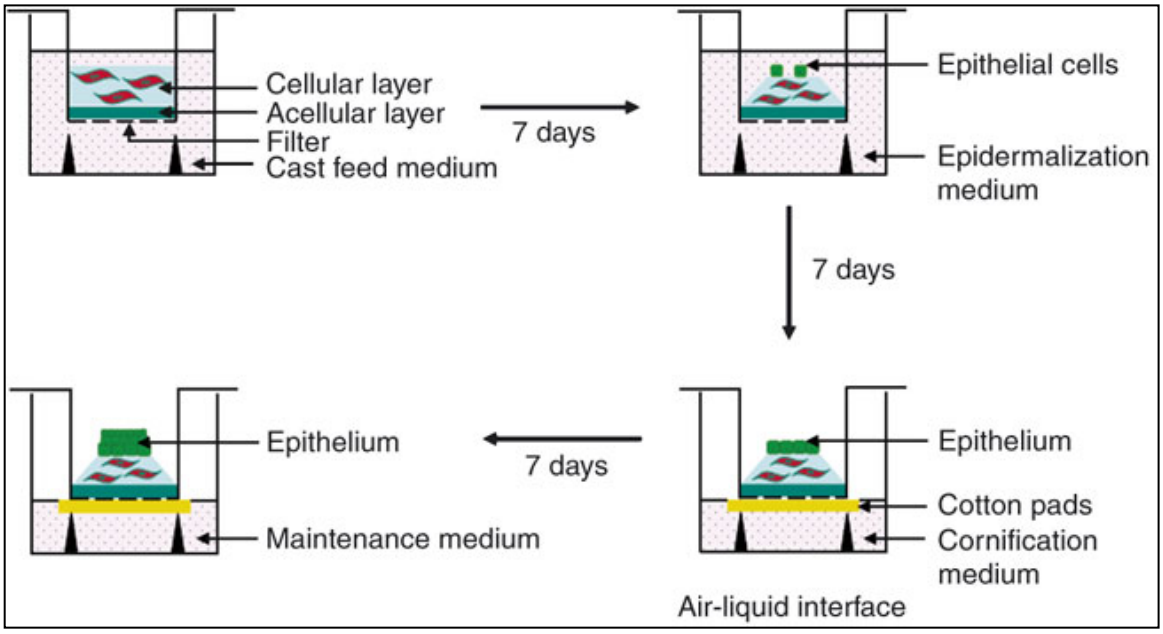

Figure 16: Chart detailing the process of the air-liquid interface. (Gangatirkar, 2007).

2.5.4 The Pins Lab System

The current state-of-the-art tissue engineered skin culturing technique utilizes cultured keratinocytes and fibroblasts, a scaffold, and medium contained within a culturing plate. In Professor Pins' lab, tissue engineered skin is cultured within a device that provides structural support for the scaffold, as well as allowing for seeding of cells onto both sides of the graft. The device also creates a well on the top of the graft, which simplifies the seeding of keratinocytes.

The tissue engineered skin cultured in this lab is know as Microfabricated Dermal Epidermal Regeneration Matrix (µDERM). µDERM incorporates aspects of systems by both Organogenesis and Integra. The novel aspect of µDERM is the inclusion of a microfabricated dermal-epidermal junction, which serves to more

closely mimic the basal lamina in natural skin. The creation of μDERM is illustrated in Figure 17.

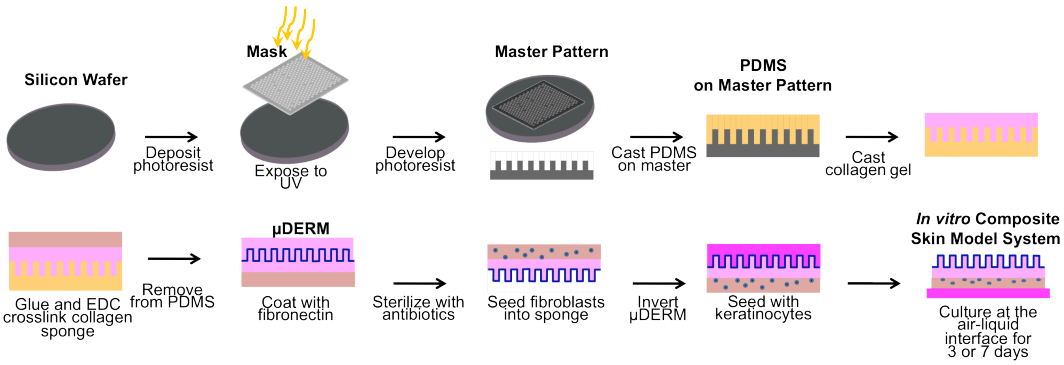

Figure 17: Illustration of the μDERM culturing process. Using photolithography, Pins' lab can create μDERM with topographical features inspired by the topography of the native basal lamina. (Courtesy of Amanda Clement)

In order to develop the dermis and epidermis, a basal lamina analog composite is utilized containing type I collagen from rat-tail tendon and adhering it to a collagen sponge from bovine hide. The fibroblasts are seeded on the bovine derived collagen sponge and are submerged for approximately 48 hours in medium. This composition forms the dermal analogue.

Following the culture of the fibroblasts, the device is flipped over and the keratinocytes are then seeded onto the collagen gel. The keratinocytes make up the epidermal portion of the graft. The device is submerged in seeding media for 24 hours, and then priming media for an additional 48 hours. At this point, the graft is removed from the device and moved to the air-liquid interface. It is then cultured at the air-liquid interface for between 3 days and 14 days (Bush, 2009). An example of a graft at air-liquid interface is shown in Figure 18.

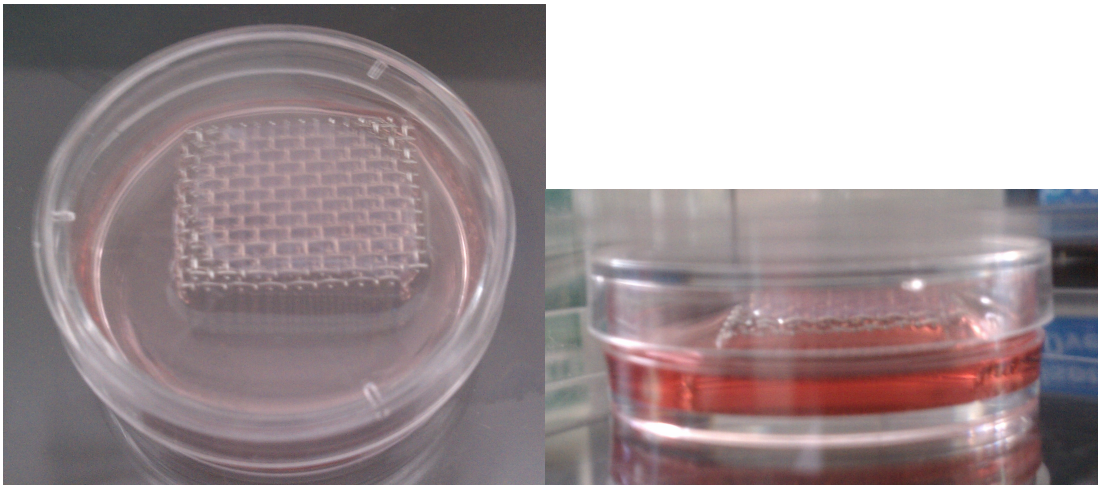

Figure 18: Example of a Graft at Air-Liquid Interface.

The current culture device used in the Professor Pins' lab was custom built to meet the growth and testing needs associated with graduate research conducted by Katie Bush, Ph.D. The device was designed specifically for controlled seeding of keratinocytes. It has been adapted for use with the additional step of seeding fibroblasts. After seeding, the grafts are removed for culturing at the air-liquid Interface. This device can be seen in Figure 19.

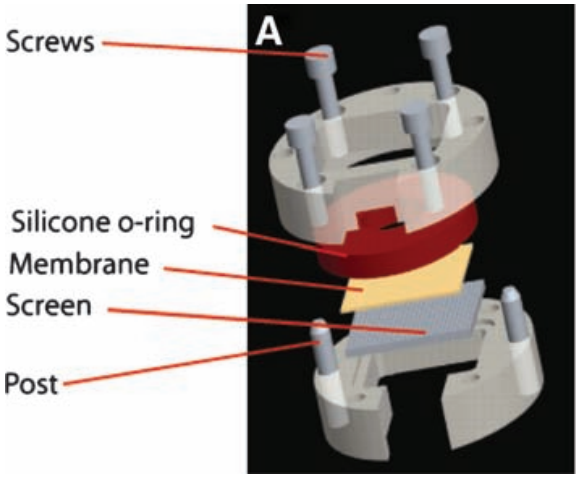

Figure 19: Schematic of the Pins Lab Culture Device (Bush, 2010).

The outer support structure is composed of polycarbonate and is designed to fit within a standard six well plate. The screen is made of surgical grade stainless steel, and is the portion onto which the collagen sponge is placed. Over this the silicone O-ring is placed and then secured in place by the top polycarbonate portion. The entire device is held together by 4 stainless steel screws (Bush, 2010).

The screen in the system allows the tissue engineered skin grafts to be suspended in the medium. It provides structural support for the graft as well as allowing for diffusion of nutrients from the medium onto all sides of the graft. The O-ring provides a tight seal around the graft, which creates a constrained area in which the cells will remain during seeding (Bush, 2010). Fibroblasts are seeded onto the dermal analogue through the screen with the epidermal side down (screen side up). Then the device is flipped and the keratinocytes are seeded onto the epidermal side of the graft. After the keratinocytes have grown sufficiently, the device is disassembled and the graft is moved to another screen upon which the air-liquid interface portion of the process takes place.

Professor Pins' lab is focused on improvements to the general process that can someday be implemented into clinical solutions. Research has focused on the use of complex topography of the basal lamina (Pins, 2000), adsorption of fibronectin onto the collagen membranes (Bush, 2010), and other related improvements.

2.6 Imaging Systems

Observing and recording results of the cultured skin graft commonly requires the use of imaging analysis to reveal quantitative data. In the case of skin-

related sectional analysis, fluorescence microscopy offers versatility and accuracy in identifying cell structures and molecular interactions as well as allowing for quantification of sections in intensity and marking of cell types. Immunohistochemistry in particular has been carried out on dermal-epidermal sectional analysis for both qualitative and quantitative conclusions.

2.6.1 Immunohistochemistry

Immunohistochemistry combines immunology, histology, and chemistry in order to exploit the antigen-antibody binding process of the immune system and attach coloration, visible under either visible light or ultraviolet light, to a specific antigen (Ramos-Vara, 2005). Special antibodies are grown in a source animal by exposing them to an antigen. Tissue samples are incubated with these antibodies during cell culture and the antibodies bind the target antigen.

In order to take advantage of this chemical reaction, a reporter molecule, also known as a "label", is attached to the antibody to serve as a chemical marker of this reaction having occurred. Many different labels are available, falling into the subtypes of chromogeneic enzymes, fluorescent compounds, and metals (Taylor, 2002). Enzymes interact with a substrate to produce a visible color, while fluorescent compounds cause the antigen-antibody binding to emit light when excited at a specific wavelength. An example of the results of this technique is shown in Figure 20. Human melanocytes fluoresce green ([alpha-9][beta-1] integrin) and red (F-actin) in places where the associated antigen-antibody process has ocurred. (Lydolph, 2009)

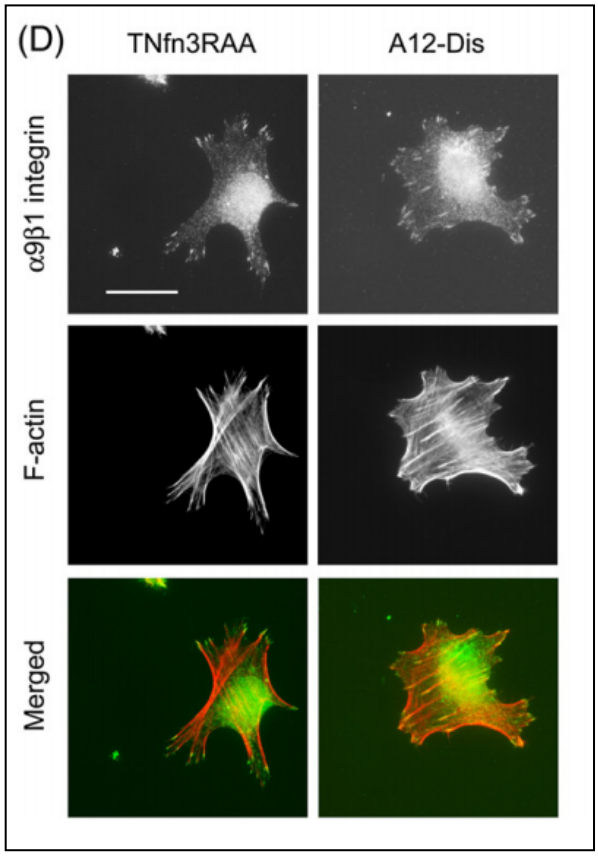

Figure 20: Immunofluorescence of epidermal melanocytes, with beta-1 integrin response in green. (Lydolph, 2009).

Immunofluorescence imaging is particularly useful for analyzing tissue sections, and the following example shows how it is appropriate for use in skin section histology. In one sectional dermatological analysis of skin epithelial tissue, a collection of skin biopsies was taken and imaged in order to identify tissue structures and lesions, and judge how effective immunostaining is at diagnosing dermal pathologies (Ranjana, 2010). Specifically, fluorescent immunoglobins IgG, IgA, IgM, and C3 were introduced to the sections, where they settled in dermo-epidermal junctions and boundaries, intercellular spaces, cytoid bodies, etc.

Fluorescence imaging and analysis of epithelial cells, as shown in Figure 21, confirmed that 77% of the biopsy image analyses matched clinical diagnoses and

conclusions reached from immunostaining of the skin sections; specifically, the presence of lupus erythematosus.

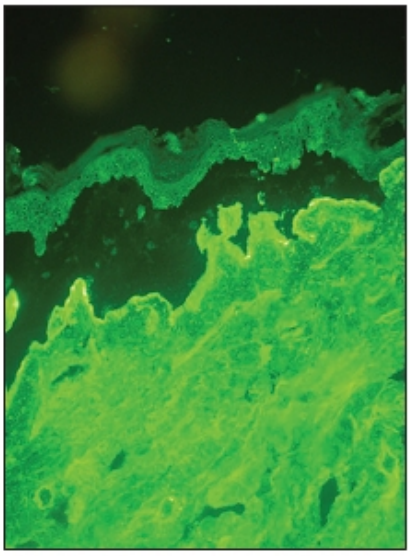

Figure 21: Fluorescence image of the dermal-epidermal junction (Ranjana, 2010).

Hence, the use of immunohistochemistry allows for the observation and diagnosis of skin pathologies, such as systemic lupus erythematosus, by highlighting immune complexes in cell structures of the epithelial tissue, thereby confirming diagnoses of the pathologies underlying the lesions and growth present in the skin biopsy section (Ranjana, 2010).

By analyzing resultant images with imaging analysis software, statistical investigation of cellular activity and presence is possible and therefore allows for quantitative analysis of cell cultures. This is invaluable in the analysis of tissue engineered skin graft viability, production, and cellular mechanisms.

2.6.2 Image Processing- Segmentation and Pixel Intensity

Processing images gained from fluorescence micrography, in order to quantify the qualitative conclusions drawn from the experiment, involves retrieving

quantitative data from a qualitative source such as a digital image. In order to establish quantitative measurements for such images, an image analyzer or similar program must be developed that detects and analyzes the pixel intensities of the captured images, and is able to pinpoint what parts of the image are under scrutiny without input from the user, i.e. automatically. With regards to images relevant to the Pins Lab, the graft section segments that are being analyzed are likely to be the cells or any part of the cell structure, such as the plasma membrane. The level of fluorescence given off by cells, or cell parts, is recorded in a digital image as the relative pixel intensity. Therefore, the program proposed for use in automatically analyzing histological fluorescence slides of the epithelium is one that can detect the cells and cell structures, and one that can quantify, analyze, and manipulate the pixel intensity values of the image that correlate with the cells.

CellProfiler (www.cellprofiler.org) is an open-source biological image analysis software package designed specifically for analyzing images of cells, called cytometry. Computer-aided cytometry is able to interpret and gather much more data than is possible by humans, as the program is able to automatically record data points such as number of cells or cell structures, average pixel intensity, the shape of all structures recorded, etc., making it extremely versatile and greatly reducing the amount of time needed to analyze and record this information (Carpenter, 2006). Additionally, it removes variables such as qualitative-only analysis by humans and image-wide scores as opposed to per-structure scores.

The main advantage of CellProfiler is its ability to perform cell segmentation. Segmentation in image processing is the usage of techniques such as thresholding

and edge detection in order to divide an image into individual regions, parts, or structures (Kueh, 2008). CellProfiler offers this process in a modular format, allowing for segmentation of nearly any structure in an image of cells depending on the options and methods input into the program. Therefore, this program offers the ability to automatically and modularly perform complicated image processing techniques in a user-friendly manner. An example of images produced by computer-aided image segmentation is shown below in Figure 22. Individual cells are identified and recorded as individual objects (along with related data), and the outlines of the objects correlating to these objects are overlaid on the original image to visualize the outcome of segmentation (Carpenter, 2006).

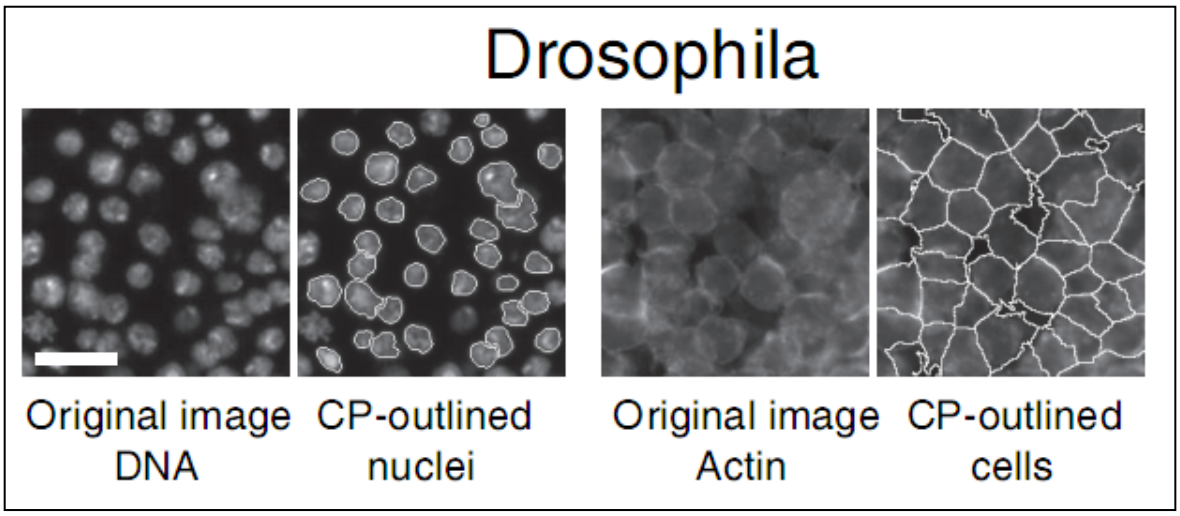

Figure 22: Segmentation of Drosophila cells in CellProfiler (Carpenter, 2006).

Due to the specific nature of the data intending to be analyzed, and the massive amount of data points being recorded, MATLAB offers the most flexibility in automatic data analysis and manipulation of the pixel intensity values of individual cell structures using pre-defined methodology. MATLAB operates on the principle of data points organized in a matrix format. This matrix format is essentially identical

to the way that pixels values are stored in digital images. MATLAB is therefore extremely useful for manipulating image data. With regards to the images used in fluorescence microscopy, MATLAB is able to identify pixels based on RGB values, which correspond to markers that fluoresce red, green, and blue light. This allows MATLAB to easily handle color images and to distinguish different types of data and objects depending on whether the data correlates to red, green, or blue fluorescence. Additionally, MATLAB commonly comes with modules and scripts meant specifically for image processing, including but not limited to edge detection, Fourier transforms, spatial filtering, watershed processing, and object identification (McAndrew, 2004).

2.6.3 Current Imaging System and Limitations

Currently, the Pins lab uses fluorescence microscopy and immunohistochemistry of histological sections as its method of cell analysis and imaging. The current device does not allow for longitudinal, non-destructive study under the fluorescence microscope due to clearance issues and an inability to remain sterile outside of a proper cell culturing environment, and therefore whole-mount imaging is not performed. The inability to perform whole-mount imaging can be resolved by removing the graft from the device before being studied under the microscope. In order to be compatible with whole-mount longitudinal study using the current imaging system, the device must either be flat enough to fit comfortably under a fluorescence microscope or allow for graft removal. In either case, neither imaging nor removal must be destructive or result in contamination of the graft.

The laboratory of Fiona Watt used whole-mount imaging and staining for β1 integrin to identify keratinocyte stem cells in the basal layer. According to the research, cells at the extremities of the dermal papillae, the plateau or well-like structures in sections, have a high degree of β1 integrin expression. Since a high level of β1 integrin is associated with keratinocyte stem cells, by using fluorescent detection and thresholding the results could be used to identify stem cells (Watt, 1999).

Additionally, the fact that stem cells are likely to be far away from non-stem cells – in this case, nearer to the basal layer – can be confirmed by colocalization with a cell nuclei marker specifically designed to highlight stem cells (Watt, 1999). Quantitative analysis can therefore be performed by highlighting the areas in which this marker appears and analyzing the pixel intensity in the digital images, and manipulating the data to reveal average pixel intensity, the location of cells versus their average intensity, etc. The average intensity of these cells can then be compared to the cells that are identified as stem cells, with the goal of equating high β1 expression with stem cell status. If this is possible, this will allow for analysis of the effect of microtopography on stem cell localization.

3 Project Strategy

There are five major steps in going from the initial project statement given from the client up through the creation of a final design. These five steps are shown in the five boxes on the right in Figure 23.

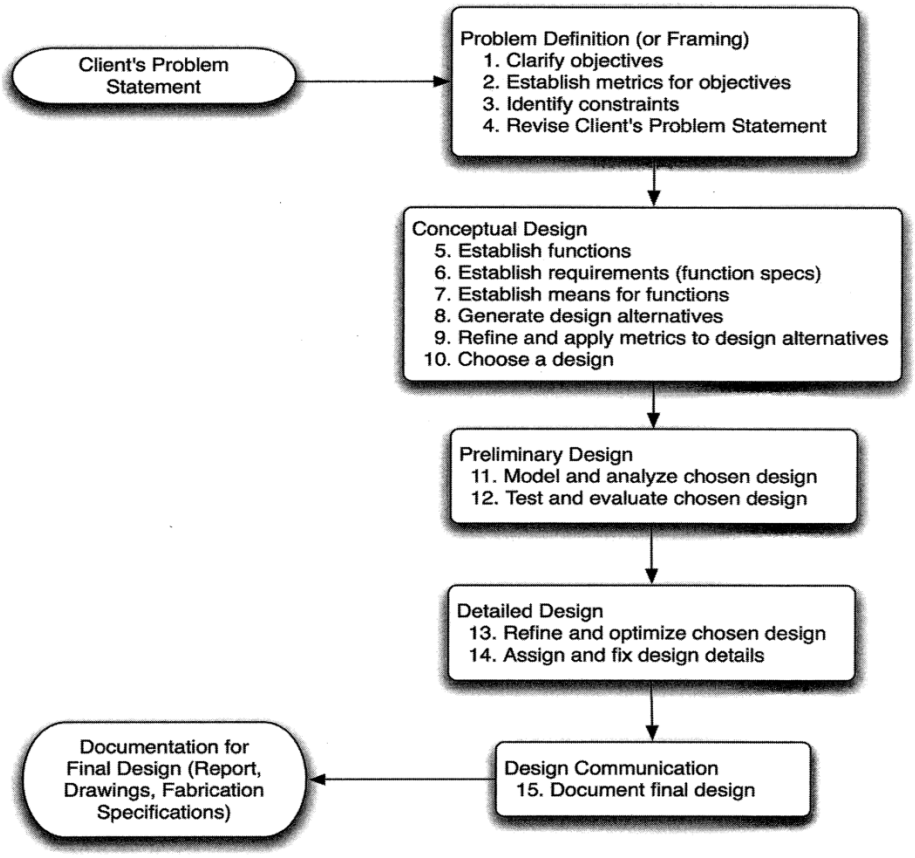

Figure 23: Five-Stage Outline of the Design Process (Dym, 2009).

Also contained within each box are some of the more specific steps to be taken in the design process. This chapter focuses mainly on the first box on the right: problem definition.

3.1 Project Motivation

Although the system used in Professor Pins' lab has been used for this culturing process for several years there are many problems associated with its use. The system is not user friendly and without training can be susceptible to contamination. Specifically, the screws that hold the device together require a significant amount of handling to remove, and therefore makes the device difficult to disassemble. The disassembly process must take place in a sterile environment, and therefore a difficult process increases the possibility for contamination. In addition, due to the highly proliferative nature of the keratinocytes, they can begin to grow up the sides of the O-ring. This means that removal of the graft from the device is likely to result in destruction of the graft (Correspondence with Amanda Clement).

Because of the many issues associated with the use of this device, a new device for creation of these grafts is needed. It will need to incorporate all of the functions of the current device described above, but address and improve upon the many drawbacks. It is the job of our design team to design and evaluate this device. The design and testing of our new device are detailed in the following chapters.

3.2 Stakeholders

In the design of the device the major stakeholders are the client, the user, and the designer. For this project the client is Professor George Pins. The user is graduate student Amanda Clement. The designers are the MQP team: Daniel Barrows, Timothy Haufler, Rebecca Paz, and Lauren Walsh.

3.3 Client Statement

Design and develop a system to improve the skin graft culturing system to simplify the graft production, culture, manufacturing, and evaluation process.

3.4 Design Goals

To arrive at the best possible design for the device, it is important to take into account what each of the stakeholders (client, user, designers) need in the device and in the project as a whole. It is also important to consider their wants, and also any constraints they have on the design or the process. We gained this info based upon the general information given to us in the project syllabus as well as a preliminary discussion with the client and user. A listing of these can be seen in Table 2.

Table 2: Initial Needs, Wants, and Constraints

Initial Needs, Wants, and Constraints
Design Team 1) Needs: a) Need to finish project by the end of C term b) Need to create a working prototype by the end of C term c) Need to follow the design process d) Need to consult with client and user e) Need to make weekly reports to client and user f) Needs to validate the success of the project g) Need to create presentation for MQP day 2) Wants a) Want the device to be an improvement over the current system b) Want the system to be used in the lab upon completion c) Want to successfully present the project 3) Constraints a) Budget of $524 b) Time (until end of C term) c) Limited to equipment found in the client's lab
Client 1) Needs: a) Needs a redesigned skin graft culture system b) Needs to provide consultation for design c) Needs weekly and quarterly reports d) Needs device created to not be prohibitively expensive e) Needs system to be reproducible 2) Wants: a) Wants to be able to image the grafts non-destructively b) Wants device to be cost effective c) Wants consistency in grafts produced 3) Constraints: a) Limited to equipment found in lab b) Availability to design team
User 1) Needs: a) Needs to be able to use system effectively b) Needs to provide feedback on design process c) Needs device to be sterile d) Needs system to not easily cause contamination 2) Wants: a) Wants the system to be easy to use b) Wants to image grafts nondestructively c) Wants system to minimize total processing time d) Wants consistency in grafts produced 3) Constraints a) Time

Keeping an open mind and being unbiased are also critical components of the design process. This is especially true in a project such as this where an existing system is being redesigned. It is important to leave open the ability to consider all solutions to the problem and not limit the design space to what is already being used in the lab. By keeping the objectives, constraints, functions, and means independent of the current system we can consider all possibilities and arrive at the best possible solution.

3.5 Objectives

To help identify the objectives of the project we conducted a client interview. The questions and answers from this interview can be seen in Appendix A: Client Interview Questions With Answers. From these answers we formed a list of objectives and organized them as seen in Figure 24.

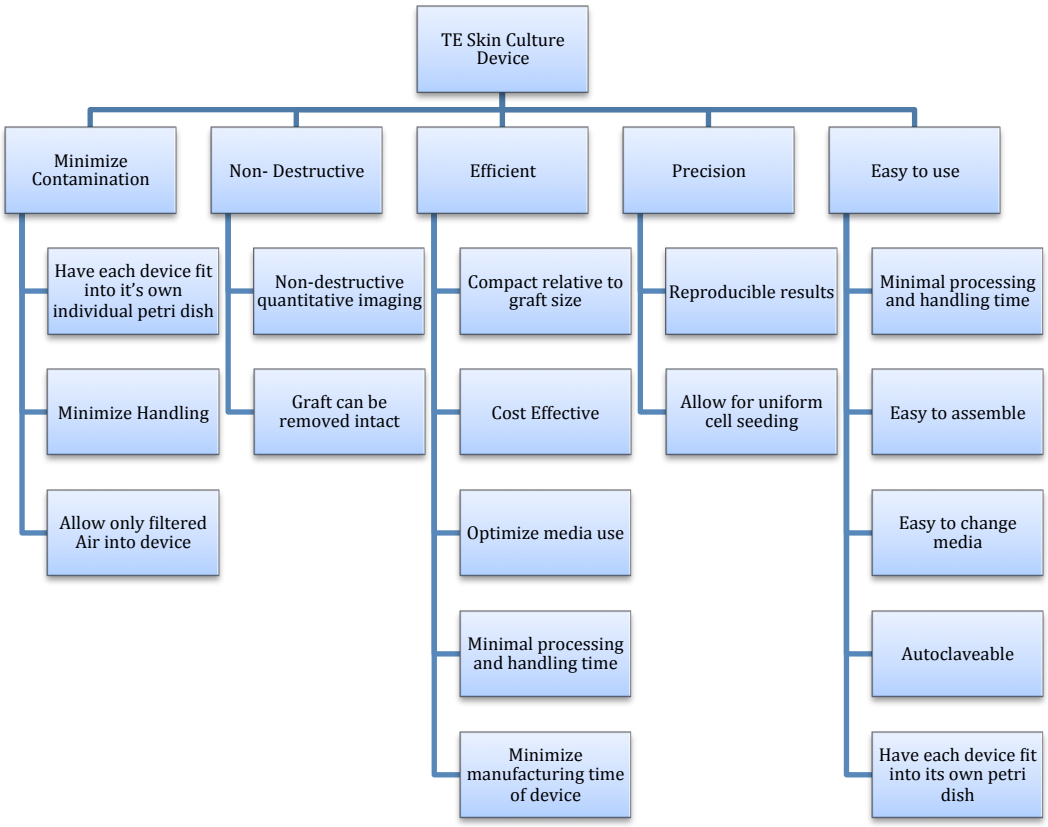

Figure 24: Objectives Tree

As seen in Figure 24, we decided upon five major objectives: Minimize opportunity for contamination, non-destructive, efficient, precise, and easy to use. Listed under each of these are second level objectives that compose the main objectives.

3.6 Ranked Objectives

To determine which objectives were most important we came up with six pairwise comparison charts. The first compared all of the first level objectives. The next five compared the sub-objectives for each of the main objectives respectively. The client, user, and designers each completed the pairwise comparison charts

individually. The responses are shown in Appendix B: Pairwise Comparison Responses

From the responses to pairwise comparison charts we were able to calculate weights for each of the objectives. Each design team member filled out a chart individually and then the average was taken. The average score from the team members for each of the main objectives are shown in the "Team Average" column of Table 3. The results from Amanda and Professor Pins' pairwise comparisons are listed in their respective columns of Table 3.

Table 3: Weighting of main objectives in order from most important to least important

Main Objectives					
Totals	**Team Average**	**Amanda**	**Prof. Pins**	**Average**	**Weighted Total**
Minimize opportunity for contamination	2.125	3.5	4	3.208	0.318
Precise	2.25	3	2	2.417	0.240
Easy To Use	1.375	2	2	1.792	0.178
Efficient	1.625	1	2	1.542	0.153
Non-Destructive	2.875	0.5	0	1.125	0.112

In the design process, it is critical to remember that the goal is to meet the needs and wants of the client and user. To make sure that these needs would be met it was decided that entire design team's responses would be averaged and are listed in the "Team Average" column. The results of pairwise comparisons from the user, Amanda, and the client, Professor Pins, are listed in the next two columns respectively. The design team decided that each of these columns was given ⅓ of the total weighting. This represents the greater importance of the needs and wants of

the client and user by giving them a combined ⅔ say in the rankings while still giving the design team input as well. To get the weighed total we added up the values for each objective and then divided by the total of all possible points. Based upon this analysis, the objectives are ranked as follows:

1. Minimize opportunity for contamination
2. Precise
3. Easy to use
4. Efficient
5. Non- destructive

The client and user valued minimizing the opportunity for contamination above all of the others because any contamination will destroy the samples. Contamination would ruin the results as well as waste valuable time and resources. Accuracy and precision were found to be the next most important. To have any significant results come from the production of these grafts, it is critical that the results are reproducible, meaning that they display a good degree of accuracy and precision. Non-destructive was ranked least important mainly in reference to the imaging sub-objective. The client and user both believed that the easy production of consistent grafts was more important than the ability to image them non-destructively. The team initially ranked non-destructive imaging as the most important, however, the preventing contamination and producing consistent results were relatively close.

Table 4: Efficient Sub- Objectives

Efficient Sub- Objectives					
	Team Average	**Amanda**	**Prof. Pins**	**Average**	**Weighted Total**
Minimal processing and handling time	3.5	3.5	4	3.667	0.373
Compact relative to graft size	1.375	3	3	2.458	0.250
Optimize media use	2	0.5	2	1.500	0.153
Minimize manufacturing time of device	0.75	2.5	1	1.417	0.144
Cost of $1-2 per graft	1.875	0.5	0	0.792	0.081

Shown in Table 4 are the weightings for the efficient sub-objectives. In order of ranking they are:

1. Minimal processing and handling time
2. Compact relative to graft size
3. Optimize media use
4. Minimize manufacturing time of device
5. Cost of $1-2 per graft

These results highlight the importance of consulting with the client and user in the design process. The design team, user, and client all had minimal processing and handling time as the most important, however, the second position in the rankings is where the results begin to differ. The design team considered optimizing media use and the cost of $1-2 per graft as being the next two most important objectives. The user and client had these listed as close to their least important

objectives. If the client and user's needs were not taken into account, the design team could have come up with a design that did not actually meet all of their priorities.

Table 5: Easy to use Sub-Objectives

Easy to use Sub- Objectives					
	Team Average	**Amanda**	**Prof. Pins**	**Average**	**Weighted Total**
Easy to assemble	3	3.5	3	3.167	0.317
Minimal Processing and handling time	2.33	3.5	3	2.943	0.294
Easy to change media	3.33	2	3	2.777	0.278
Autoclavable	0.833	1	0	0.611	0.061
Have each device fit into its own petri dish	0.5	0	1	0.500	0.050

The results of the pairwise comparison for the Easy to Use sub objectives are shown in Table 5. These objectives ranked in order based upon the results are as follows:

1. Easy to Assemble
2. Minimal Processing and Handling Time
3. Easy to Change Media
4. Autoclavable
5. Have each device fit into its own petri dish

While all of the objectives were based upon desires of the client or user, it was clear from this comparison that some were far more important than the others.

Objectives one, two, and three in the above list were clearly the most important sub-objectives in this category to the client, user, and design team. Each of these has a weighted total of approximately 0.30 (30%) of the importance for this category. Objectives 4 and 5 from this list combined only made up approximately 10%. Based upon this analysis it is more important that the device be easy to assemble, have minimal processing and handling time, and make it easy to change media than for it to be autoclavable or contained within an individual petri dish.

Table 6: Minimize Opportunity for Contamination Sub- Objectives

Minimize Opportunity for contamination Sub objectives					
	Team Average	**Amanda**	**Prof. Pins**	**Average**	**Weighted Total**
Minimize handling	1.75	1.5	1.5	1.583	0.528
Allow only filtered air into device	1.25	1.5	1.5	1.417	0.472
Have each device fit into its own petri dish	0	0	0	0	0

The results from the pairwise comparisons of the Minimize Opportunity for Contamination Sub-Objectives are shown in Table 6. Minimizing handling was found to be the most important of these, closely followed by allowing only filtered air into device.

Table 7: Non-Destructive Sub- Objectives

Non- Destructive Sub-objectives					
	Team Average	**Amanda**	**Prof. Pins**	**Average**	**Weighted Total**
Graft Can be removed from device intact	1	1	1	1	1
Non-destructive quantitative imaging	0	0	0	0	0

The ranking of Non-Destructive Sub-Objectives seen in Table 7, was the only unanimous decision out of all the pairwise comparisons. Everyone believed that it is more important that the graft can be removed from the device intact than the ability of the graft to be imaged non-destructively.

The Precision Sub- Objectives are shown in Table 8. It was found that allowing for uniform cell seeding was more important than reproducible results. The difference between their weighting, however, is very minimal based upon the pairwise comparison results.

Table 8: Precision Sub- Objectives

Precision Sub-Objectives					
	Team Average	**Amanda**	**Prof. Pins**	**Average**	**Weighted Total**
Allow for uniform cell seeding	0.125	1	0.5	0.542	0.542
Reproducible Results	0.875	0	0.5	0.458	0.458

Figure 25 is the weighted objectives tree. This chart allows us to compare each sub-objective against all of the others. This means that all of the major

objectives are being compared to each other in that number. The number on the left of each box is that objectives importance compared to others in the same category. This means that each sub-objective is compared to the other sub-objectives in that category. The number on the right represents the overall importance of each sub-objective compared to all the sub-objective regardless of main objective.

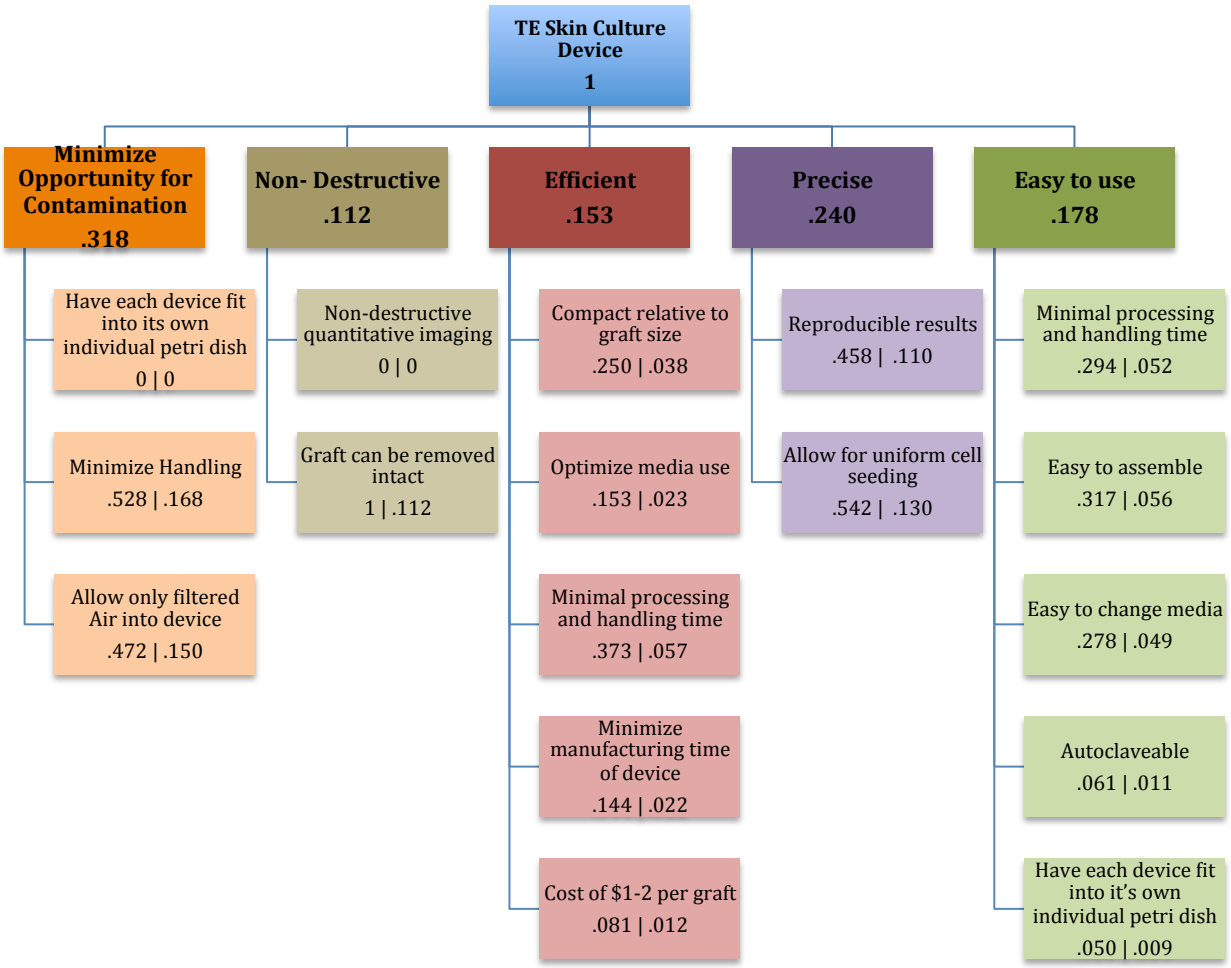

Figure 25: Weighted Objectives

Based upon Figure 25 the sub-objectives in order of importance are:

1. Minimize handling
2. Allow only filtered air into device
3. Allow for uniform cell seeding

4. Graft can be removed intact
5. Reproducible results
6. Minimal processing and handling time (efficient)
7. Easy to assemble
8. Minimal processing and handling time (easy to use)
9. Easy to change media
10. Compact relative to graft size
11. Optimize media use
12. Minimize manufacturing time of device
13. Cost of $1-2 per graft
14. Autoclavable
15. Have each device fit into its own petri dish
16. Non- destructive quantitative imaging

3.7 Revised Client Statement

The revised client statement is composed as a way to relay to the client the design team's understanding of the problem. Through the client interview and other meetings with the client and user many needs, wants, objectives, and constraints have been identified, and it is important to ensure that the problem is understood correctly. Presenting the revised client statement to the client and user gives them greater insight into how the design team views the problem to make sure that the created system will adequately meet the needs and wants of the client and user. The revised client statement for the Tissue Engineered Skin Culture device is as follows:

Design and develop a safe, easy to use improvement of the skin graft culturing system. The system needs to accommodate a graft of ~1-1.5 cm x ~2-2.5 cm. It will have a cost of $1-2 per graft and optimize the use of media. It needs to be seeded on one side with fibroblasts in submerged culture and facilitate seeding of keratinocytes on the other side. It must then allow the graft to be removed and placed in air/liquid interface culture. The grafts must be able to be imaged and analyzed as non-destructively as possible.

3.8 Constraints

Constraints are the limits that are placed on the design. While objectives define what the designed system should be, constraints serve to outline what the system cannot be (Dym, 2009). The constraints represent conditions that if not met, would cause the design to be unsuccessful. The constraints gathered by the design team based upon the client interview and other correspondences are listed in Table 9.

Table 9: Constraints

Constraints
• Must be sterilizable • Must be cytocompatible • Allows for the removal of the graft without damage • Budget of $524 • Fit in currently available tissue culture product or be self-contained • Must fit graft of size ~1-1.5 cm x ~2-2.5 cm • Must be able to seed on both sides • Safe

For any tissue culture device it is critical that it can maintain a sterile environment, and not have a harmful effect on the cells being cultured. The budget

of $524 dollars is set by the BME department ($156 for each of the four team members minus a $100 dollar lab fee). The device must fit in current tissue culture products or be completely self- contained. This will make it a closed system that will keep in the cells and medium, but keep out contamination. The grafts that are grown in the lab are approximately 1-1.5 cm by 2-2.5 cm. These are the grafts for which the device is being created, and therefore if it did not fit these grafts it would be useless. Additionally, an important step in the process of growing cultured skin substitutes is the ability to seed fibroblasts on one side of the graft and keratinocytes on the other (see 2.5.4 The Pins Lab System).

4 Alternative Designs

4.1 Generation of Alternative Designs

4.1.1 Functions and Specifications

Functions are defined by Dym and Little as "the action for which a person or thing is specifically fitted or used or for which a thing exists" (Dym, 2009). The functions of a device define specifically what it will do. Functions must be as specific and quantitative as possible. Functions of the skin graft culturing device were determined through incorporation of client and user needs with the intent and objectives of the skin culturing process. After analysis and discussion, the finalized functions are listed below, in Table 10.

Table 10: Functions

Functions
• Cultures keratinocytes in submerged culture then at the air/liquid Interface • Cultures fibroblasts in submerged culture • Holds graft in place for culturing • Produces consistent skin grafts • Allows for removal of graft without destruction • Provides structural support for graft • Creates well on top of device for seeding keratinocytes (watertight seal) • Holds media in place • Provides access to medium for daily media change • Provides access to both sides of graft for controlled, reproducible seeding • Leaves enough room between device and lid of culture tray to prevent contamination • Allows for non-destructive quantitative imaging • Maintain device integrity throughout process (hold it together)

The functions are intended to be the basis off of which the rest of the design process. For this reason, the creation of an accurate functions list is critical. In the next sections, these functions will be matched up with means to accomplish them.

Fulfillment of these function criteria imply the creation of specifications for the device as a means of detailing to what extent they are fulfilled. Hard numbers, dimensions, and details were developed given the use of the device, requirements of the culturing process, and adherence to the objectives and constraints previously outlined in the paper. The finalized specifications for the culturing device are compiled in Table 11.

Table 11: Specifications

Specifications	
Parameter	**Specification**
Graft size	Approximately 2-2.5 by 1-1.5 cm
Maximum Height	Approximately 10 mm
Temperature Range	20°C - 121°C
Submersion	Culture time up to 28 days 3 Different types of culture
Air Liquid Interface	Culture time up to 28 days
Attachment	Graft held in place for entire process Stays in place when Submerged or in air
Material	Cytocompatible Doesn't degrade in culture media Sterilizable
Total Media Volume	Minimum of 5 ml
Viability of grafts	At least 90%

In summary, the device should hold up to a 2.5 by 1.5 centimeter scaffold in place, should allow for submerged culture and air-liquid interface culture for up to 28 days in at least 5 mL of media. It should prevent contamination and destruction

of the graft by being sterilizable, while also remaining cytocompatible and retaining structural integrity in culture media. It should consistently produce skin grafts at a viability rate of at least 90%.

4.1.2 Brainstorming

After creation of functions and specifications, several general and part-specific designs for the device were created and are included here with their related write-ups.

Pressing Skin Design

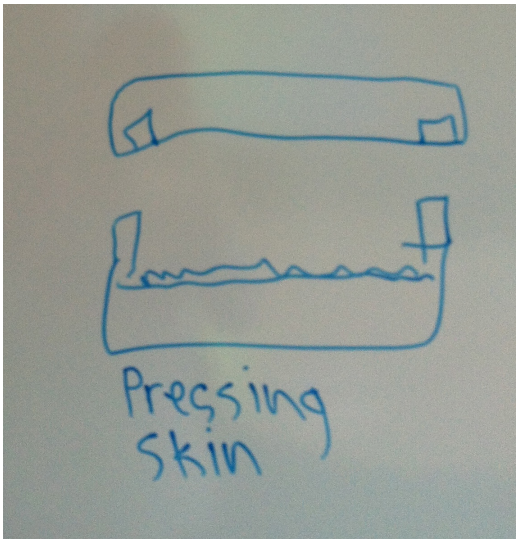

Figure 26: Pressing Skin Design, Side View

This device has two interlocking pieces that contain and immobilize the screen and the graft as shown in Figure 26. The device is locked by using properties of self-adhesion found in certain polymers. The interlocking pieces will be made into a cross shape and another with many sides to increase surface area for attachment and increase the strength of the seal. It provides a seal with minimal area that can be contaminated, but it may not provide a strong enough seal. The design is easy to

handle and manufacture, but the strength of the seal may make it hard to disassemble.

Screw-Top Design

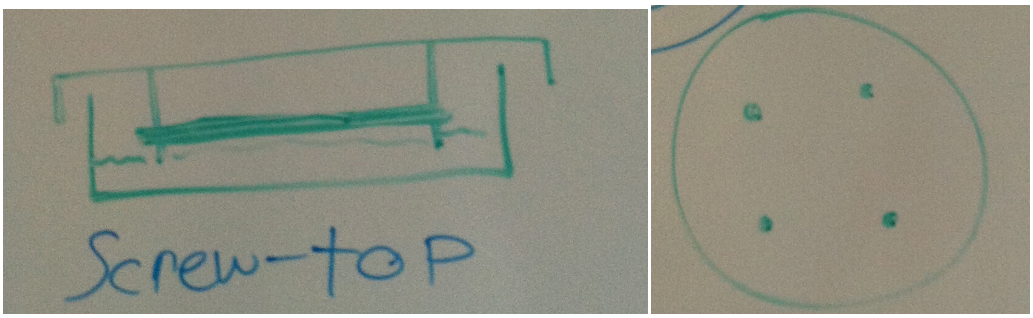

Figure 27: Screw-Top Design. Left: Side View, Right: Top View

In the Screw-Top design, shown in Figure 27, there are four metal pegs going through the top of the lid of the Petri dish. The scaffold will have four holes cut into it to allow it to be attached and also removed from the four metal pegs. There would be threads in both the lid and base of the Petri dish to allow the two to be screwed together. Media can be changed by simply removing the lid, but the design does not allow for a press-fit to seal the graft. The scaffold can be removed from the pegs, which does not allow for a well effect on the top of the scaffold.

Sandwich Device Design

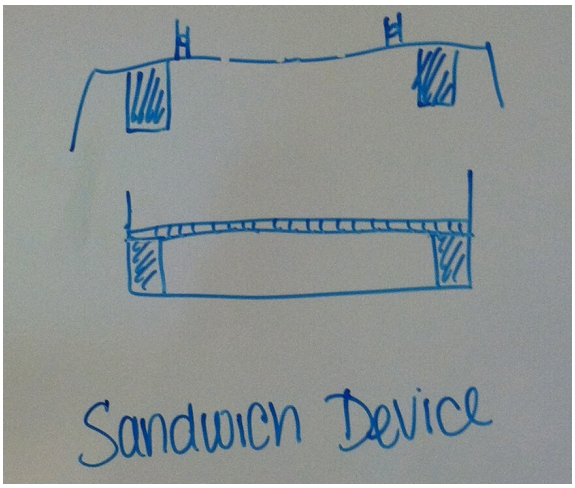

Figure 28: Sandwich Device, Side View

In the Sandwich Device design, there is a thick O-ring on the bottom of the base of the Petri dish, allowing for media to be kept. The scaffold would sit on top of the O-ring as seen in Figure 28. On the lid of the Petri dish, there would be another thick O-ring in order to create a well effect for cells to grow on. There would be two to four metal rods going through the Petri dish with a set of pegs going through a hole within these rods to keep them seal tight. There would be some air holes at the top of the Petri dish within the inner portions of the O-rings. This design allows for a press-fit seal for the graft as well as a well effect above the scaffold and media storage, but could be difficult to disassemble and move the graft in case of media change or imaging. In addition the holes in the top of the Petri dish could lead to contamination.

Concentric Rings Design

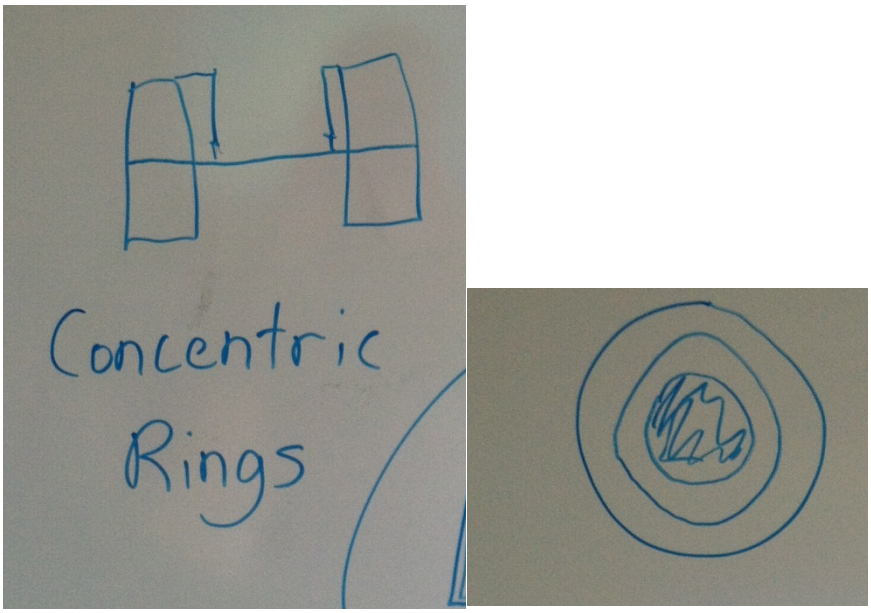

Figure 29: Concentric Rings Design. Left: Cross-section, Right: Top View

This design is composed of two concentric rings and a screen, as shown in Figure 29. The larger ring has a screen embedded in it, which will support the graft. The smaller ring fits inside and forms a seal over the graft. It is simple and easy to manufacture, but may not properly form a seal over the graft.

Twist-And-Lock Design

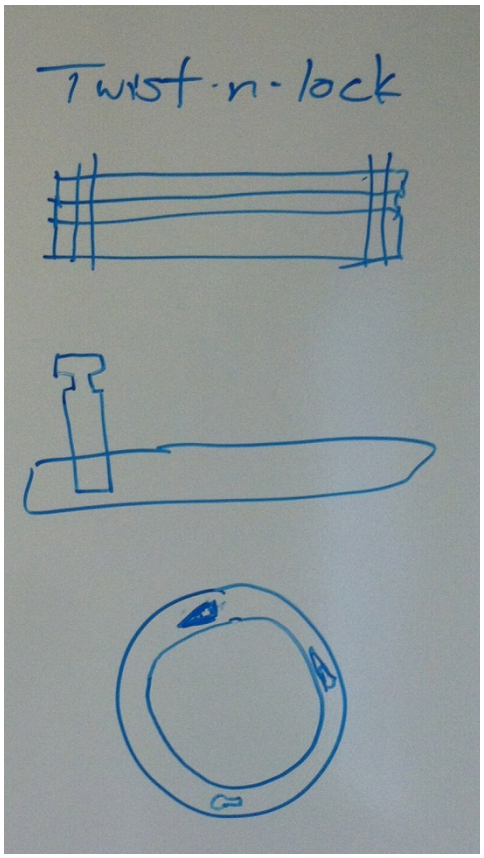

Figure 30: Twist-and-Lock Design

This is a method of holding the device together. Several of the pegs shown in the middle image will stick through the holes in the ring pictured on the bottom of Figure 30. When this is rotated, it will lock the pins in place, and therefore hold the entire device together. It only takes one motion to lock the device together and no shear force would disrupt the graft, but may be difficult to use and anchoring of the pins could be problematic.

Post-and-Pin/Post-and-Snap/Post-and-Twist

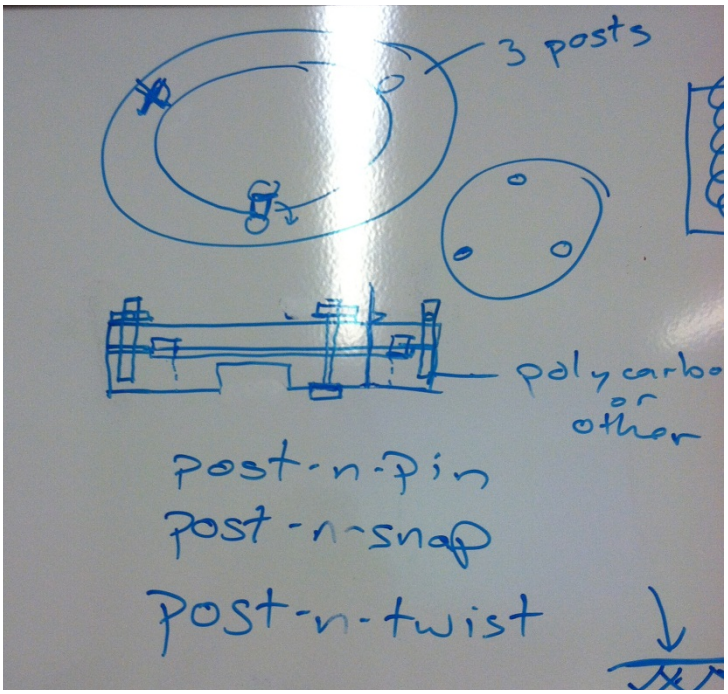

Figure 31: Post-and-Pin/Snap/Twist Design

This design incorporates three iterations of the same design, all shown in Figure 31. In the post-n-pin, a post passes through the entire structure and then a pin is inserted through a hole in the side of the post to hold it in place. In the post-n-twist, a post has a bar on the top, that when rotated, keeps it from falling into the hole and locks it in place. All of these designs incorporate three or more pins depending on the number that will be needed to provide enough force to hold the device together. This method uses screws instead of pins for ease of use and manufacture, but may be difficult to use due to manipulating small parts.

Snap-To-It

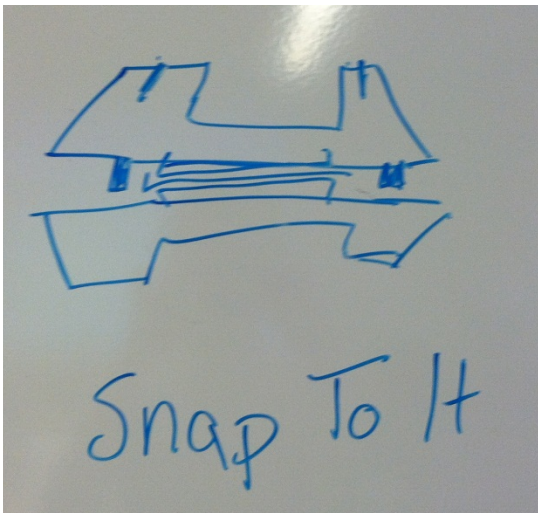

Figure 32: Snap-to-it Design

The snap-to-it is a method for closure shown in Figure 32. It functions essentially like a plastic buckle. By pinching the two slits at the top with forceps it could be put in place or removed. The device would be easy to assemble with a minimal amount of handling, and there are no small parts to manipulate.

Split Model Design

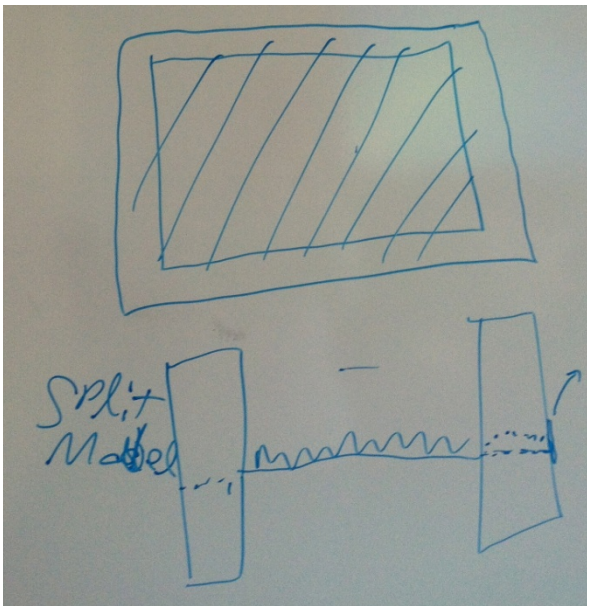

Figure 33: Split Model Design

The split model, shown in Figure 33, is a method of retrieving the graft, whereby the device splits apart. This requires moving and disassembly of the device, but is the simplest method for retrieval.

Slot Model Design

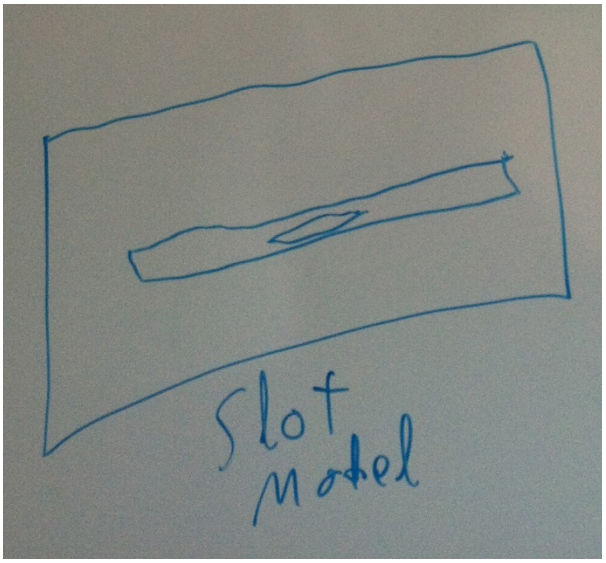

Figure 34: Slot Model Design

The slot model, shown in Figure 34, is a method of retrieving the graft whereby the support for the graft slides out of the device, allowing the graft to be removed. It is more complicated than other methods, but allows for less manipulation of the device and a lower clearance for use in microscopy.

Teflon-Like Gasket Design

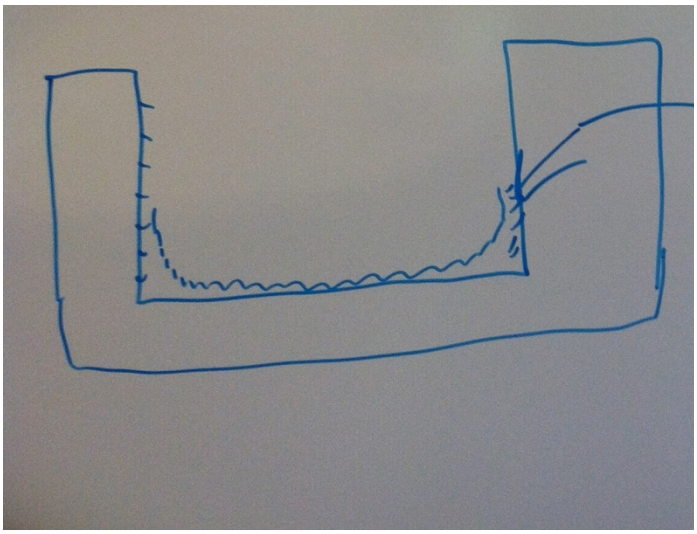

Figure 35: Teflon-Like Gasket Design

The Teflon-like gasket, shown in Figure 35, is a gasket for use in restricting where the cells can grow. It constrains cell growth to a certain area as well as not allowing cells to grow up along the walls. This will negate the possibility of tearing the graft upon removal.

Drape-And-Punch

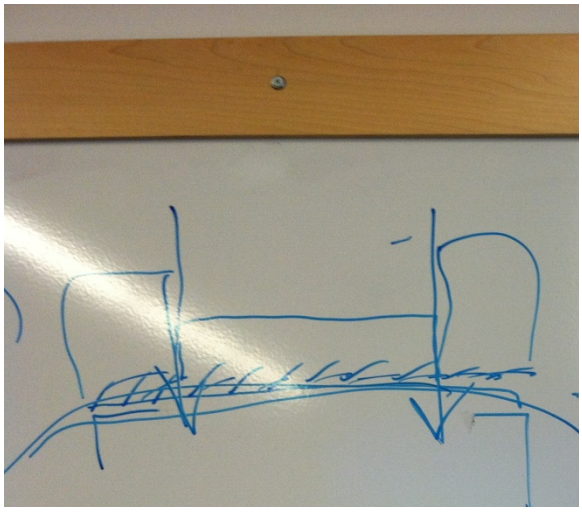

Figure 36: Drape and Punch Design

In the drape and punch, shown in Figure 36, the graft is supported by a disposable material that is stretched across the growth area. When the graft is ready for removal, it can be punched out of the device alongside the support material. This has the advantage of making graft retrieval simple and easy. The main disadvantage is that this method creates the need for design and production of proprietary tooling.

4.1.3 Materials Selection

When developing the devices for the culturing of keratinocytes, one of the most important decisions is the material to be used in the system. Some of the requirements for these materials are that they must be biocompatible, sterilizable, autoclavable and have necessary mechanical properties to build the device. The majority of these devices are made from thermoplastic polymers.

One of the main materials used in the making of these devices is polycarbonate. Polycarbonate is a popular choice because of its durability, its ability to be shaped and molded, and its transparency. This material can be made into almost any shape to form the bioreactor, and can aid in the imaging of the graft due to its transparent nature. The durability and the high melting temperature (267 C) of the material also allows for the device to be reusable and autoclavable. Polycarbonate has an elastic modulus of 2.0-2.4 GPa, a tensile strength of 55-75 MPa, a flexural strength of 82 MPa, a density of 1.20-1.22 g/cm^3, shear strength of 63 MPa and a compressive strength of about 80 MPa. The mechanical properties of

this thermoplastic appear to be adequate for the construction of the bioreactor. An example of this material can be seen in Figure 37.

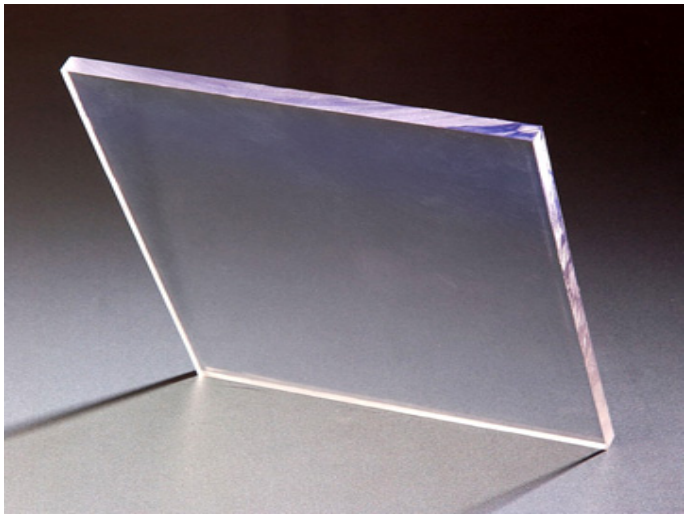

Figure 37: Sheet of Polycarbonate (http://www.livsupplies.co.uk/admin1/image/5316solid.jpg)

Another material choice for culturing devices is polystyrene. It is another thermoplastic polymer with many similar characteristics to polycarbonate. They are both durable and very moldable. One disadvantage of polystyrene is that its thermal properties are not as good as those of polycarbonate. It has a melting temperature of only about 220 C and a glass transition temperature of about 95 C. While inside an autoclave, polystyrene may become rubbery due to its lower glass transition temperature or may even partially melt. It has an elastic modulus of 3000-3600 MPa, a tensile strength of 40-60 MPa and a density of 1.05 g/cm^3. Polystyrene has lower weight and less beneficial mechanical properties. In addition it is not as optically clear as polycarbonate, which can make imaging in the device difficult. A sheet of polystyrene is shown in Figure 38.

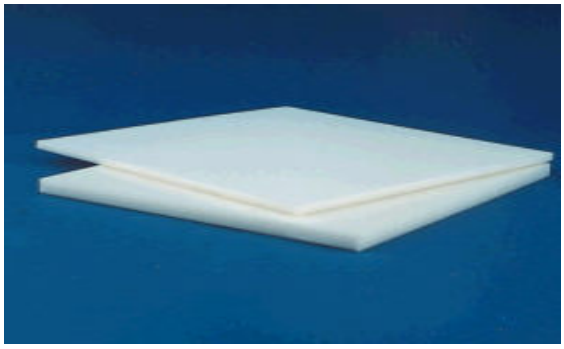

Figure 38: Sheet of Polystyrene (http://www.usplastic.com/catalog/ImageViewer.aspx?description=High+Impact+Styrene+Sheet&curimage=sheet%2f43330p.jpg&image=sheet%2f43330p.jpg&catid=715&itemid=22883).

Polyoxymethylene (POM), also known as Delrin is another very promising material to construct the housing for the graft. An image of a Delrin rod is shown in Figure 39. Delrin is a thermoplastic, like polycarbonate, that is used to make precision parts that require high stiffness, low friction, and great dimensional stability. This material has outstanding mechanical properties. It has a tensile strength of approximately 68 MPa, a flexural strength of 98 MPa, compressive strength of 124 MPa, and shear strength of 65 MPa. These values show that Delrin is much stronger mechanically than polycarbonate or polystyrene. This material also possesses high density of 1.41-1.42 g/cm^3, and adequate heat resistance with a melting point of 175 degrees Celsius (Penick, 2005). As seen in Figure 39 below, Delrin does not possess the same amount of optical clarity as polycarbonate because of its highly crystalline structure. With Delrin's superior mechanical properties, adequate thermal properties and the ability to be machined into small, very intricate parts, Delrin appears to be the top choice for building material.

After researching the effects of Delrin on cell culture, it was determined to be safe for cell growth and poses no obvious hazard to the graft. One possible concern for Delrin is overheating from repeated autoclaving or excessive heat from

machining the parts. However, according to Penick et al, "the use POM material as a culture-medium wetted component appears to be innocuous, even after machining and repeated autoclaving" (Penick, 2005). Based on this paper and other research the use of delrin is deemed safe for cell culture.

Figure 39: Delrin Rod (http://www.plasticsintl.com/images/products/white-delrin-rod.jpg).

4.1.4 Morph Chart

The morph chart displays the results of our brainstorming session. The chart is shown in Table 12. It displays all of the means that the design team, client, and use came up with arranged by function. By selecting one means for each function, a number of alternative designs can be created.

The focus of much of the brainstorming session ended up being different methods with which to hold the device together. This is reflected in the morph chart, as there are more means for this function than for any other.

Table 12: Morph Chart

Function	Means								
Allow for non-destructive imaging of device	Image in device	Split model	Slot model						
Keep cells from sticking to device	Teflon- like gasket	Punch							
Hold Device together	Single Piece	Screw-top	Self-Adhesive material	Press- fit	Twist-n-lock	Post-n-pin	Post-n-twist	Snap	Waffle iron
Facilitate changing of media	Access from top for manual change	Continuous perfusion	Drip and drain system	Remove device from well					
Provide structural support for graft	Stainless steel screen	Polymer screen							
Facilitate air-liquid interface culture	In device	Device partially disassembles	Graft completely removed						
Device Material	316L Stainless Steel	Polycarbonate	PDMS	Delrin					
Shape/ form	Circular	Square	Rectangular	Triangular					

4.2 Selecting a Design

4.2.1 Creation of Metrics

Metrics are important in the selection of a final alternative design. The metrics serve as a consistent guideline against which all of the designs can be judged. The correct selection of metrics is essential for the picking of a good design.

The final metrics were based directly off of the objectives list. For each sub-objective the team came up with a way to objectively judge how well each solution met them. The objectives were done on a scale with higher numbers representing better achievement of the function. Because not every objective had the same number of possible scores, dividing each score by the highest possible score for that objective normalized the results. The full list of metrics can be seen in Appendix C.

Listed in Appendix D: Initial Metrics are the initial metrics that the team had devised. When it came to scoring the functions, the team had difficulty in coming up with scores. The only way to accurately score each one of them would be to build several devices and test them against the metrics which was deemed to be too time consuming. For this reason the new set of metrics was created. These new metrics were specifically designed to be subjectively evaluated without the need for building each possible device.

4.2.2 Numerical Evaluation Matrix

After the creation of metrics, the design team created a numerical evaluation matrix to use these metrics to pick a final design. The numerical evaluation matrix is a systematic means to choosing the best means to perform each function and to

choose the overall best design. This allows for an unbiased way to choose the best design and the best combination of designs. The full chart showing the numerical evaluations is shown in Appendix E: Numerical Evaluation Matrix.

Each row in the matrix contains an objective or a constraint, while the columns contain different designs for each function. The teams' matrix had the constraints in the top rows. These are answered either yes or no, and anything that has a no in the constraints is eliminated from contention. None of the design team's potential designs failed any of the constraints. Below the constraints are the objectives. These objectives all have metrics attached to them, which allow the scoring of individual parts of the device. These are then combined to make the best possible design. Although not every metric applied directly to every type of potential design, the metrics were applied evenly to everything. This resulted in some good designs and functions with low scores on certain metrics, but it evened out after everything was scored. To even the scores, they were normalized and weighted. In the end, several designs had very similar scores and a choice between a few designs was required.

Table 13: Morph Chart Scores for Various Closure Methods

Design	Score
Single Piece	313.00
Self-Adhesive Material	313.00
Snap	309.30
Post-n-Pin	309.25
Post-n-Twist	309.25
Twist-n-Lock	290.80
Waffle Iron	290.00
Screw-Top	248.50

This first category of designs being ranked was the various methods for keeping the device closed when in use. As seen in Table 13, many of the methods of closure were feasible and had very similar scores. These numbers are based off of metrics designed to test non-destructive imaging, efficiency, easy to use, precise and to minimize the risk for contamination. The highest scoring designs were the single piece, the self-adhesive material, the post-n-pin, post-n-twist, and the snap. These all scored close enough that they were assumed to be approximately equal. The design team chose the winning design from these based on feasibility and simplicity. The post-n-pin was the simplest concept to produce. The self-adhesive device and single piece device had major complexities to overcome. With the self-adhesive device, finding material to seal adequately and release on command was problematic. The single piece had concerns because of the difficulty of coming up with a design that would form an adequate seal on the graft. The post-n-pin was chosen as the best design because of its simplicity, perceived effectiveness, and availability of adequate materials.

Table 14: Facilitate Changing of Media Means

Design	Score
Access from the top for manual media change	291.33
Continuous Perfusion	253.17
Drip and Drain System	258.17
Remove Device from Well	255.67

The second category ranked was the various systems to empty and replace media to nourish the cells. There were only four ideas for media change and they are shown in Table 14. With a score of 291.33, emptying the media from the top was a

clear winner. It was more feasible than the drip and drain and continuous perfusion, while less contaminating than the removal of the device. After going through the matrix, emptying the media from the top was a clear choice.

Table 15: Facilitate Air- Liquid Interface Culture

Design	Score
Air-Liquid In Device	313.00
Device Partially Disassembles for Air-Liquid	283.33
Graft Completely Removed for Air-Liquid	283.33

This next category was the culturing of the graft at air-liquid interface, shown in Table 15. In the current system, the device must be disassembled to perform air-liquid interface culture. With a score of 313, culture in the device is regarded as the best solution. Performing air-liquid in the device would be easier and have less risk of contamination caused by movement of the graft. Because of the nature of the device, if air liquid interface culture in the device were possible, it would also be possible to remove the graft and culture it following the current procedure. After consultation with the client and the user it was determined that it would also be acceptable to maintain their current air-liquid interface protocol as it had been proven effective. Therefore our device simply had to allow for the removal of the graft so that it could be moved to the air-liquid interface.

Table 16: Keep Cells from Sticking to Device Means

Design	Score
Teflon-like Gasket	313.00
Punch Out	301.25

This category was to deal with the problem of cell overgrowth onto the device. Two ideas were put forward, the Teflon gasket and the punch out. The results are shown in Table 16. The Teflon gasket worked by providing a surface outside the graft that cells could not adhere to and the punch out worked like a cookie cutter and cut out the graft without regard of overgrowth. With a score of 313, the Teflon gasket was chosen as the better idea. A big reason the gasket was chosen is because of the need to machine a custom tool for the punch out.

Table 17: Provide Structural Support for Graft Means

Design	Score
Stainless Steel Screen	291.33
Polymer Screen	287.58

The next category is the structural support for the graft, shown in Table 17. The two choices were a stainless steel screen and a polymer screen. With a slight edge the steel screen won with a score of 291.33. There was not much difference between the two, but the stainless steel screen is already used and is known to work well. The only main advantage of the polymer screen is it is softer and works better in tandem with the punch out device.

4.2.3 Alternative Designs

In the creation of alternative designs, one means is selected for each function and they are combined to form one alternative design. These combinations of ideas lead to designs that will meet all the required functions and objectives of the device. The alternative designs that we created focused on different methods of holding the device together due to the close scoring of these sections. We considered a single

piece design, a self-adhesive material design, a post-and-pin design, and a post-and-twist design. They all utilized a Teflon-like gasket, stainless steel screen, round shape, and manual changing of media. The single piece was deemed infeasible because it would not be able to successfully form a seal over the surface of the graft. The self-adhesive material design was deemed infeasible because of concerns about the material providing enough force to create a seal on the surface of the graft. The post-and-pin was selected because it is a simple system that is objectively easy to use. It would also allow for greater ease of manufacturing than the other methods.

4.3 Selected Design

The selected design for the skin graft culturing device utilizes the mechanism of the "Post-n-Pin" system. It can be seen in Figure 40. The top and bottom portion of the device, composed of Delrin, have an outer diameter of approximately 3.3 cm to accommodate a 6-well tissue culture plate. The hole in the center of the top is approximately 1.3 cm by 1.7 cm to accommodate the graft.

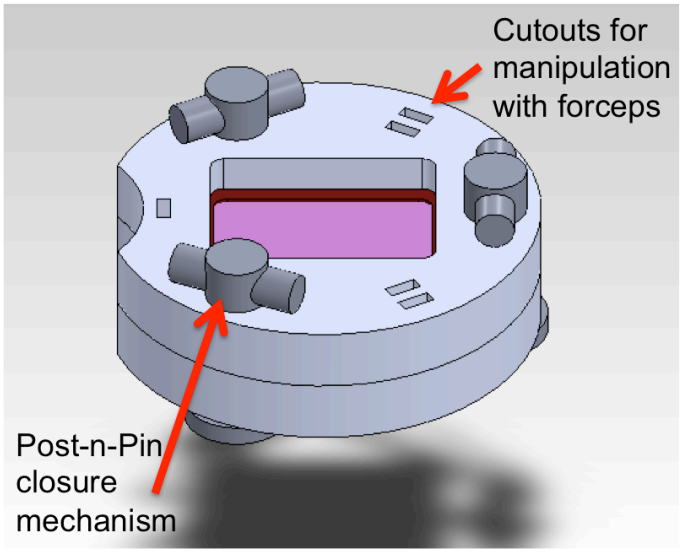

Figure 40: Solidworks model of selected device design

Three posts extend through the device and the holes in the posts allow for insertion of pins to lock the device together. The heads of the posts on the bottom allow for approximately 2 mm of clearance between the bottom of the device and the petri dish to allow media to flow and to not cause suction upon aspiration. This is shown in Figure 41.

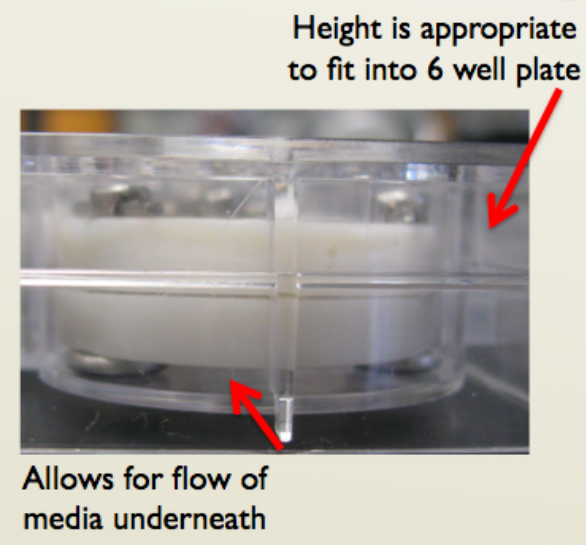

Figure 41: Side view of device

The assembly of the device will result in a seal around the top of the graft. This will allow for more efficient seeding of keratinocytes onto the graft. Additionally, after the grafts are grown, the user will be able to remove the pins with forceps and disassemble the device without contamination of the graft.

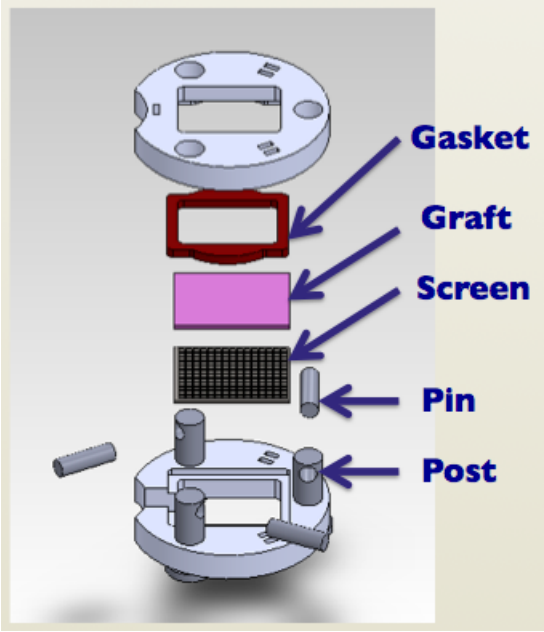

Figure 42: Exploded view of Solidworks model

One of the distinct features of this device is that the top and bottom portions of the device contain several cutouts. Each set of two allows for the insertion of forceps to easily remove the top portion of the device without contamination of the graft. Another feature of the device is the semicircular cutout on the side of the device to allow for both aspiration and replacement of media in the petri dish. These features are pointed out in Figure 43.

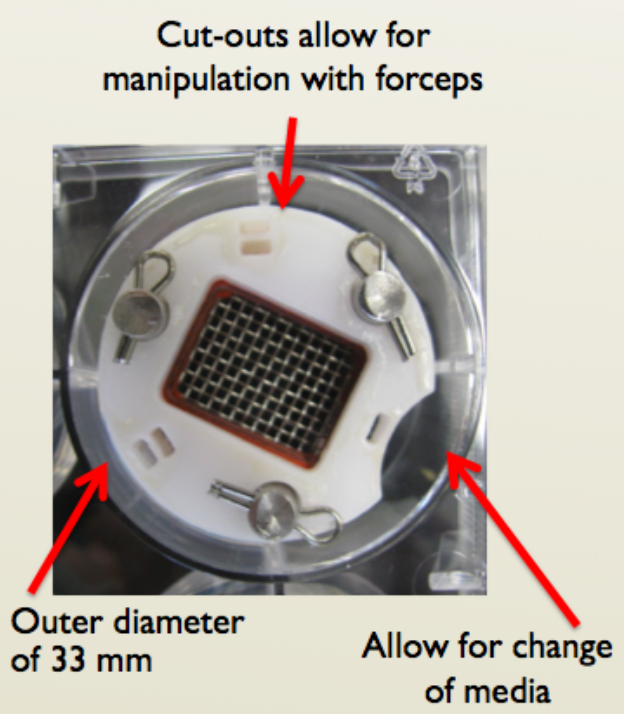

Figure 43: Top view of device

The inner portion of the device features a silicone rubber gasket 2.3mm in thickness. This material was chosen due to its good biocompatibility and the ability to be autoclaved. The gasket allows a seal to be created around the graft. This creates a well on the top surface of the graft, which makes seeding of keratinocytes easier. The screen adds mechanical support and stability to the graft during the culturing process and is made from grade 316 surgical stainless steel. This screen is porous enough to permit the diffusion of media within the device, while still providing adequate mechanical support.

The Delrin parts were made using a combination of laser cutting and CNC machining. The main outline and cutouts were made using laser cutting. The ledges upon which the screen and gasket would sit were cut using a CNC machine. The outlines of the silicone rubber gaskets were made using the laser cutter, and they

were then cut by hand with a razor blade. The screens were cut by hand using metal snips. The pins were also cut to the appropriate length using metal snips. The specifications for the raw materials used are shown in Table 18.

Table 18: Raw Material Specifications

Material	Dimensions	Vendor	Part Number
Delrin- Acetal Sheet, ASTM D6100, White	3/16" thick 12"x12" sheet	Small Parts	SDE-0187-C
Pins- 316 Stainless Steel Cotter Pin,	1/16" Diameter 1/2" Length	McMaster Carr	98355A010
Posts- 316 Stainless Steel	3/16" diameter 1/2" length	Grainger	1WGF8
Gasket -FDA-Compliant Silicone Rubber, Plain Back, 60A Durometer	12"x12" sheet 3/32" thick	McMaster Carr	86045K123
Screen- Particle-Sifting Woven Wire Cloth, Type 316	20x20 mesh .014" wire diameter 12"x12" sheet	McMaster Carr	9238T704

4.3.1 Teflon-like Gasket

One of the issues with the previous device was that cells could begin to grow onto the gasket. This could cause for destruction of the graft and separation of the two layers upon disassembly of the device. In order to solve this problem, a number of options were researched.

As the title implies, the first material considered was Teflon. Unfortunately for this application it was necessary to have a flexible and compressible gasket, and we could not find a Teflon product that meets these requirements. Some Teflon-coated gaskets do exist, however not in the shape or form necessary for our application.

The next idea researched was that a coating could be applied to silicone rubber to change the properties and decrease chance of cells adhering to the gasket. One coating that is commonly used is parylene. This is a biocompatible coating that is hydrophobic and resistant to cell adhesion (Fortin, 2003). There are however some major drawbacks to the use of this material. First, the process for coating is relatively complicate and expensive. Additionally the coating is only physically bonded and on a flexible surface such as our gaskets, this could lead to cracking of the surface, making it essentially useless (Surface Solutions Group, 2012).

Another family of commonly used materials are silicone lubricants. These materials decrease friction and do not inhibit the mechanical properties of the silicone rubber. This material, however, has a few drawbacks as well (Surface Solutions Group, 2012). First, it is expensive, and second as a liquid it could migrate from the gasket. This migration could result in contamination of the grafts and therefore this is not an ideal solution.

A newer material that shows potential for this type of application is called Slick Sil, manufactured by Surface Solutions Group, LLC. This material is designed specifically as a friction reducing coating for silicone rubber. It is designed to match the mechanical properties of silicone rubber, and flex with the material. (Surface Solutions Group, 2012). The main drawback of this material is that it is not available as a coating, and is only done as a contract coating operation. For the relatively small number of parts we will be using as well as the constraints of our budget, this option was deemed unfeasible.

4.3.2 Assembly Tools

Through initial assembly testing there were a number of issues that the design team addressed through the inclusion of additional assembly tools. The first problem encountered was in lining up the posts for assembly. It was determined that it would be beneficial to be able to align the posts so that the Delrin pieces could simply by slide over them. For this reason the team designed the assembly guide shown in Figure 44.

Figure 44: PDMS Assembly Guide

Through the suggestion of the user it was decided to create the guide out of PDMS. This material was already used in the lab, is sterilizable, and reusable. The design team created a mold made from ABS plastic utilizing the WPI rapid prototyping machine. This mold is shown in Figure 45. The use of this mold would allow for new assembly guides to be made simply and reproducibly when necessary.

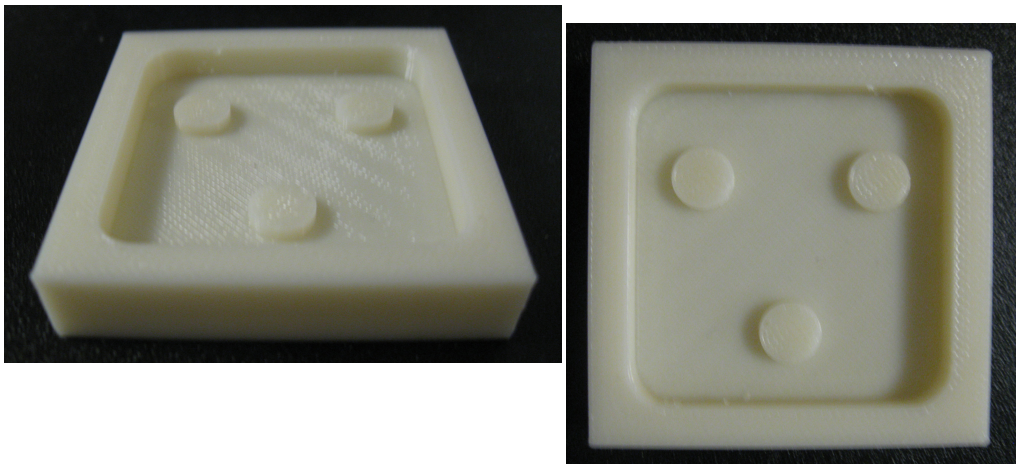

Figure 45: ABS negative mold for the assembly guide

When designing the mold for the assembly guide, there was some discussion about what the best diameter and depth of the holes. To determine this, the mold shown in Figure 46 was created. This had five different combinations of depth and height. Through consultation with the client and user, the best depth and height were chosen.

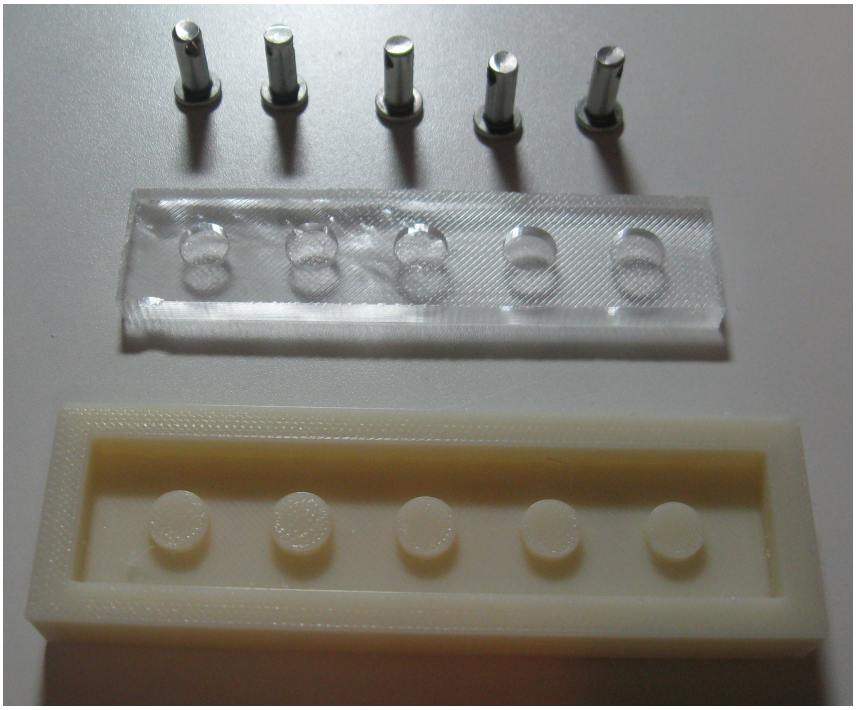

Figure 46: Top to bottom- posts, test guide, test guide mold

The inclusion of the assembly guide made this process simpler. It also decreased the likelihood of contamination caused by the additional handling that was initially necessary during assembly.

The second issue discovered during assembly is that a mechanism was needed to provide compression of the gasket while the pins were inserted through the posts. Initially the easiest way to provide this compression was using the hands. After consultation with Professor Pins and Amanda, it was determined that this would not be acceptable because touching the device would compromise its sterility. This necessitated a sterilizable tool with which to provide this compression. This would eliminate the need to touch the device with the hands.

The first attempts at providing this compression were done using forceps and a previous generation of the device, however applying pressure on only part of

the device made it prone to flipping. This uncontrolled flipping of the device is not acceptable as it could cause contamination or other damage to the graft. For this reason a tool was needed that would provide uniform compression around the device. The current version of this tool is shown in Figure 47.

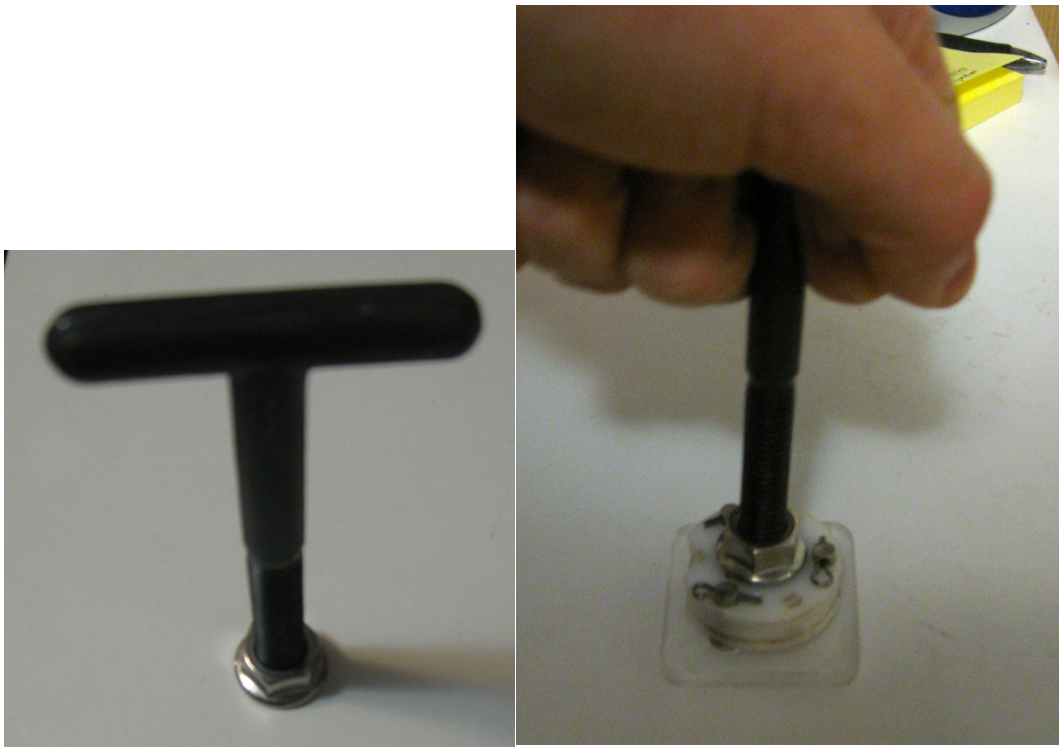

Figure 47: Compression Tool- alone on left, in use on right

This tool provides equal and adequate compression to the gasket, while at the same time eliminating the need to touch the device with the fingers. However, this tool could not be autoclaved, and therefore was unsuitable.

Upon further testing with the newest version of the device and the assembly guide, it was determined that pressure with forceps would be sufficient. Due to more careful machining of the parts, less force was needed for assembly, which solved the previously encountered flipping issue.

One issue that is inherent in this process is that seeding is much easier with a seal around the graft, however when the device is submerged, air cannot be trapped up against the graft. To prevent air from being trapped when keratinocytes were being seeded, a cutout had to be made in the bottom piece of the device to allow adequate flow of medium and air. This is shown in Figure 48.

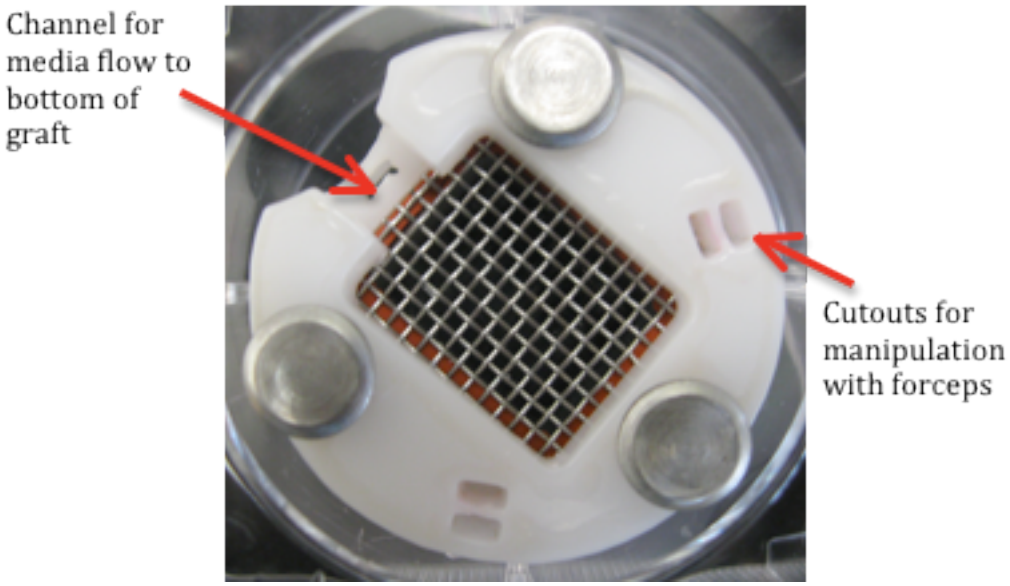

Figure 48: Bottom view of device

As an attempt to solve this problem, the design team came up with a few possible solutions. The first two ideas are shown in Figure 49. On the right, the center portion of this would be cut out. This would then serve as a removable gasket to hold in the media during seeding. On the left, the bulk of the piece will be removed, leaving only the piece that will plug the channel cut into the bottom piece of the device. This removable gasket only blocks the major opening from which the cells and media would escape.

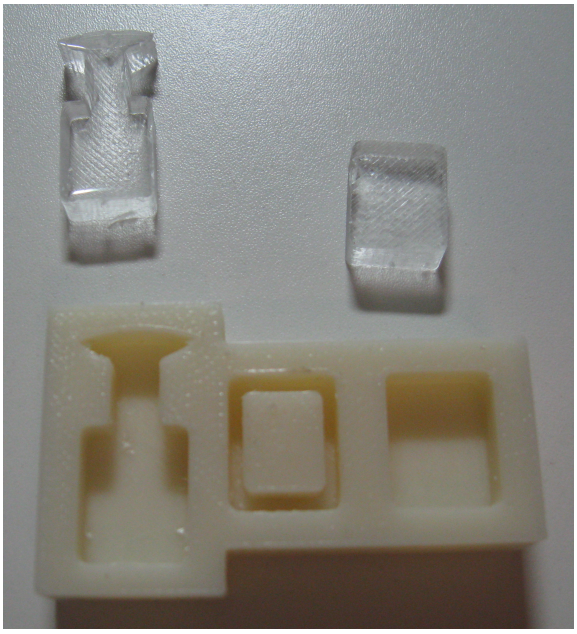

Figure 49: Removable gaskets and mold

4.3.3 Design Iterations

Throughout the process of arriving at this design, several changes were made to improve the eventual function of the device. Before an actual prototype was built the design team decided to have a rapid prototype made. This prototype is shown in Figure 50. After handling the rapid prototyped parts it was clear that the dimensions would need to be changed. The posts and pins proved to be too small to manipulate, and the ledge onto which the screen would be placed was not wide enough. Without enough surface area on the ledge, it would be difficult to create a sufficient seal between the gasket and the graft.

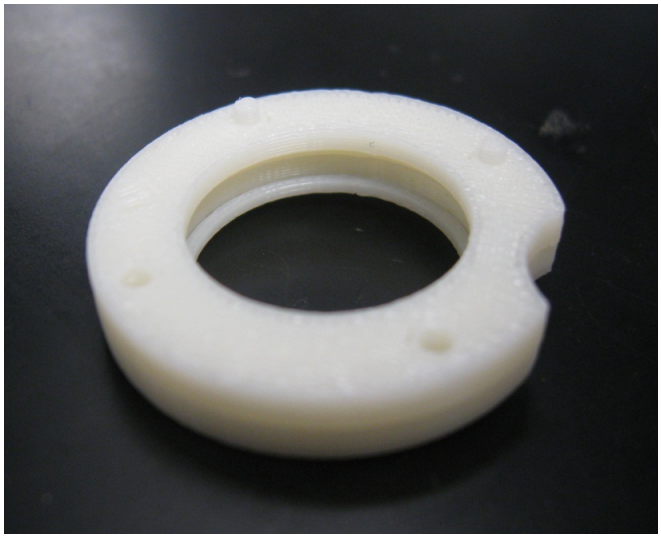

Figure 50: Rapid protoyped device

Incorporating the changes discussed after inspection of the rapid prototyped devices, the updated CAD drawings are shown in Figure 51. Upon further discussion, it was decided that the opening on the top of the device and the gasket should be changed to the rounded rectangle it is in the current device, as seen in Figure 40. This would better accommodate the shape and size of the grafts for which the device is being designed. Additionally the post-holes were made larger to accommodate the size of commercially available posts of this type.

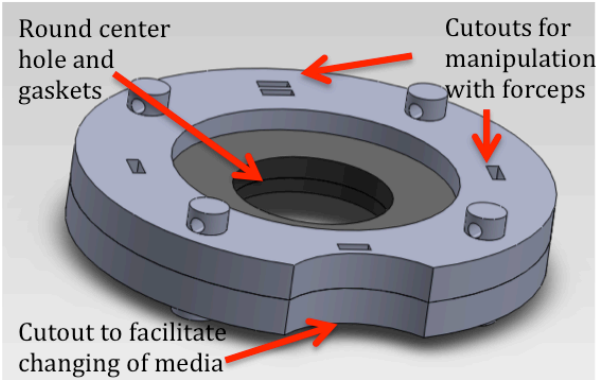

Figure 51: Solidworks model of pervious design iteration

Upon beginning the laser cutting of the first generation device it was discovered that the design of the forceps cut outs was causing problems. They were too close together, and too close to the edges of the pieces. This caused melting together of the holes and made them structurally unstable. To account for this, the holes were enlarged to their final size and moved to their final location.

After the first generation devices were fully assembled and testing began, it was clear that additional changes needed to be made. The outer diameter of the device was close enough to that of the petri dish that it did not fit easily. For this reason, the diameter was reduced from 34mm to 33mm. The final change made was the decision to switch from four posts to three. This idea was tested and determined to be faster, while still maintaining the same sealing ability as the four-post device.

Another problem recognized after initial testing was that the size of the opening would not be large enough to accommodate the grafts sufficiently. The change from 4 pins to 3 allowed for the inclusion of a larger seeding window on the top of the device. A comparison of the previous generation of our device and the current generation is shown in Figure 52. This comparison clearly demonstrates the change in size of the center hole as well as the change from four posts to three.

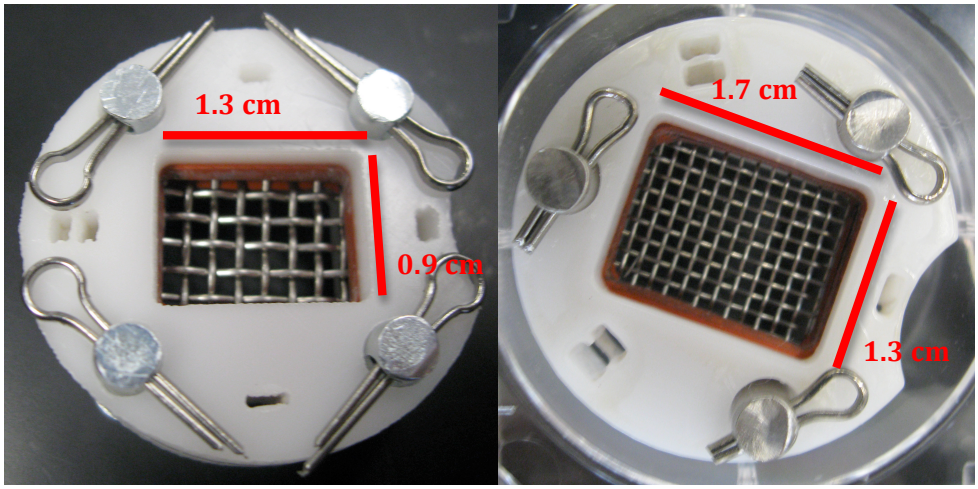

Figure 52: Left- Previous generation of our device, Right- Current generation

4.4 Selected Image Processing System

The chosen approach to automated image analysis of the skin graft sections is a combination of CellProfiler and MATLAB application. CellProfiler performs cell segmentation on the marked fluorescence micrographs, individualizing the relevant objects and recording the object data. CellProfiler's pipeline and module configurations load the red, green, and blue channel images to be analyzed, thus allowing for identification of cell membranes, filtering of these membranes based on correlation with p63 nuclear transcription factor marker, and output of object data. The object data obtained from CellProfiler can be overlaid onto and correlated with the analyzed image in MATLAB, which will record average pixel intensity and centroid position for each object. The MATLAB script will produce a bar graph of average pixel intensity of each object versus the related x-coordinate of the centroid of each object, resulting in an intensity plot in the x-direction of each object. The objects overlaid onto the image will then be color-coded based on the level of average pixel intensity of each object, divided into terciles, and the total average

intensity data for each object is output into Excel file format. Additionally, the script marks cell membranes that have a matching p63 marker according to CellProfiler, and notes whether that object is of stem-cell status or not.

A CellProfiler pipeline and module configuration was established by loading the red, green, and blue channel images. First, the dermal-epidermal junction of the original image is isolated in order to define the region within cell membranes will be analyzed as shown in Figure 53.

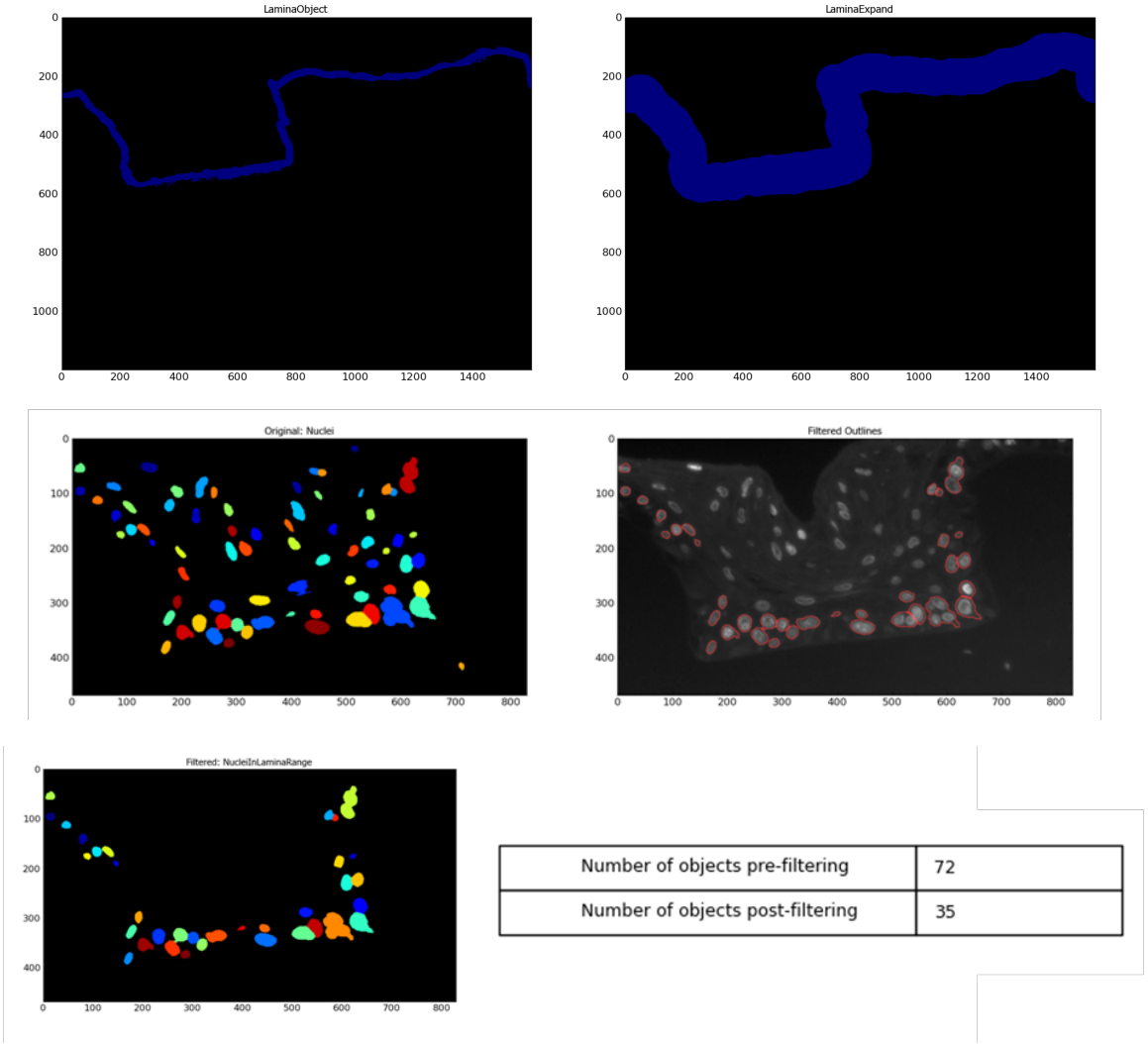

Figure 53: Top row: Expansion of laminar range. Right: boundary, Left: Expanded boundary layer. Middle row: identified cell nuclei. Left: original, Right: Identified by layer. Bottom row: filtered cell nuclei within laminar range.

The blue channel images were thresholded and segmented to identify the cell nuclei in the selected area. These cell nuclei were used as seeds from which the nucleus and cytoplasm of each cell could be identified in the red channel images. These nucleus-and-cytoplasm objects represent each whole cell in the red channel image minus the cell membrane. Next, the nucleus-and-cytoplasm objects were enlarged in CellProfiler by three to four pixels to define the entirety of the cells in the image, including the cell membrane, thereby defining whole cell objects. Isolating the cell membranes was achieved by subtracting the nucleus-and-cytoplasm objects from the whole cell objects leaving behind only the cell membranes as the final objects. An example of these objects outlined on the original image can be seen in Figure 54.

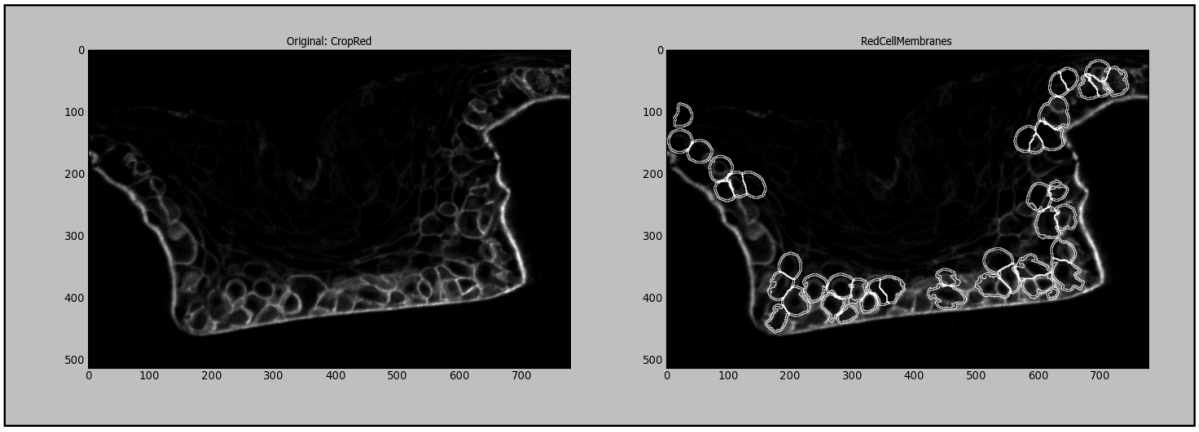

Figure 54: CellProfiler image of cell membrane objects overlaid onto the input image.

The MATLAB script developed for analyzing the object and image data produced by CellProfiler utilizes the regionprops() command to extract centroid and average pixel intensity for each object as compared to the input image. By extrapolating and compiling the x-position data of the centroid for each object, the MATLAB script can optionally plot the x-coordinate value versus the average

intensity for each object to produce an x-direction intensity plot for each cell in the image, as shown in Figure 55.

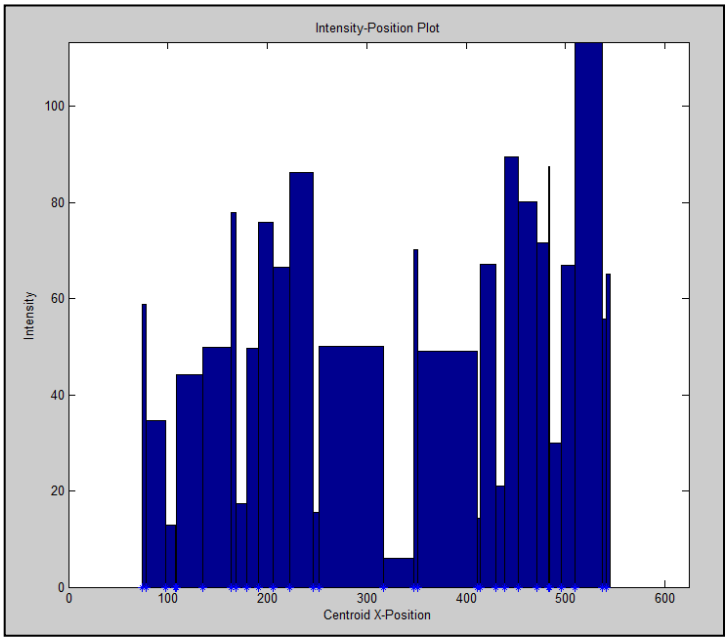

Figure 55: Intensity Position Plot generated by MATLAB script for the objects found by CellProfiler.

After compiling the data for average pixel intensity per object, each object can be divided into three groups based on their qualification as a low, medium, or high intensity object. Concatenating and normalizing the image data created from the three groups of objects can create a reproduction of the original image, with cell membranes outlined and color coordinated with blue being high intensity, green being medium intensity, and red being low intensity. The resultant image shows qualitative evidence of higher average expression near the dermal-epidermal junction. A comparison of images before and after analysis is shown in Figure 56. This image highlights the presence of high β1 expression near the bottom of the well, while the cells that are present in the higher non-well area generally have low

expression. Additionally, it notes whether or not a cell membrane is associated with presence of p63 factor, as denoted by the pink dot.

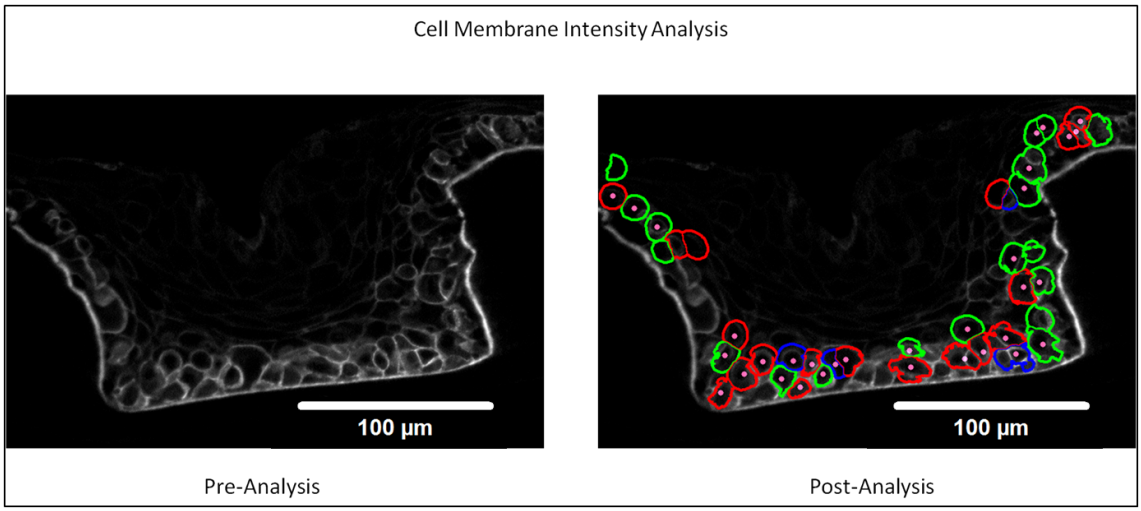

Figure 56: Comparison of epithelial stem cell well images before and after analysis. Red represents β1 bright, green represents β1 mid, blue represents β1 dim, and pink dot represents p63+.

This intensity data is recorded onto an Excel file unique to each image analyzed, which can then be used for statistical analysis. Therefore, this MATLAB and CellProfiler combination results in a strong quantitative set of data based off of qualitative information and images. An example of this is shown in Table 19.

Table 19: Example of resultant Excel chart of average cell intensities and stem cell status.

Object Number	X Coordinate	Y Coordinate	Average Intensity	Stem Cell Status
1	696.4876712	30.88767123	31.52876712	Yes
2	651.4169381	44.88273616	13.81758958	Yes
3	728.0446097	52.50743494	44.67472119	Yes
4	628.6472393	59.00613497	11.30674847	Yes
5	698.1978417	60.1618705	51.01438849	Yes
6	676.3057143	63.85714286	23.75142857	Yes
7	26.59360731	106.8789954	2.901826484	No
8	627.4352159	102.1013289	32.910299	Yes
9	622.0656109	141.0791855	41.42533937	Yes
10	574.952	142.7493333	11.216	No

5 Design Verification

In order to ensure that our design met all of needs of the clients, a number of tests needed to be performed. These tests would test the individual components of the design as well as the function of the design as a whole. The results from our testing are shown in Tables 20-24. The tests are discussed in detail in the following sections.

Table 20: Assembly Results

Old Device		New Device	
Operator	*Time (s)*	*Operator*	*Time (s)*
1	141	1	124
	89		126
	120		120
	110		79
2	140	2	171
	136		170
	142		160
	134		151
Average	**126.5**	Average	**137.6**
Std Dev	16.7	Std Dev	29.4

Table 21: Disassembly Results

Old Device		New Device	
Operator	*Time (s)*	*Operator*	*Time (s)*
1	145	1	32
	209		38
	160		45
	190		42
2	189	2	27
	162		27
	167		24
	180		27
Average	**175.3**	Average	**32.8**
Std Dev	18.1	Std Dev	7.4

Table 22: Flip Test Results

Old Device		New Device	
Operator	*Time (s)*	*Operator*	*Time (s)*
1	18	1	10
	18		6
	17		10
	18		7
2	14	2	10
	19		4
	15		8
	18		6
Average	**17.1**	Average	**7.6**
Std Dev	1.6	Std Dev	2.2

Table 23: Gasket Test Results

Old Device		New Device	
5 Minutes	No Leakage	5 Minutes	No Leakage
1 Hour	No Leakage	1 Hour	No Leakage

Table 24: Media Test Results

Old Device		New Device	
Media Change	Pass	Media Change	Pass
Media Contacting Bottom of Graft	Pass	Media Contacting Bottom of Graft	Pass

5.1 Well Creation

In order for efficient and even seeding, it is important for the device to create a well on the surface of the graft that maintains a watertight seal without leakage. To test this aspect of the device, a piece of chicken skin was used to simulate an actual skin graft and colored water was used to simulate media. The entire device

and experiment was tested inside a petri dish for a better simulation. The chicken skin was placed in the device on the screen and assembled normally. Then approximately 1-2 ml of the colored water was placed in the upper well. The assembled device is then left in the petri dish for two hours or until the seal breaks (water leaks out). In the early versions of the new device, the seal only lasted about 2 minutes before failure. The subsequent generations of the device, both three and four hole, have all passed this test. The current device was also tested and passed the test as well. The success of this test is paramount for the device because if a tight seal is not achieved, the ability to seed cells would be severely diminished.

5.2 Autoclavability

Another important aspect of the device is the ability to be autoclaved. All the materials used in the device are confirmed to be autoclavable by various research articles and the information posted by the part manufacturers and it was confirmed that none of the materials selected would fail after a short span of time due to autoclaving. For this reason we determined it unnecessary to perform our own tests specifically for autoclavability. However, all components were autoclaved for the completion of design validation testing, and no adverse effects were observed.

5.3 Simulated Use

One of the main reasons for this project was to improve the ease of use of the device. In order to do this, we simulated use of the device throughout the most important aspects of the process.

5.3.1 Assembly Test

The first of the tests that were preformed was the assembly test. In this test, the device is assembled in the same way it would be done in the lab for starting a skin graft. This test was not done in a biosafety cabinet due to the fact that it is typically not assembled in that environment. The test is repeated four times to obtain an average and the times are recorded. A current device was also tested in the same manner and five times were recorded for its assembly as well. After repeated assemblies of the two devices, the new device had consistently faster times than the old device. The mean for the new device had no statistically significant difference from the old device.

5.3.2 Disassembly Test

The next test for the simulation of use is the disassembly test. The device is disassembled in the same way it would be done in the lab. The time is recorded after each trial and four trials were preformed per person. This test mimicked a sterile environment. The device was required to remain in a larger petri dish. In addition to that, the hands could not come into contact with the device, or any part of the tools that will contact the device. Finally, the hands should pass over the device as little as possible. In disassembly, the new device was 435% faster. As with the first test, the standard deviations were large to account for adapting to the devices. The higher times associated with the old device are due to the difficulties with turn and manipulating the small screws holding the device together. This is especially true for the testers, having little to no experience with this device

previously. However, the test has shown that the new device is significantly faster to take apart which allows the device less time exposed to open air and thus lowers the risk of contamination.

5.3.3 Flip Test

When actually culturing the skin graft, the device must be flipped over to allow growth on both sides. When being turned over, the device is exposed to open air. This exposure should be minimized as much as possible to lessen the likelihood of contamination. To flip the device, it is first taken out of the six-well plate it was housed in and placed onto a sterile field (petri dish). Next the device is flipped upside down rested back onto the petri dish. Finally, the device is picked up and placed back into the six-well (now upside down). The time was recorded after each test and was repeated four times. The device is also only handled with forceps or other sterile tools. This test was relative quick and easy to perform and the improvement on the current device was not as pronounced as the previous tests. The average time to flip the current device was 17.25s with a 1.9s standard deviation, while the new device had an average time of 13.5s with a 4.92s standard deviation. Flipping of the post-n-pin device was approximately 124% faster than the current device, which is very important to minimizing contamination.

5.3.4 Medium Change

Another important test for the device design is that it is able to have its medium aspirated efficiently. For this test, the device was assembled and placed into a six-well plate. Approximately 5-6 ml of colored water was placed into the well

to simulate the addition of media. The water was added with a serological pipet. After the well is full and water is settled into it, the "medium" is aspirated out of the well with a Pasteur pipet. The primary objective of this test was to complete these tasks without the pipets contacting the device or the side of the well causing contamination. The ability of the device to be aspirated is crucial because if the media cannot be aspirated sterilely, then the device will either be contaminated or it will run out of media causing the cells die from malnourishment. Initially the device was too big to fit the pipet past it. It was subsequently modified to have a bigger recess cut into the front of the device big enough to allow the pipets easy access.

5.3.5 Medium Contact

The last test that was performed to simulate the use of the device was to test the medium contact on the bottom of the device when submerged in medium. This test is very important because if medium does not wet the bottom of the device the cells will starve. For proper feeding of the graft, the medium must touch the bottom of the graft. The device is assembled with a piece of chicken skin to simulate the actual graft and was placed into a petri dish. Approximately 100 ml of colored water was added to the petri dish until it was almost completely submerged. If the water has contacted the bottom of the skin, it will be dyed with the food coloring. The assembled device was submerged in the water and left to sit for approximately 20 minutes. When the time is up, the water is aspirated out and the bottom is checked for wetting. The first round of these tests failed. There was zero wetting on the bottom of the devices. The problem was quickly discovered. In the initial design of

the device, the group utilized a double gasket both above and below the screen and graft. This bottom gasket created a well effect under the water therefore trapping a bubble under the device. This prevented the water from contacting the bottom surface at all. In the next round of testing, the bottom gasket was removed and the bottom portions of skin turn red due to the dye. The initial failed test led the team to slightly redesign and improve the device. The current design now only has top gaskets, and passed the test.

5.4 Statistics

Statistical analysis was conducted using a one-way student t-test utilizing StatPlus: mac LE. This test allows the means of two populations to be compared to determine statistical significance. This test assumes that the variances of each set of data are not equal. The null hypothesis is that there is no difference between the means of the two populations. For each test we performed, n=8. *p*-values ≤.05 were considered statistically significant. (AnalystSoft, 2010). The detailed results of the statistical analysis can be seen in Appendix F: Statistical test results.

6 Final Design Validation

The function of the final device was validated by seeding and culturing cells in the device. For these tests only fibroblasts were utilized. Keratinocytes were not used do to limited availability and prohibitively high cost. It was determined through suggestion of the client and user that the use of fibroblasts in place of keratinocytes to validate seeding would not sufficiently affect the results.

6.1 Experimental Design

Two different test groups and two controls were prepared to demonstrate the seeding effectiveness. One test group involved seeding onto the epidermal side of the graft in a new device. The second test group involved seeding onto the dermal side of the graft in a new device. As a control, cells were also seeded onto grafts using the old device. After culturing for approximately 48 hours to ensure the cells attached, the grafts were analyzed using microscopy to ensure seeding.

After the grafts had been analyzed, the devices were disassembled and the grafts were removed. They were then moved to the air-liquid interface where they were cultured for an additional 7 days. At this point it would be apparent if the grafts had become contaminated during the process.

6.2 Results

In the first test performed, human dermal fibroblasts were utilized. Due to changing culture conditions between the laboratory space in which the cells were originally cultured and the space in which the testing was going to take place, the initial seeding density was much lower than expected. Additionally, due to inexperience in use with the old devices, we were not able to hold a seal for the

control test, and therefore, this seeding was inhibited. Because there were fewer cells than expected and the controls failed, no significant conclusions could be made.

The test was then repeated using NIH/3T3 mouse fibroblasts. Approximately 48 hours after seeding, the grafts were photographed using a Zeiss AxioCamMR3. A comparison between the control, seeding on the bottom of the new device, and seeding on the top of the new device is shown in Figure 57. Based upon these images, nearly complete coverage was observed for several regions on each graft. Qualitatively, there is very little difference between the observations from the control and the test groups.

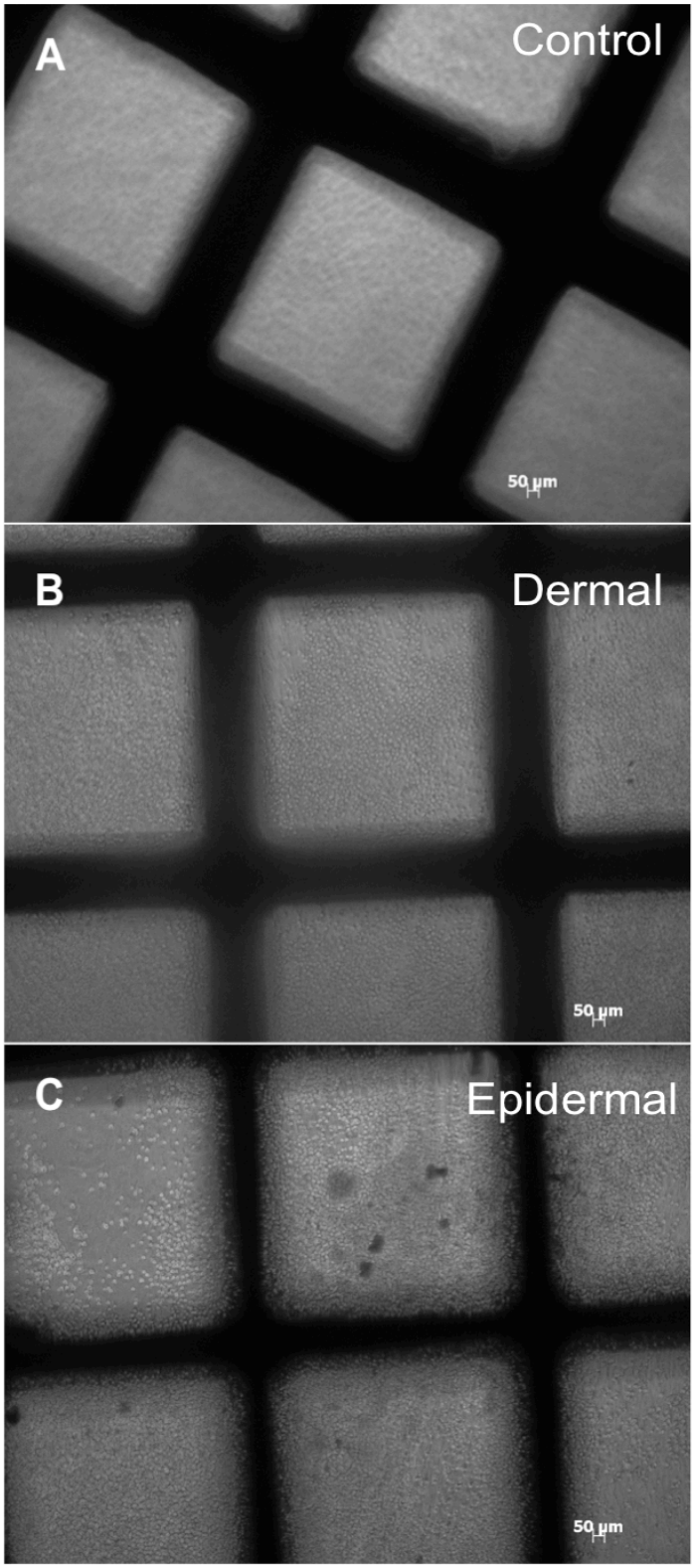

Figure 57: Images of seeded fibroblasts at 4X after 48hrs. The black shadows are shadows from the screen on which the grafts are cultured. (A) Control, (B) New device seeded on the bottom, (C) New device seeded on the top.

The design team decided to extend the initial seeding study for an additional 24 hours as an additional assurance that our device would support the growth and continued attachment of cells. Figure 58 shows an example of the sustained cell concentration after any remaining suspended cells had been aspirated out of the wells. The presence of cells at this time point proves that our device does not prevent the growth of cells.

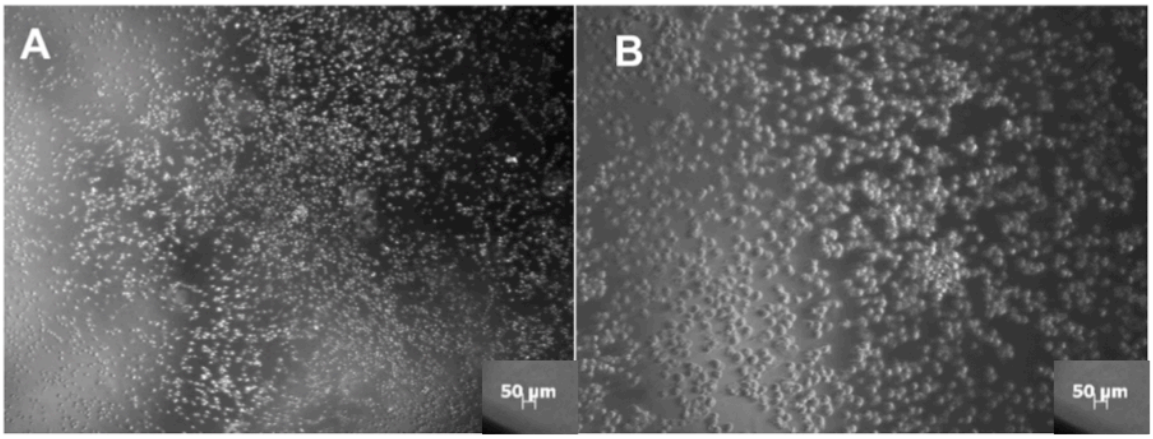

Figure 58: Image of a graft in new device at 72 hrs. (A) 4X magnification, (B) 10X Magnification

The purpose of the contamination validation was to prove that our device allowed for disassembly and removal of a graft without the introduction of contamination. In the first test, when the grafts were removed they were moved to the air liquid interface and all placed in different wells of a six well plate. Because of this, when one well became contaminated, the entire plate had to be disposed of, destroying the rest of the specimens. The design team determined that this contamination was likely due to inexperience in tissue culture, and not an innate problem with the device.

This test was also repeated using the samples from the second seeding test after that test was concluded. To prevent the destruction of additional samples

unnecessarily, each sample for this test was placed in an individual p35 petri dish. For easier handling, multiple p35s were then placed inside larger p150 plates. This would mean that if a single sample became contaminated, only that sample would have to be discarded, and not the whole plate.

After 7 days, cultures still remained uncontaminated. This proves that our system can be assembled and disassembled without contamination. This result is even more significant due to the fact that the team had very little tissue culture experience, therefore, in the hands of a more experienced user the risk of contamination should be even less.

7 Discussion

7.1 Project Discussion

The purpose of this design project was to create an improved device in which skin grafts and potentially other types of grafts could be cultured. The selected design for the device allows for a more user-friendly interface that does not compromise the graft in conjunction with decreasing the amount of disassembly time. This new device met one of the three objectives and functions that the team established prior to design. The first objective of this project was to create a device that minimized the handling time associated with cell culture, allow for uniform cell seeding/creates a watertight seal, be able to remove graft without destruction, and minimized the amount of manufacturing time. This objective was met with various components of the final design.

Minimizing the handling time associated with cell culture of a skin graft was achieved by the Post-n-Pin technology, which allows the user to utilize forceps and remove the pins that reside within each post. This design is a great advancement over the previously used system, which required the user to use an Allen wrench to unscrew four screws within a sterile environment without directly contacting the device. By having pins and cutouts on the device, it can be manipulated, assembled, and disassembled with forceps, severely decreasing the amount of time required by the user. Also, the use of the custom assembly guide allows for a quicker, easier assembly of the device.

Allowing for uniform cell seeding was accomplished by utilizing a silicone gasket and establishing the overall height of the device so that a watertight seal

could be made around the graft. When the graft is placed inside of the device, the silicone gasket makes a tight seal creating a well on the surface of the graft. This allows for the seeding of keratinocytes on the top of the scaffold and keeping them from spreading to the rest of the device. To this point, the problem of creating a sealed well on the dermal side has not yet been completely solved.

Allowing for the removal of the graft without destruction was achieved also by the Post-n-Pin technology in conjunction with the overall shape of the device. The Post-n-Pin technology allows the pins to be removed as well as the stainless steel screen to be removed by forceps, leading to less contamination. The overall screen shape and size allows for easy removal without contamination.

Minimizing manufacturing time was accomplished by utilizing a simple design in which several components can be purchased as well as using materials that could be created with faster tooling, such as a laser cutter and CNC machine. The design for this device is comprise of three stainless steel posts, pins, and screen that were purchased. The two Delrin pieces as well as the two silicone gaskets were the only pieces that were machined, leading to less manufacturing time.

7.2 Impact Analysis

Due to the fact that this device will be utilized by not only the Pins lab but possibly several other labs, it is important to determine the political, ethical, societal, environmental ramifications as well as understand the impact on health and safety, sustainability and manufacturability.

7.2.1 Economics

Due to the fact that the ongoing research for which the device will be utilized is limited to *in vitro* research within the lab, there is virtually no economical impact. If the process utilized in the lab were to be used as a treatment for diabetic ulcers or burn victims, then there would be an increased demand for these devices. The increase of available treatment could also lead to an increase of cost for insurance companies as well as patients seeking this form of treatment. One of the benefits of the device is its low cost to manufacture, helping to alleviate some of the possible financial burdens associated with these forms of treatment. The cost per device was approximately $4.68. The specific breakdown by piece is shown in Table 25: Cost analysis

Table 25: Cost analysis

	Pieces per unit	Unit Price	Price per piece	Pieces per device	Cost per device
Delrin	49	$22.96	$0.47	2	$0.94
Gasket	144	$16.57	$0.12	1	$0.12
Screen	400	$13.80	$0.03	1	$0.03
Posts	10	$11.21	$1.12	3	$3.36
Pins	100	$7.60	$0.08	3	$0.23
					Total= $4.68

7.2.2 Environmental Impact

This device has a minimal effect on the environment. One of the negative impact is the use of collagen as a scaffold for cell seeding, which is derived primarily from bovine. Since this is a miniscule amount, there is virtually no negative impact to the environment from this device.

Another environmental impact would be the creation of the Delrin used in our device. Since Delrin is a form of plastic, which requires large forms of energy to create, this could have an impact on the environment. Additionally, one of them main components of Delrin is formaldehyde, which in its non-polymerized form can be harmful to human health. The assumption is that the manufacturers have in place ways to mitigate this risk effectively. Since this device uses no energy during use and requires only a small amount of Delrin, it has no major impact on the environment.

7.2.3 Societal Impact

This device has the potential to greatly impact society. The technology that this device will help develop will improve the quality of life for patients suffering from ulcers and burns. Decreasing the amount of time to use the device and limiting the amount of contamination can help create more grafts, leading to the increased amount of grafts ultimately made. Also, by shortening the time to assemble and disassemble, this device can assist in the efficiency within the laboratory. This increase in laboratory efficiency can speed up the innovation regarding these skin grafts that is being done within the Pins lab.

7.2.4 Political Ramifications

Due to the fact that the technology for which the device is utilized is in primarily a laboratory setting, there are virtually no political ramifications. The use of this device would be helpful in the culturing of cells within a graft within other

laboratories, but due to its confined setting it would have a relatively small impact on the global and international markets alike.

7.2.5 Ethical Concerns

The ethical concerns attributed to this device are minimal. This device is utilized for creating skin grafts to be used ultimately from patients suffering from diabetic ulcers and burns. One ethical concern toward this device is toward the origin of the collagen utilized as a scaffold for which the cells adhere to. Since collagen is derived primarily from bovine source, some might have an ethical concern regarding materials chosen for a scaffold.

One improvement in terms of ethics is the fact that skin grafts can be used in research in place of animal testing in some cases. For this reason, our device will actually eliminate some ethical concerns in other areas.

7.2.6 Health and Safety Issues

This project has the ability to greatly improve the health of patients suffering from diabetic ulcers and burns. This device helps to create a consistent skin graft in a quicker and more efficient process in order to improve the quality of life of the patient. The improvements made to this device eliminated all need for direct contact, negating possible instances for contamination that would compromise the safety of the patient.

7.2.7 Manufacturability

This device was designed to be very easy to reproduce, in regards to both manufacturability and assembly. With the SolidWorks models for the device as well as the correct machining, this device is easily recreated for a minimal cost. In addition, the tooling required for utilization of the device, including the assembly guide and compression tool, all experimentation conducted during design verification can be repeated. The chosen materials for our device are also readily available and have a small cost associated with them.

7.2.8 Sustainability

Due to the fact that the device needed to be created of a material that would not instigate a cellular reaction that could compromise the integrity of the graft, Delrin was chosen at it is cheap and would not cause any form of reaction. The downside of this is that Delrin is a plastic, which requires large forms of energy to create which could be a difficult material to sustain in the future. If this design project was intended on being sustainable, perhaps another type of material could have been chosen that also would not cause a cellular reaction. The device was additionally designed to be reusable. This decreases the need for constant production of devices and presents a more sustainable option than single-use devices would. Since this device uses no energy during use, is reusable, requires only a small amount of Delrin, there are no foreseeable impact regarding sustainability of the device.

8 Conclusions and Recommendations

The design concept for this device improves the skin graft culturing system by making the device more efficient, minimizing handling, and mitigating the risk of contamination. Additionally progress was made towards improving the current imaging and analysis methods. The device is an improvement of the current graft system with the introduction of the "Post-n-Pin" technology, the cutouts for both removal of graft and seal of device, and the elevated device from the petri dish to prevent the formation of suction upon removal of media. This system can accommodate a graft the size of 1-1.5cm x 2-2.5 cm, and has the ability to be autoclaved and reused, therefore minimizing production costs. The usage of the media will essentially be the same as the prior culturing device

Possibly the most important improvement in our device is the new post-and-pin closure mechanism. A major drawback with the previous system was the time and difficulty that was necessary to disassemble the device. This resulted in increased likelihood of contamination. Our improvements have led to a 435% decrease in the time that it takes to disassemble the device.

8.1 Miniaturization

When this project was undertaken, there was a difference in some minor final goals between the user, Amanda Clement, and the client, George Pins. One of these areas was in the area of size. Professor Pins wanted a device that would fit into a p35 tissue culture dish. This would allow each graft to be completely secluded from all the others, which would reduce the risk of cross contamination. Amanda had concerns over the use of individual p35 dishes because their small size could

make movement more problematic. She preferred the use of a 6-well tissue culture plate. The diameter difference between the two is negligible, and it is only the height that is substantially different.

In an attempt to build a device that would fit properly into either a p35 or a 6-well plate, the size of available posts ended up being a limiting factor. The smallest commercially available 316 stainless steel posts found after extensive searching are approximately 1.25 cm. For this reason, the device fits into a 6-well plate but not a p35. Eventually it could be beneficial in the future to miniaturize the closure mechanism to allow it to fit into a smaller plate.

8.2 Sealing Both Sides of the Graft

When the old version of the device was designed, there was no intention of being able to seed cells onto both sides of the device. When the device was adapted for use in the current process, it made seeding onto the bottom of the graft more difficult than on the top of the device do to the lack of a constrained area on the bottom of the device.

In designing the new device the design team attempted to create a device that sealed properly on both sides of the graft. After testing the devices, however it was found that sealing the bottom of the graft created additional problems. When the device was face up and media was introduced to the well, a bubble of air was being trapped under the graft. When the device is in this configuration, there are fibroblasts on the bottom of the graft that need to be nourished by the media, which is impossible if the media cannot contact the graft because of air entrapment. This is a serious problem because if the fibroblasts are not nourished, they will die.

Due to these issues the design team considered a number of possible solutions. It was decided that the only feasible solution short of a complete redesign was removing the bottom gasket. This solved the problem, however means that seeding on the bottom of the graft is still not ideal. The design team began development of a removable gasket composed of PDMS as outlined in the design chapter. Sufficient testing, however, has not been done to confirm this function. A future improvement to the device, therefore, to further test this solution, or to establish an alternative method by which the bottom of the device can create seal for seeding purposes, while allowing air to escape during other functions.

8.3 Automation

During the initial portion of the design process, the team discussed the possibility of creating a continuous perfusion system. Currently, changing media in the grafts must be done daily. This involves the aspiration of old media and the addition of new media. This process could be made easier by switching to a continuous perfusion system. This would mean that the medium would be either continuously cycled, or added and removed automatically at prescribed times. This idea, however, was deemed to be outside of the scope of the current project. It is possible that this could be implemented in the future as well.

8.4 In-Device Longitudinal Imaging

Another aspect of the project that was not fully addressed was the ability to assess progress of the devices in process. Currently imaging is an end-state process and cannot be done without destroying the grafts. Therefore, a future improvement will be to incorporate longitudinal imaging within the device into the process.

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Appendices

Appendix A: Client Interview Questions With Answers

1. What type of scaffold are the cells cultured on? (material)

   The cells are cultured on either basal lamina analogs or composites containing basal lamina analogs. The basal lamina analog is a rat tail tendon type I collagen gel (final concentration is ~8mg-ml created from neutralizing a 10mg-ml solution). It is optically clear and can be micropatterned with various topographies.

   The composites are created by adhering a porous collagen sponge (bovine, similar to Integra-DRT) to a basal lamina analog. The sponges are porous, but not optically clear. Imaging through them is difficult, but there is some penetration. Keratinocytes are seeded on the basal lamina analog and fibroblasts can be seeded in the sponge, which functions as a dermal analog. If you would like to see samples of these, you should come see me in the lab. We also culture cells on acellular dermis and opaque collagen/GAG membranes.

   See pages 105 and 115 of Katie Bush's dissertation (available at http://www.wpi.edu/Pubs/ETD/Available/etd-012909-194611/) for more method details.

2. What should the cost per device be?

   This will depend in part on whether you decide to do a reusable, partially reusable or disposable device. Ideally I think the cost per graft should be no

more than $1-2. So if the device could be reused 10 times, then $10-20 each would be reasonable.

However, a KEY component associated with the cost of the device is the time/labor to make new devices. A $1 device that takes a week's work of time to make does not help us.

3. Should the devices be reusable or disposable?

   Either. There are certainly advantages and disadvantages to both. The key is a trade of with the cost per device.

4. How big should the device the (dimensions)?

   The device needs to hold our grafts which are ~1-1.5 cm x ~2-2.5 cm. It needs to fit inside an available tissue culture product or function as its own isolator. The small the device, relative to the graft, the better it is. Bigger tissue culture devices take up more space and use more media.

5. Should the device be designed for use in a six well plate, or an individual petri dish?

   Ideally an individual petri dish. 6 well plates are a concern because if one well gets contaminated, it's not isolated from your other grafts – so an entire plate is compromised. Individual petri dish can be a pain to transport to and from the incubator, especially because they are shallower than 6 well plates. I'm not tied to either product. If you device is compatible with another commercially available and comparably priced tissue culture product, that better meat your objectives, that's fine.

6. Is there a specific material that the device should be made out of?

No. But the device should be biocompatible and easily sterilizable (preferably autoclavable).

7. Is there a minimum volume of medium that we need to contain in the scaffold?

   Currently, the grafts are cultured in ~5 ml of media prior to going to air liquid and ~3.5-4.5 ml of air liquid media. The media needs to be changed daily. As such, without a bioreactor system to exchange the media automatically, I would be hesitant to use less.

8. Is there a maximum volume of medium that should be used?

   Not a specific number, but media is expensive, so media waste should be minimized. There is an upper limit on volume relative to the height of the scaffolds. If the scaffolds are submerged in excessive quantities of media, it will inhibit oxygen diffusion to the cells.

9. How often are the current devices moved during the whole culturing process, and what is the ideal amount of movement during the process?

   Grafts are removed from the incubator and the media is exchanged once per day. On the day they move from submerged to A/L culture, there is additionally handling during the media exchange. I generally try to avoid moving them any more than I have to. Especially once the grafts are at the A/L, because at that point it is critical to keep media from sloshing onto the surface of the grafts. The current devices are also handled once to facilitate seeding of fibroblasts into dermal components, before flipping over to seed with keratinocytes.

10. What kind of media is used in the process, and what kind of nutrients are contained in it?

    We use a total of 4 different types of media throughout the keratinocyte growth and graft creation process.

    Briefly:

    1. Keratinocytes plated in "Keratinocyte Media w/o EGF" (2 days)

    1b. Keratinocytes fed and maintained with "Keratinocyte Media + EGF" (3-5 days)

    2. Keratinocytes harvested and seeded on grafts in "Seeding Media" (1 day)
    3. Grafts fed with "Priming Media" (2 days)
    4. Grafts moved to air-liquid and fed with "A/L Media" (up to 14 days, may go out to 28)

    Detailed media formulations can be found on pages 108-109 of Katie Bush's thesis.

    Briefly, the base of all of the medias is a 3:1 mixture of DMEM/Hams F12. Keratinocyte media contains 10% serum, seeding media contains 1% serum, priming media contains 1% serum + BSA, and A/L media is serum free. Both priming media and A/L media contain a mixture of fatty acids to facilitate barrier formation.

Appendix B: Pairwise Comparison Responses

Professor Pins-

Prof. Pins- Main Objectives						
	Minimize opportunity for contamination	Non-Destructive	Efficient	Accurate and Precise	Easy To Use	Totals
Minimize opportunity for contamination	X	1.0	1.0	1.0	1.0	4.0
Non-Destructive	0.0	X	0.0	0.0	0.0	0.0
Efficient	0.0	1.0	X	0.5	0.5	2.0
Accurate and Precise	0.0	1.0	0.5	X	0.5	2.0
Easy To Use	0.0	1.0	0.5	0.5	X	2.0

Prof. Pins- Efficient Sub- Objectives						
	Compact relative to graft size	Cost of $1-2 per graft	Optimize media use	Minimal processing and handling time	Minimize manufacturing time of device	Totals
Compact relative to graft size	X	1.0	1.0	0.0	1.0	3.0
Cost of $1-2 per graft	0.0	X	0.0	0.0	0.0	0.0
Optimize media use	0.0	1.0	X	0.0	1.0	2.0
Minimal processing and handling time	1.0	1.0	1.0	X	1.0	4.0
Minimize manufacturing time of device	0.0	1.0	0.0	0.0	X	1.0

Prof. Pins- Easy to use Sub- Objectives						
	Minimal Processing and handling time	Easy to assemble	Easy to change media	Autoclavable	Have each device fit into its own petri dish	Totals
Minimal Processing and handling time	X	0.5	0.5	1.0	1.0	3.0
Easy to assemble	0.5	X	0.5	1.0	1.0	3.0
Easy to change media	0.5	0.5	X	1.0	1.0	3.0
Autoclavable	0.0	0.0	0.0	X	0.0	0.0
Have each device fit into its own petri dish	0.0	0.0	0.0	1.0	X	1.0

Prof. Pins- Minimize Opportunity for contamination Sub objectives				
	Have each device fit into its own petri dish	Minimize handling	Allow only filtered air into device	Totals
Have each device fit into its own petri dish	X	0	0	0
Minimize handling	1	X	0.5	1.5
Allow only filtered air into device	1	0.5	X	1.5

Prof. Pins- Non- Destructive Sub-objectives			
	Non-destructive quantitative imaging	Graft Can be removed from device intact	Totals
Non-destructive quantitative imaging	X	0	0
Graft Can be removed from device intact	1	X	1

Prof. Pins- Precision Sub-Objectives			
	Reproducible Results	Allow for uniform cell seeding	Totals
Reproducible Results	X	0.5	0.5
Allow for uniform cell seeding	0.5	X	0.5

Amanda-

Amanda- Main Objectives						
	Minimize opportunity for contamination	Non-Destructive	Efficient	Accurate and Precise	Easy To Use	Totals
Minimize opportunity for contamination	X	1	1	1	0.5	3.5
Non-Destructive	0	X	0.5	0	0	0.5
Efficient	0	0.5	X	0	0.5	1
Accurate and Precise	0	1	1	X	1	3
Easy To Use	0.5	1	0.5	0	X	2

Amanda- Efficient Sub- Objectives						
	Compact relative to graft size	Cost of $1-2 per graft	Optimize media use	Minimal processing and handling time	Minimize manufacturing time of device	Totals
Compact relative to graft size	X	1	1	0	1	3
Cost of $1-2 per graft	0	X	0.5	0	0	0.5
Optimize media use	0	0.5	X	0	0	0.5
Minimal processing and handling time	1	1	1	X	0.5	3.5
Minimize manufacturing time of device	0	1	1	0.5	X	2.5

Amanda- Easy to use Sub- Objectives						
	Minimal Processing and handling time	Easy to assemble	Easy to change media	Autoclavable	Have each device fit into its own petri dish	Totals
Minimal Processing and handling time	X	0.5	1	1	1	3.5
Easy to assemble	0.5	X	1	1	1	3.5
Easy to change media	0	0	X	1	1	2
Autoclavable	0	0	0	X	1	1
Have each device fit into its own petri dish	0	0	0	0	X	0

Tim-

Tim- Main Objectives						
	Minimize opportunity for contamination	Non-Destructive	Efficient	Accurate and Precise	Easy To Use	Totals
Minimize opportunity for contamination	X	1	1	0	1	3
Non-Destructive	0	X	1	0	1	2
Efficient	0	0	X	0	1	1
Accurate and Precise	1	1	1	X	1	4
Easy To Use	0	0	1	0	X	1

Tim- Efficient Sub- Objectives						
	Compact relative to graft size	Cost of $1-2 per graft	Optimize media use	Minimal processing and handling time	Minimize manufacturing time of device	Totals
Compact relative to graft size	X	0	0	0	0	0
Cost of $1-2 per graft	1	X	0	0	0	1
Optimize media use	1	1	X	0	0	2
Minimal processing and handling time	1	1	1	X	1	4
Minimize manufacturing time of device	1	1	1	0	X	3

Tim- Easy to use Sub- Objectives						
	Minimal Processing and handling time	Easy to assemble	Easy to change media	Autoclavable	Have each device fit into its own petri dish	Totals
Minimal Processing and handling time	X	1	0	1	1	3
Easy to assemble	0	X	0	1	1	2
Easy to change media	1	1	X	1	1	4
Autoclavable	0	0	0	X	0	0
Have each device fit into its own petri dish	0	0	0	1	X	1

Tim- Minimize Opportunity for contamination Sub objectives				
	Have each device fit into its own petri dish	Minimize handling	Allow only filtered air into device	Totals
Have each device fit into its own petri dish	X	0	0	0
Minimize handling	1	X	1	2
Allow only filtered air into device	1	0	X	1

Tim- Non- Destructive Sub-objectives			
	Non-destructive quantitative imaging	Graft Can be removed from device intact	Totals
Non-destructive quantitative imaging	X	0	0
Graft Can be removed from device intact	1	X	1

Tim- Precision Sub-Objectives			
	Reproducible Results	Allow for uniform cell seeding	Totals
Reproducible Results	X	1	1
Allow for uniform cell seeding	0	X	0

Lauren-

Lauren- Main Objectives						
	Minimize opportunity for contamination	Non-Destructive	Efficient	Accurate and Precise	Easy To Use	Totals
Minimize opportunity for contamination	X	1	1	0.5	1	3.5
Non-Destructive	0	X	1	0.5	1	2.5
Efficient	0	0	X	0	1	1
Accurate and Precise	0.5	0.5	1	X	1	3
Easy To Use	0	0	0	0	X	0

Lauren- Efficient Sub- Objectives						
	Compact relative to graft size	Cost of $1-2 per graft	Optimize media use	Minimal processing and handling time	Minimize manufacturing time of device	Totals
Compact relative to graft size	X	0	1	0	1	2
Cost of $1-2 per graft	1	X	1	0.5	1	3.5
Optimize media use	0	0	X	0	0	0
Minimal processing and handling time	1	0.5	1	X	1	3.5
Minimize manufacturing time of device	0	0	1	0	X	1

Lauren- Easy to use Sub- Objectives						
	Minimal Processing and handling time	Easy to assemble	Easy to change media	Autoclavable	Have each device fit into its own petri dish	Totals
Minimal Processing and handling time	X	0	0	1	1	2
Easy to assemble	1	X	.5	1	1	3.5
Easy to change media	1	.5	X	1	1	3.5
Autoclavable	0	0	0	X	.5	.5
Have each device fit into its own petri dish	0	0	0	.5	X	.5

Rebecca-

Rebecca- Main Objectives						
	Minimize opportunity for contamination	Non-Destructive	Efficient	Accurate and Precise	Easy To Use	Totals
Minimize opportunity for contamination	X	0	1	1	0	2
Non-Destructive	1	X	1	1	1	4
Efficient	0	0	X	1	1	2
Accurate and Precise	0	0	0	X	0	0
Easy To Use	1	0	0	1	X	2

Rebecca- Efficient Sub- Objectives						
	Compact relative to graft size	Cost of $1-2 per graft	Optimize media use	Minimal processing and handling time	Minimize manufacturing time of device	Totals
Compact relative to graft size	X	1	0	0	1	2
Cost of $1-2 per graft	0	X	1	1	1	3
Optimize media use	1	0	X	0	1	2
Minimal processing and handling time	1	0	1	X	1	3
Minimize manufacturing time of device	0	0	0	0	X	0

Rebecca- Easy to use Sub- Objectives						
	Minimal Processing and handling time	Easy to assemble	Easy to change media	Autoclavable	Have each device fit into its own petri dish	Totals
Minimal Processing and handling time	X	0	0	1	1	2
Easy to assemble	1	X	0.5	1	1	3.5
Easy to change media	1	0.5	X	0	1	2.5
Autoclavable	0	0	1	X	1	2
Have each device fit into its own petri dish	0	0	0	0	X	0

Rebecca- Minimize Opportunity for contamination Sub objectives				
	Have each device fit into its own petri dish	Minimize handling	Allow only filtered air into device	Totals
Have each device fit into its own petri dish	X	0	0	0
Minimize handling	1	X	1	2
Allow only filtered air into device	1	0	X	1

Rebecca- Non- Destructive Sub-objectives			
	Non-destructive quantitative imaging	Graft Can be removed from device intact	Totals
Non-destructive quantitative imaging	X	0	0
Graft Can be removed from device intact	1	X	1

Rebecca- Precision Sub-Objectives			
	Reproducible Results	Allow for uniform cell seeding	Totals
Reproducible Results	X	0.5	0.5
Allow for uniform cell seeding	0.5	X	0.5

Dan-

Dan- Main Objectives						
	Minimize opportunity for contamination	Non-Destructive	Efficient	Accurate and Precise	Easy To Use	Totals
Minimize opportunity for contamination	X	0	0	0	0	0
Non-Destructive	1	X	0	1	1	3
Efficient	1	1	X	1/2	0	2.5
Accurate and Precise	1	0	1/2	X	1/2	2
Easy To Use	1	0	1	1/2	X	2.5

Dan- Efficient Sub- Objectives						
	Compact relative to graft size	Cost of $1-2 per graft	Optimize media use	Minimal processing and handling time	Minimize manufacturing time of device	Totals
Compact relative to graft size	X	1	0	0	1/2	1.5
Cost of $1-2 per graft	0	X	0	0	0	0
Optimize media use	1	1	X	1	1	4
Minimal processing and handling time	1	1	0	X	1/2	3.5
Minimize manufacturing time of device	1/2	1	0	1/2	X	2

Dan- Easy to use Sub- Objectives						
	Minimal Processing and handling time	Easy to assemble	Easy to change media	Autoclavable	Have each device fit into its own petri dish	Totals
Minimal Processing and handling time	X	0	0	1	1	2
Easy to assemble	1	X	1/2	1	1	3.5
Easy to change media	1	1/2	X	1	1	3.5
Autoclavable	0	0	0	X	1/2	.5
Have each device fit into its own petri dish	0	0	0	1/2	X	.5

Dan- Minimize Opportunity for contamination Sub objectives				
	Have each device fit into its own petri dish	Minimize handling	Allow only filtered air into device	Totals
Have each device fit into its own petri dish	X	0	0	0
Minimize handling	1	X	0	1
Allow only filtered air into device	1	1	X	2

Dan- Non- Destructive Sub-objectives			
	Non-destructive quantitative imaging	Graft Can be removed from device intact	Totals
Non-destructive quantitative imaging	X	0	0
Graft Can be removed from device intact	1	X	1

Dan- Precision Sub-Objectives			
	Reproducible Results	Allow for uniform cell seeding	Totals
Reproducible Results	X	1	1
Allow for uniform cell seeding	0	X	0

Appendix C: Final Metrics

1. Minimize Opportunity for Contamination- Weight 32%
    a. Have each device fit into it's own petri dish- measured by what it can fit into
        1. Requires specialized housing
        2. Fits into 6 well plate
        3. Fits into p35 and 6 well plate
    b. Minimize Handling
    c. Allow only filtered air into device
        1. Graft exposed to outside air
        2. Graft exposed only to filtered air
2. Precise- Weight 24%
    a. Reproducible results- based on device not interfering with process
        1. Device causes damage to graft
        2. Device is difficult to use/ prone to mistakes that damage graft
        3. Device does not interfere with the process
    b. Allow for uniform cell seeding- measured by access to graft
        1. Device must be disassembled for seeding
        2. Partial access to graft for seeding
        3. Complete access to graft for seeding
3. Easy to use- 18%
    a. Minimize processing and Handling time- Measured by exposure to unfiltered air compared to current system
        1. Exposed to air longer than current system
        2. Exposed to air the same amount as current system
        3. Exposed to air less than current system
    b. Easy to assemble- based on assembly steps
        1. 9+ steps to assemble
        2. 7 steps to assemble
        3. 5 steps to assemble
        4. 3 steps or less to assemble
    c. Easy to change media- based on access to media
        1. Device must be removed to change media
        2. Device must be partially disassembled to change media
        3. Unobstructed access to media
    d. Autoclavable- ease of sterilization

1. Cannot be sterilized
2. Can be sterilized by a method other than autoclaving
3. Can be autoclaved

4. Efficient- 15%
    a. Compact relative to graft size- measured by amount of hood space it takes up
        1. Device takes up excessive amount of space
        2. Device takes up minimal amount of space
    b. Optimize media use
        1. Excessive amount of media used
        2. Greater than 5ml of media at a time
        3. Approximately 5 ml of media at a time
    c. Minimize processing and handling time- Measured by time spent manipulating device compared to current device
        1. More time handling than current device
        2. Same time handling as current device
        3. Less time handling than current device
    d. Minimize manufacturing time of device
        1. More than 2 weeks to produce
        2. More than 1 week to produce
        3. More than 1 day to produce
        4. 1 Day or less to produce
    e. Cost of $1-2 per graft- Measured by estimate of cost per graft
        1. Much more than $1-2 per graft
        2. $1-2 or less per graft
5. Non- Destructive- 11%
    a. Non-destructive quantitative imaging- destroyed or not
        1. Graft is destroyed in order to be imaged
        2. Graft is not destroyed
    b. Graft can be removed intact- destroyed or not
        1. Graft is destroyed during removal
        2. Graft is not destroyed during removal

Appendix D: Initial Metrics

1. Have each device fit into its own individual petri dish
    a. 100: fits into p35, 50: fits into six well plate, 0: requires production of specialized housing
2. Watertight seal
    a. Yes/No
3. Non-destructive quantitative imaging
    a. 100: Non-destructive, 50: Destructive, 0: Does not allow for imaging
4. Minimal Handling
    a. 100: 2 or less moves of device from start to finish, 50: 3 or more moves, 0: 5 movements

5. Compact relative to graft size
    a. 100: Efficient height/width/depth, 50: Maximum height/width/depth, 0: More than Maximum
6. Optimize media use
    a. 100: 5 mL of media, 50: >5 mL of media, 0: extremely large amount of media OR <5 mL
7. Minimal processing and handling time
    a. 100: 30 sec or less, 50: 1 min or less, 0: more than 1 min
    b. Time of exposure to air, based on use by a novice
8. Minimize manufacturing time of device
    a. 100: One day or less, 50: One week, 0: 3 or more weeks
9. Cost of $1-2 per graft
    a. 100: $1 or less per graft, 50: $2 or less, 0: more than $2
10. Allow for uniform cell seeding (accessibility)
    a. 100: Unobstructed graft OR 2 or less steps to uncover graft, 50: partially obstructed OR 3 steps to uncover graft, 0: totally obstructed OR more than 3 steps to uncover graft
11. Easy to assemble
    a. 100: 3 steps or less for assembly, 50: 5 steps or less for assembly, 0: more than 5 steps for assembly
12. Easy to change media
    a. 100: Less than one minute for aspiration and replacing media, 50: 90 sec or less, 0: More than 90 sec
13. Autoclaveable (if reusable)
    a. 100: Autoclaveable, 50: Sterilizable by other method, 0: Not-Sterilizable
14. Able to be Imaged
    a. Number of steps- 100: Can be imaged as is, 50: Partial disassembly needed for imaging, 0: Full disassembly needed for imaging
    b. Destructivity- 100: Imaged in real- time, 50: Imaged at end-state non-destructively, 0: Imaged at end-state destructively

Appendix E: Numerical Evaluation Matrix

Hold Device Together			Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized
	Objectives (O) and Constraints (C)	Single Piece			Screw-top			Self- Adhesive material			press- fit			twist-n-lock			post-n-pin	
C	Must be sterilizable	Y			Y			Y			Y			Y			Y	
C	Must be cytocompatible	Y			Y			Y			Y			Y			Y	
C	Allows for the removal of the graft without damage	Y			Y			Y			Y			Y			Y	
C	Budget of $524	Y			Y			Y			Y			Y			Y	
C	Fit in currently available tissue culture product or be self-contained	Y			Y			Y			Y			Y			Y	
C	No more than $1-2 per graft	Y			Y			Y			Y			Y			Y	
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y			Y			Y			Y			Y	
C	Must be able to seed on both sides	Y			Y			Y			Y			Y			Y	
	Minimize Opportunity for Contamination		3	96		3	96		3	96		3	96		3	96		3
O	Have each device fit into its own petri dish	3	1		3	1		3	1		3	1		3	1		3	1
O	Minimize Handling	3	1		3	1		3	1		3	1		3	1		3	1
O	Allow only filtered Air into device	2	1		2	1		2	1		2	1		2	1		2	1
	Precise		2	48		1	24		2	48		2	48		1.67	40		2
O	Reproducible Results	3	1		2	0.6667		3	1		3	1		2	0.67		3	1
O	Allow for uniform seeding	3	1		1	0.3333		3	1		3	1		3	1		3	1
	Easy to Use		4	72		2.1667	39		4	72		4	72		3.42	61.5		4
O	Minimize processing and Handling time	3	1		1	0.3333		3	1		3	1		2	0.67		3	1
O	Easy to assemble	4	1		2	0.5		4	1		4	1		3	0.75		4	1
O	Easy to change media	3	1		1	0.3333		3	1		3	1		3	1		3	1
O	autoclavable	3	1		3	1		3	1		3	1		3	1		3	1
	Efficient		5	75		4.5	67.5		5	75		5	75		4.75	71.25		4.75
O	Compact relative to graft size	2	1		2	1		2	1		2	1		2	1		2	1
O	Optimize media use	3	1		3	1		3	1		3	1		3	1		3	1
O	Minimize processing and Handling time	3	1		3	1		3	1		3	1		3	1		3	1
O	Minimize manufacturing time of device	4	1		2	0.5		4	1		4	1		3	0.75		3	0.75
O	Cost of $1-2 per graft	2	1		2	1		2	1		2	1		2	1		2	1
	Non- Destructive		2	22		2	22		2	22		2	22		2	22		2
O	Non- destructive quantitative imaging	2	1		2	1		2	1		2	1		2	1		2	1
O	Graft can be removed intact	2	1		2	1		2	1		2	1		2	1		2	1
	TOTAL			313			248.5			313			313			290.8		

Hold Device Together			Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized
	Objectives (O) and Constraints (C)	Single Piece			Screw-top			Self- Adhesive material			press- fit			twist-n-lock			post-n-pin	
C	Must be sterilizable	Y			Y			Y			Y			Y			Y	
C	Must be cytocompatible	Y			Y			Y			Y			Y			Y	
C	Allows for the removal of the graft without damage	Y			Y			Y			Y			Y			Y	
C	Budget of $524	Y			Y			Y			Y			Y			Y	
C	Fit in currently available tissue culture product or be self-contained	Y			Y			Y			Y			Y			Y	
C	No more than $1-2 per graft	Y			Y			Y			Y			Y			Y	
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y			Y			Y			Y			Y	
C	Must be able to seed on both sides	Y			Y			Y			Y			Y			Y	
	Minimize Opportunity for Contamination		3	96		3	96		3	96		3	96		3	96		3
O	Have each device fit into its own petri dish	3	1		3	1		3	1		3	1		3	1		3	1
O	Minimize Handling	3	1		3	1		3	1		3	1		3	1		3	1
O	Allow only filtered Air into device	2	1		2	1		2	1		2	1		2	1		2	1
	Precise		2	48		1	24		2	48		2	48		1.67	40		2
O	Reproducible Results	3	1		2	0.6667		3	1		3	1		2	0.67		3	1
O	Allow for uniform seeding	3	1		1	0.3333		3	1		3	1		3	1		3	1
	Easy to Use		4	72		2.1667	39		4	72		4	72		3.42	61.5		4
O	Minimize processing and Handling time	3	1		1	0.3333		3	1		3	1		2	0.67		3	1
O	Easy to assemble	4	1		2	0.5		4	1		4	1		3	0.75		4	1
O	Easy to change media	3	1		1	0.3333		3	1		3	1		3	1		3	1
O	autoclavable	3	1		3	1		3	1		3	1		3	1		3	1
	Efficient		5	75		4.5	67.5		5	75		5	75		4.75	71.25		4.75
O	Compact relative to graft size	2	1		2	1		2	1		2	1		2	1		2	1
O	Optimize media use	3	1		3	1		3	1		3	1		3	1		3	1
O	Minimize processing and Handling time	3	1		3	1		3	1		3	1		3	1		3	1
O	Minimize manufacturing time of device	4	1		2	0.5		4	1		4	1		3	0.75		3	0.75
O	Cost of $1-2 per graft	2	1		2	1		2	1		2	1		2	1		2	1
	Non- Destructive		2	22		2	22		2	22		2	22		2	22		2
O	Non- destructive quantitative imaging	2	1		2	1		2	1		2	1		2	1		2	1
O	Graft can be removed intact	2	1		2	1		2	1		2	1		2	1		2	1
	TOTAL			313			248.5			313			313			290.8		

Facilitate Changing of Media			Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized	Weighted
	Objectives (O) and Constraints (C)	access from top for manual change			continuous perfusion			drip and drain system			remove device from well		
C	Must be sterilizable	Y			Y			Y			Y		
C	Must be cytocompatible	Y			Y			Y			Y		
C	Allows for the removal of the graft without damage	Y			Y			Y			Y		
C	Budget of $524	Y			Y			Y			Y		
C	Fit in currently available tissue culture product or be self-contained	Y			Y			Y			Y		
C	No more than $1-2 per graft	Y			Y			Y			Y		
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y			Y			Y		
C	Must be able to seed on both sides	Y			Y			Y			Y		
	Minimize Opportunity for Contamination		2.66666667	85.333333		2.33333333	74.666667		2.33333333	74.666667		2.33333333	74.666667
O	Have each device fit into its own petri dish	3	1		1	0.33333333		1	0.33333333		3	1	
O	Minimize Handling	2	0.66666667		3	1		3	1		1	0.33333333	
O	Allow only filtered Air into device	2	1		2	1		2	1		2	1	
	Precise		2	48		2	48		2	48		1.66666667	40
O	Reproducible Results	3	1		3	1		3	1		2	0.66666667	
O	Allow for uniform seeding	3	1		3	1		3	1		3	1	
	Easy to Use		3.66666667	66		3.66666667	66		3.66666667	66		3	54
O	Minimize processing and Handling time	2	0.66666667		2	0.66666667		2	0.66666667		2	0.66666667	
O	Easy to assemble	4	1		4	1		4	1		4	1	
O	Easy to change media	3	1		3	1		3	1		1	0.33333333	
O	autoclavable	3	1		3	1		3	1		3	1	
	Efficient		4.66666667	70		2.83333333	42.5		3.16666667	47.5		4.33333333	65
O	Compact relative to graft size	2	1		1	0.5		1	0.5		2	1	
O	Optimize media use	3	1		1	0.33333333		2	0.66666667		3	1	
O	Minimize processing and Handling time	2	0.66666667		3	1		3	1		1	0.33333333	
O	Minimize manufacturing time of device	4	1		2	0.5		2	0.5		4	1	
O	Cost of $1-2 per graft	2	1		1	0.5		1	0.5		2	1	
	Non- Destructive		2	22		2	22		2	22		2	22
O	Non- destructive quantitative imaging	2	1		2	1		2	1		2	1	
O	Graft can be removed intact	2	1		2	1		2	1		2	1	
	TOTAL			291.33333			253.16667			258.16667			255.66667

Facilitate Air/ Liquid Interface Culture			Normalized	Weighted		Normalized	Weighted		Normalized	Weighted
	Objectives (O) and Constraints (C)	In device			device partially disassembles			graft completely removed		
C	Must be sterilizable	Y			Y			Y		
C	Must be cytocompatible	Y			Y			Y		
C	Allows for the removal of the graft without damage	Y			Y			Y		
C	Budget of $524	Y			Y			Y		
C	Fit in currently available tissue culture product or be self-contained	Y			Y			Y		
C	No more than $1-2 per graft	Y			Y			Y		
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y			Y		
C	Must be able to seed on both sides	Y			Y			Y		
Contamination			3	96		2.66666667	85.333333		2.66666667	85.333333
O	Have each device fit into its own petri dish	3	1		3	1		3	1	
O	Minimize Handling	3	1		2	0.66666667		2	0.66666667	
O	Allow only filtered Air into device	2	1		2	1		2	1	
Precise			2	48		1.66666667	40		1.66666667	40
O	Reproducible Results	3	1		2	0.66666667		2	0.66666667	
O	Allow for uniform seeding	3	1		3	1		3	1	
Easy to Use			4	72		3.66666667	66		3.66666667	66
O	Minimize processing and Handling time	3	1		2	0.66666667		2	0.66666667	
O	Easy to assemble	4	1		4	1		4	1	
O	Easy to change media	3	1		3	1		3	1	
O	autoclavable	3	1		3	1		3	1	
Efficient			5	75		4.66666667	70		4.66666667	70
O	Compact relative to graft size	2	1		2	1		2	1	
O	Optimize media use	3	1		3	1		3	1	
O	Minimize processing and Handling time	3	1		2	0.66666667		2	0.66666667	
O	Minimize manufacturing time of device	4	1		4	1		4	1	
O	Cost of $1-2 per graft	2	1		2	1		2	1	
Non- Destructive			2	22		2	22		2	22
O	Non- destructive quantitative imaging	2	1		2	1		2	1	
O	Graft can be removed intact	2	1		2	1		2	1	
	TOTAL			313			283.33333			283.33333

Keep Cells From Sticking to Device			Normalized	Weighted		Normalized	Weighted
	Objectives (O) and Constraints (C)	teflon- like gasket			punch		
C	Must be sterilizable	Y			Y		
C	Must be cytocompatible	Y			Y		
C	Allows for the removal of the graft without damage	Y			Y		
C	Budget of $524	Y			Y		
C	Fit in currently available tissue culture product or be self-contained	Y			Y		
C	No more than $1-2 per graft	Y			Y		
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y		
C	Must be able to seed on both sides	Y			Y		
Contamination			3	96		3	96
O	Have each device fit into its own petri dish	3	1		3	1	
O	Minimize Handling	3	1		3	1	
O	Allow only filtered Air into device	2	1		2	1	
Precise			2	48		1.66666667	40
O	Reproducible Results	3	1		2	0.66666667	
O	Allow for uniform seeding	3	1		3	1	
Easy to Use			4	72		4	72
O	Minimize processing and Handling time	3	1		3	1	
O	Easy to assemble	4	1		4	1	
O	Easy to change media	3	1		3	1	
O	autoclavable	3	1		3	1	
Efficient			5	75		4.75	71.25
O	Compact relative to graft size	2	1		2	1	
O	Optimize media use	3	1		3	1	
O	Minimize processing and Handling time	3	1		3	1	
O	Minimize manufacturing time of device	4	1		3	0.75	
O	Cost of $1-2 per graft	2	1		2	1	
Non- Destructive			2	22		2	22
O	Non- destructive quantitative imaging	2	1		2	1	
O	Graft can be removed intact	2	1		2	1	
	TOTAL			313			301.25

Provide Structural Support for Graft			Normalized	Weighted		Normalized	Weighted
	Objectives (O) and Constraints (C)	stainless steel screen			polymer screen		
C	Must be sterilizable	Y			Y		
C	Must be cytocompatible	Y			Y		
C	Allows for the removal of the graft without damage	Y			Y		
C	Budget of $524	Y			Y		
C	Fit in currently available tissue culture product or be self-contained	Y			Y		
C	No more than $1-2 per graft	Y			Y		
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y		
C	Must be able to seed on both sides	Y			Y		
Contamination			2.66666667	85.333333		2.6666667	85.333333
O	Have each device fit into its own petri dish	3	1		3	1	
O	Minimize Handling	2	0.66666667		2	0.6666667	
O	Allow only filtered Air into device	2	1		2	1	
Precise			2	48		2	48
O	Reproducible Results	3	1		3	1	
O	Allow for uniform seeding	3	1		3	1	
Easy to Use			3.66666667	66		3.6666667	66
O	Minimize processing and Handling time	2	0.66666667		2	0.6666667	
O	Easy to assemble	4	1		4	1	
O	Easy to change media	3	1		3	1	
O	autoclavable	3	1		3	1	
Efficient			4.66666667	70		4.4166667	66.25
O	Compact relative to graft size	2	1		2	1	
O	Optimize media use	3	1		3	1	
O	Minimize processing and Handling time	2	0.66666667		2	0.6666667	
O	Minimize manufacturing time of device	4	1		3	0.75	
O	Cost of $1-2 per graft	2	1		2	1	
Non- Destructive			2	22		2	22
O	Non- destructive quantitative imaging	2	1		2	1	
O	Graft can be removed intact	2	1		2	1	
	TOTAL			291.33333			287.58333

Allow for non-destructive	imaging of	Normalized	Weighted		Normalized	Weighted		Normalized	Weighted		Normalized	Weighted
Objectives (O) and Constraints (C)	Image in device			Split model			slot model			window		
C Must be sterilizable	Y			Y			Y			Y		
C Must be cytocompatible	Y			Y			Y			Y		
C Allows for the removal of the graft without damage	Y			Y			Y			Y		
C Budget of $524	Y			Y			Y			Y		
C Fit in currently available tissue culture product or be self-contained	Y			Y			Y			Y		
C No more than $1-2 per graft	Y			Y			Y			Y		
C Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y			Y			Y		
C Must be able to seed on both sides	Y			Y			Y			Y		
Contamination		2.5	80		2.16666667	69.33333		2.16666667	69.333333		3	96
O Have each device fit into its own petri dish	3	1		3	1		3	1		3	1	
O Minimize Handling	3	1		2	0.66666667		2	0.66666667		3	1	
O Allow only filtered Air into device	1	0.5		1	0.5		1	0.5		2	1	
Precise		2	48		1.66666667	40		1.66666667	40		2	48
O Reproducible Results	3	1		2	0.66666667		2	0.66666667		3	1	
O Allow for uniform seeding	3	1		3	1		3	1		3	1	
Easy to Use		3.66666667	66		3.66666667	66		3.41666667	61.5		3.66666667	66
O Minimize processing and Handling time	2	0.66666667		2	0.66666667		2	0.66666667		2	0.66666667	
O Easy to assemble	4	1		4	1		3	0.75		4	1	
O Easy to change media	3	1		3	1		3	1		3	1	
O autoclavable	3	1		3	1		3	1		3	1	
Efficient		5	75		4.66666667	70		4.41666667	66.25		5	75
O Compact relative to graft size	2	1		2	1		2	1		2	1	
O Optimize media use	3	1		3	1		3	1		3	1	
O Minimize processing and Handling time	3	1		2	0.66666667		2	0.66666667		3	1	
O Minimize manufacturing time of device	4	1		4	1		3	0.75		4	1	
O Cost of $1-2 per graft	2	1		2	1		2	1		2	1	
Non- Destructive		2	22		2	22		2	22		2	22
O Non-destructive quantitative imaging	2	1		2	1		2	1		2	1	
O Graft can be removed intact	2	1		2	1		2	1		2	1	
TOTAL			291			267.3333			259.08333			307

Shape/ Form		circular	Normalized	Weighted	square	Normalized	Weighted	rectangular	Normalized	Weighted	triangular	Normalized	Weighted
	Objectives (O) and Constraints (C)												
C	Must be sterilizable	Y			Y			Y			Y		
C	Must be cytocompatible	Y			Y			Y			Y		
C	Allows for the removal of the graft without damage	Y			Y			Y			Y		
C	Budget of $524	Y			Y			Y			Y		
C	Fit in currently available tissue culture product or be self-contained	Y			Y			Y			Y		
C	No more than $1-2 per graft	Y			Y			Y			Y		
C	Must fit graft of size ~1-1.5 cm x ~2-2.5 cm	Y			Y			Y			Y		
C	Must be able to seed on both sides	Y			Y			Y			Y		
Contamination			2.66666667	85.333333		2.66666667	85.333333		2	64		2	64
O	Have each device fit into its own petri dish	3	1		3	1		1	0.33333333		1	0.33333333	
O	Minimize Handling	2	0.66666667		2	0.66666667		2	0.66666667		2	0.66666667	
O	Allow only filtered Air into device	2	1		2	1		2	1		2	1	
Precise			2	48		2	48		2	48		2	48
O	Reproducible Results	3	1		3	1		3	1		3	1	
O	Allow for uniform seeding	3	1		3	1		3	1		3	1	
Easy to Use			3.66666667	66		3.66666667	66		3.66666667	66		3.66666667	66
O	Minimize processing and Handling time	2	0.66666667		2	0.66666667		2	0.66666667		2	0.66666667	
O	Easy to assemble	4	1		4	1		4	1		4	1	
O	Easy to change media	3	1		3	1		3	1		3	1	
O	autoclavable	3	1		3	1		3	1		3	1	
Efficient			4.66666667	70		4.33333333	65		3.5	52.5		3.5	52.5
O	Compact relative to graft size	2	1		2	1		1	0.5		1	0.5	
O	Optimize media use	3	1		2	0.66666667		1	0.33333333		1	0.33333333	
O	Minimize processing and Handling time	2	0.66666667		2	0.66666667		2	0.66666667		2	0.66666667	
O	Minimize manufacturing time of device	4	1		4	1		4	1		4	1	
O	Cost of $1-2 per graft	2	1		2	1		2	1		2	1	
Non- Destructive			2	22		2	22		2	22		2	22
O	Non- destructive quantitative imaging	2	1		2	1		2	1		2	1	
O	Graft can be removed intact	2	1		2	1		2	1		2	1	
	TOTAL			291.33333			286.33333			252.5			252.5

Appendix F: Statistical Test Results

Assembly

Comparing Means [t-test assuming unequal variances (heteroscedastic)]			
Descriptive Statistics			
VAR	*Sample size*	*Mean*	*Variance*
Old Device	8	126.5	312.5824
New Device	8	137.625	863.71332
Summary			
Degrees Of Freedom	11	*Hypothesized Mean Difference*	0
Test Statistics	0.91746	*Pooled Variance*	588.14786

Two-tailed distribution			
p-level	0.37858	*t Critical Value (5%)*	2.20099
One-tailed distribution			
p-level	0.18929	*t Critical Value (5%)*	1.79588

Disassembly

Comparing Means [t-test assuming unequal variances (heteroscedastic)]			
Descriptive Statistics			
VAR	*Sample size*	*Mean*	*Variance*
Old Device	8	175.25	369.7929
New Device	8	32.75	54.9081
Summary			
Degrees Of Freedom	9	*Hypothesized Mean Difference*	0.E+0
Test Statistics	19.55772	*Pooled Variance*	212.3505
Two-tailed distribution			
p-level	1.10581E-08	*t Critical Value (5%)*	2.26216
One-tailed distribution			
p-level	5.52906E-09	*t Critical Value (5%)*	1.83311

Flip Test

Comparing Means [t-test assuming unequal variances (heteroscedastic)]			
Descriptive Statistics			
VAR	*Sample size*	*Mean*	*Variance*
	8	17.125	2.5921
	8	7.64	4.7524
Summary			
Degrees Of Freedom	13	*Hypothesized Mean Difference*	0.E+0
Test Statistics	9.89923	*Pooled Variance*	3.67225
Two-tailed distribution			
p-level	2.02336E-07	*t Critical Value (5%)*	2.16037
One-tailed distribution			
p-level	1.01168E-07	*t Critical Value (5%)*	1.77093

Appendix G: MATLAB Code

```
%% Rebecca Paz
%% CellProfiler Quantification

%% Clear variables, open windows, command window
clear all; clear all; close all; clc;

%% Check for existence of MATLAB Output folder
if ~exist('MATLAB Output', 'dir')
  mkdir('MATLAB Output');
end
```

```
%% Create list of files to be used in script
imagefiles = dir('Output\Cropped Red Images\Red*Outlines.tiff');
objectfiles = dir('Output\Red Objects\Red*Objects.tiff');
filterfiles = dir('Output\Red Filtered Objects\Red*Filtered.tiff');
stemfiles = dir('Output\Red Stem Seeds\Red*Seeds.tiff');

%% Preallocate list of maximum and minimum intensities per file
maxlist = zeros([1 numel(imagefiles)]);
minlist = zeros([1 numel(imagefiles)]);

for k = 1:numel(imagefiles)

    %% Define what files in list to use
    image = imread(['Output/Cropped Red Images/',imagefiles(k).name]);
    objects = imread(['Output/Red Objects/',objectfiles(k).name]);
    filters = imread(['Output/Red Filtered Objects/',filterfiles(k).name]);
    seeds = imread(['Output/Red Stem Seeds/',stemfiles(k).name]);

    %% Find mean intensities of the objects in the image files
    stats = regionprops(objects,image,'MeanIntensity','Centroid');

    %% Create matrix table of intensity statistics from statistics cells
    table = cell2mat(struct2cell(stats).');

    %% Find maximum and minimum mean intensities
    int = table(:,3);
    maxlist(1,k) = max(int);
    minlist(1,k) = min(int);

end

    %% Define range as average maximum and average minimum of intensities
    range = [mean(maxlist) mean(minlist)];

%% Main section, for finding average intensities and overlaying objects
for k = 1:numel(imagefiles)

    %% Define what files in list to use
    image = imread(['Output/Cropped Red Images/',imagefiles(k).name]);
    objects = imread(['Output/Red Objects/',objectfiles(k).name]);
    filters = imread(['Output/Red Filtered Objects/',filterfiles(k).name]);
    seeds = imread(['Output/Red Stem Seeds/',stemfiles(k).name]);

    %% Define data for resultant file naming
    input = imagefiles(k).name;
    [pathstr, name, ext] = fileparts(input);
    output = fullfile(pathstr, [name '.xls']);
    figput = fullfile(pathstr, [name '.png']);

    %% Split objects between those with a matching seed, and those without
    noseeds = objects - (filters.*255);
    withseeds = objects - (noseeds.*255);
```

```
    %% Find statistics of all objects, objects with seeds, and objects without
seeds
    fullstats = regionprops(objects,image,'MeanIntensity','Centroid');
    withseedsstats = regionprops(withseeds,image,'MeanIntensity');
    noseedstats = regionprops(noseeds,image,'MeanIntensity');

    %% Convert statistics from cell format to table matrix format
    table = cell2mat(struct2cell(fullstats).');
    withseedtable = cell2mat(struct2cell(withseedsstats).');
    noseedtable = cell2mat(struct2cell(noseedstats).');

    %% Find NaNs as a result of absent objects in line 72
    nans = isnan(withseedtable);
    %% Preallocate cells for presence of with-seed objects
    stemstatus = {};

    %% Convert NaNs in line 76 to 'No', numbers to 'Yes'
    for q = 1:numel(withseedtable);
        if nans(q) == 1; % following comment suppresses warning in line 83
            stemstatus{q} = 'No'; %#ok<*SAGROW>
        else stemstatus{q} = 'Yes';
        end
    end

    %% Transpose table for spreadsheet purposes
    stemstatus = stemstatus';

    %% Find centroids and x-positions of cell objects
    xy = [table(:,1) table(:,2)];
    int = table(:,3);
    xpos = xy(:,1);

    %% Optional: Output X-position of cell objects
%     fig1 = figure(1);
%     q = bar(xpos,int,'histc');
%     title('Intensity-Position Plot');
%     xlabel('Centroid X-Position');
%     ylabel('Intensity');
%     axis([0 size(image,2) 0 max(int)]);
%     print(fig1,'-dpng',['Output\' figput]);

    %% Define linear range for bright, mid-bright, dim cells
    linpoints = linspace(min(range), max(range), 4);

    %% Find dim cells
    idr = find([stats.MeanIntensity] <= linpoints(2));
    red = ismember(objects, idr);


    %% Find bright cells
    idb = find([stats.MeanIntensity] >= linpoints(3));
    blu = ismember(objects, idb);

    %% Find mid-bright cells
    idg = find([stats.MeanIntensity] >= 0);
```

```
    all = ismember(objects, idg);
    grn = im2bw((all - red - blu));

    %% Create image of sorted objects
    I = zeros(size(all,1), size(all,2), 3);
    I(:, :, 1) = red;
    I(:, :, 2) = grn;
    I(:, :, 3) = blu;

    %% Overlay sorted objects onto original image
    flip = ~all;
    noobj = image.*(im2uint8(flip)/255);
    rnoob = cat(3,noobj,noobj,noobj);
    outlines = (im2double(rnoob) + I);

    %% Add stem cell markers onto original image (not optimized)
    seeds = seeds.*255;
    seeds = im2double(seeds);
    outlines(:,:,1) = outlines(:,:,1)+seeds;
    seeds = seeds/255;

    seeds = seeds.*105;
    seeds = im2double(seeds);
    outlines(:,:,2) = outlines(:,:,2)+seeds;
    seeds = seeds/105;

    seeds = seeds.*180;
    seeds = im2double(seeds);
    outlines(:,:,3) = outlines(:,:,3)+seeds;
    seeds = seeds/180;

    %% Write final image to MATLAB Output folder
    imwrite(outlines, ['MATLAB Output\' imagefiles(k).name]);

    %% Create Excel file for statistical analysis
    xl = rot90(1:size(table),3);
    xl(:,2) = table(:,1);
    xl(:,3) = table(:,2);
    xl(:,4) = table(:,3);

    %% Title data columns in Excel file
    titles = {'Object Number', 'X Coordinate', 'Y Coordinate', 'Average
Intensity', 'Stem Cell Status'};

    %% Write columns to Excel file
    xlswrite(['MATLAB Output\' output], titles, 1, 'A1');
    xlswrite(['MATLAB Output\' output], xl(:,1), 1, 'A2');
    xlswrite(['MATLAB Output\' output], xl(:,2), 1, 'B2');
    xlswrite(['MATLAB Output\' output], xl(:,3), 1, 'C2');
    xlswrite(['MATLAB Output\' output], xl(:,4), 1, 'D2');
    xlswrite(['MATLAB Output\' output], stemstatus, 1, 'E2');
end
```

Appendix H: CellProfiler Pipeline Code

```
CellProfiler Pipeline: http://www.cellprofiler.org
Version:1
SVNRevision:11000

LoadImages:[module_num:1|svn_version:\'10951\'|variable_revision_number:11|show_
window:False|notes:\x5B\'Load red, green, blue, and lamina images.\'\x5D]
    File type to be loaded:individual images
    File selection method:Text-Regular expressions
    Number of images in each group?:3
    Type the text that the excluded images have in common:Do not use
    Analyze all subfolders within the selected folder?:None
    Input image file location:Default Input Folder\x7C.
    Check image sets for missing or duplicate files?:No
    Group images by metadata?:No
    Exclude certain files?:No
    Specify metadata fields to group by:
    Select subfolders to analyze:
    Image count:4
    Text that these images have in common (case-sensitive):Red..tif
    Position of this image in each group:d0.tif
    Extract metadata from where?:None
    Regular expression that finds metadata in the file name:None
    Type the regular expression that finds metadata in the subfolder path:None
    Channel count:1
    Group the movie frames?:No
    Grouping method:Interleaved
    Number of channels per group:2
    Load the input as images or objects?:Images
    Name this loaded image:OrigRed
    Name this loaded object:Nuclei
    Retain outlines of loaded objects?:No
    Name the outline image:NucleiOutlines
    Channel number:1
    Rescale intensities?:Yes
    Text that these images have in common (case-sensitive):Blue..tif
    Position of this image in each group:2
    Extract metadata from where?:None
    Regular expression that finds metadata in the file
name:^(?P<Plate>.*)_(?P<Well>\x5BA-P\x5D\x5B0-9\x5D{2})_s(?P<Site>\x5B0-9\x5D)
    Type the regular expression that finds metadata in the subfolder
path:.*\x5B\\\\/\x5D(?P<Date>.*)\x5B\\\\/\x5D(?P<Run>.*)$
    Channel count:1
    Group the movie frames?:No
    Grouping method:Interleaved
    Number of channels per group:3
    Load the input as images or objects?:Images
    Name this loaded image:OrigBlue
    Name this loaded object:Nuclei
    Retain outlines of loaded objects?:No
    Name the outline image:LoadedImageOutlines
    Channel number:1
    Rescale intensities?:Yes
    Text that these images have in common (case-sensitive):Green..tif
    Position of this image in each group:3
    Extract metadata from where?:None
```

```
    Regular expression that finds metadata in the file
name:^(?P<Plate>.*)_(?P<Well>\x5BA-P\x5D\x5B0-9\x5D{2})_s(?P<Site>\x5B0-9\x5D)
    Type the regular expression that finds metadata in the subfolder
path:.*\x5B\\\\/\x5D(?P<Date>.*)\x5B\\\\/\x5D(?P<Run>.*)$
    Channel count:1
    Group the movie frames?:No
    Grouping method:Interleaved
    Number of channels per group:3
    Load the input as images or objects?:Images
    Name this loaded image:OrigGreen
    Name this loaded object:Nuclei
    Retain outlines of loaded objects?:No
    Name the outline image:LoadedImageOutlines
    Channel number:1
    Rescale intensities?:Yes
    Text that these images have in common (case-sensitive):Lamina..tif
    Position of this image in each group:4
    Extract metadata from where?:None
    Regular expression that finds metadata in the file
name:^(?P<Plate>.*)_(?P<Well>\x5BA-P\x5D\x5B0-9\x5D{2})_s(?P<Site>\x5B0-9\x5D)
    Type the regular expression that finds metadata in the subfolder
path:.*\x5B\\\\/\x5D(?P<Date>.*)\x5B\\\\/\x5D(?P<Run>.*)$
    Channel count:1
    Group the movie frames?:No
    Grouping method:Interleaved
    Number of channels per group:3
    Load the input as images or objects?:Images
    Name this loaded image:Lamina
    Name this loaded object:Nuclei
    Retain outlines of loaded objects?:No
    Name the outline image:LoadedImageOutlines
    Channel number:1
    Rescale intensities?:Yes

Crop:[module_num:2|svn_version:\'10804\'|variable_revision_number:2|show_window:
False|notes:\x5B\'Crop red picture. This cropping will be used for all
images.\'\x5D]
    Select the input image:OrigRed
    Name the output image:CropRed
    Select the cropping shape:Rectangle
    Select the cropping method:Mouse
    Apply which cycle\'s cropping pattern?:Every
    Left and right rectangle positions:38,788
    Top and bottom rectangle positions:73,600
    Coordinates of ellipse center:500,500
    Ellipse radius, X direction:400
    Ellipse radius, Y direction:200
    Use Plate Fix?:No
    Remove empty rows and columns?:Edges
    Select the masking image:None
    Select the image with a cropping mask:None
    Select the objects:None

Crop:[module_num:3|svn_version:\'10804\'|variable_revision_number:2|show_window:
False|notes:\x5B\x5D]
    Select the input image:OrigBlue
    Name the output image:CropBlue
```

```
    Select the cropping shape:Previous cropping
    Select the cropping method:Mouse
    Apply which cycle\'s cropping pattern?:First
    Left and right rectangle positions:38,788
    Top and bottom rectangle positions:73,600
    Coordinates of ellipse center:500,500
    Ellipse radius, X direction:400
    Ellipse radius, Y direction:200
    Use Plate Fix?:No
    Remove empty rows and columns?:Edges
    Select the masking image:None
    Select the image with a cropping mask:CropRed
    Select the objects:None

Crop:[module_num:4|svn_version:\'10804\'|variable_revision_number:2|show_window:
False|notes:\x5B\x5D]
    Select the input image:OrigGreen
    Name the output image:CropGreen
    Select the cropping shape:Previous cropping
    Select the cropping method:Mouse
    Apply which cycle\'s cropping pattern?:First
    Left and right rectangle positions:38,788
    Top and bottom rectangle positions:73,600
    Coordinates of ellipse center:500,500
    Ellipse radius, X direction:400
    Ellipse radius, Y direction:200
    Use Plate Fix?:No
    Remove empty rows and columns?:Edges
    Select the masking image:None
    Select the image with a cropping mask:CropRed
    Select the objects:None

IdentifyPrimaryObjects:[module_num:5|svn_version:\'10826\'|variable_revision_num
ber:8|show_window:False|notes:\x5B\'Find blue nuclei. \'\x5D]
    Select the input image:CropBlue
    Name the primary objects to be identified:Nuclei
    Typical diameter of objects, in pixel units (Min,Max):10,70
    Discard objects outside the diameter range?:Yes
    Try to merge too small objects with nearby larger objects?:Yes
    Discard objects touching the border of the image?:Yes
    Select the thresholding method:RobustBackground Global
    Threshold correction factor:1
    Lower and upper bounds on threshold:0.1,0.5
    Approximate fraction of image covered by objects?:0.1
    Method to distinguish clumped objects:Shape
    Method to draw dividing lines between clumped objects:Shape
    Size of smoothing filter:10
    Suppress local maxima that are closer than this minimum allowed distance:5
    Speed up by using lower-resolution image to find local maxima?:Yes
    Name the outline image:NucleiOutline
    Fill holes in identified objects?:Yes
    Automatically calculate size of smoothing filter?:Yes
    Automatically calculate minimum allowed distance between local maxima?:Yes
    Manual threshold:0.0
    Select binary image:MoG Global
    Retain outlines of the identified objects?:Yes
    Automatically calculate the threshold using the Otsu method?:Yes
```

```
    Enter Laplacian of Gaussian threshold:.5
    Two-class or three-class thresholding?:Two classes
    Minimize the weighted variance or the entropy?:Weighted variance
    Assign pixels in the middle intensity class to the foreground or the
background?:Foreground
    Automatically calculate the size of objects for the Laplacian of Gaussian
filter?:Yes
    Enter LoG filter diameter:5
    Handling of objects if excessive number of objects identified:Continue
    Maximum number of objects:500
    Select the measurement to threshold with:None

IdentifyPrimaryObjects:[module_num:6|svn_version:\'10826\'|variable_revision_num
ber:8|show_window:False|notes:\x5B\'Find green nuclei.\'\x5D]
    Select the input image:CropGreen
    Name the primary objects to be identified:GreenNuclei
    Typical diameter of objects, in pixel units (Min,Max):10,70
    Discard objects outside the diameter range?:Yes
    Try to merge too small objects with nearby larger objects?:Yes
    Discard objects touching the border of the image?:Yes
    Select the thresholding method:RobustBackground Global
    Threshold correction factor:1
    Lower and upper bounds on threshold:0.1,0.4
    Approximate fraction of image covered by objects?:0.1
    Method to distinguish clumped objects:Shape
    Method to draw dividing lines between clumped objects:Intensity
    Size of smoothing filter:10
    Suppress local maxima that are closer than this minimum allowed distance:5
    Speed up by using lower-resolution image to find local maxima?:Yes
    Name the outline image:GreenNucleiOutline
    Fill holes in identified objects?:Yes
    Automatically calculate size of smoothing filter?:Yes
    Automatically calculate minimum allowed distance between local maxima?:Yes
    Manual threshold:0.0
    Select binary image:MoG Global
    Retain outlines of the identified objects?:Yes
    Automatically calculate the threshold using the Otsu method?:Yes
    Enter Laplacian of Gaussian threshold:.5
    Two-class or three-class thresholding?:Two classes
    Minimize the weighted variance or the entropy?:Weighted variance
    Assign pixels in the middle intensity class to the foreground or the
background?:Foreground
    Automatically calculate the size of objects for the Laplacian of Gaussian
filter?:Yes
    Enter LoG filter diameter:5
    Handling of objects if excessive number of objects identified:Continue
    Maximum number of objects:500
    Select the measurement to threshold with:None

IdentifyPrimaryObjects:[module_num:7|svn_version:\'10826\'|variable_revision_num
ber:8|show_window:False|notes:\x5B\'Find lamina.\'\x5D]
    Select the input image:Lamina
    Name the primary objects to be identified:LaminaObject
    Typical diameter of objects, in pixel units (Min,Max):150,6000
    Discard objects outside the diameter range?:Yes
    Try to merge too small objects with nearby larger objects?:Yes
    Discard objects touching the border of the image?:No
```

```
    Select the thresholding method:Manual
    Threshold correction factor:1
    Lower and upper bounds on threshold:0.1,0.5
    Approximate fraction of image covered by objects?:0.1
    Method to distinguish clumped objects:None
    Method to draw dividing lines between clumped objects:Intensity
    Size of smoothing filter:10
    Suppress local maxima that are closer than this minimum allowed distance:5
    Speed up by using lower-resolution image to find local maxima?:Yes
    Name the outline image:LaminaOutline
    Fill holes in identified objects?:Yes
    Automatically calculate size of smoothing filter?:Yes
    Automatically calculate minimum allowed distance between local maxima?:Yes
    Manual threshold:0.00001
    Select binary image:MoG Global
    Retain outlines of the identified objects?:Yes
    Automatically calculate the threshold using the Otsu method?:Yes
    Enter Laplacian of Gaussian threshold:.5
    Two-class or three-class thresholding?:Two classes
    Minimize the weighted variance or the entropy?:Weighted variance
    Assign pixels in the middle intensity class to the foreground or the
background?:Foreground
    Automatically calculate the size of objects for the Laplacian of Gaussian
filter?:Yes
    Enter LoG filter diameter:5
    Handling of objects if excessive number of objects identified:Continue
    Maximum number of objects:500
    Select the measurement to threshold with:None

ExpandOrShrinkObjects:[module_num:8|svn_version:\'10830\'|variable_revision_numb
er:1|show_window:False|notes:\x5B\'Expand lamina.\'\x5D]
    Select the input objects:LaminaObject
    Name the output objects:LaminaExpand
    Select the operation:Expand objects by a specified number of pixels
    Number of pixels by which to expand or shrink:50
    Fill holes in objects so that all objects shrink to a single point?:No
    Retain the outlines of the identified objects for use later in the pipeline
(for example, in SaveImages)?:No
    Name the outline image:ShrunkenNucleiOutlines

RelateObjects:[module_num:9|svn_version:\'10300\'|variable_revision_number:2|sho
w_window:False|notes:\x5B\'Find all blue nuclei that reside within the expanded
lamina.\'\x5D]
    Select the input child objects:Nuclei
    Select the input parent objects:LaminaExpand
    Calculate distances?:None
    Calculate per-parent means for all child measurements?:No
    Calculate distances to other parents?:No
    Parent name:None

FilterObjects:[module_num:10|svn_version:\'10300\'|variable_revision_number:5|sh
ow_window:False|notes:\x5B\'Filter out nuclei that are not within lamina
range.\'\x5D]
    Name the output objects:NucleiInLaminaRange
    Select the object to filter:Nuclei
    Filter using classifier rules or measurements?:Measurements
    Select the filtering method:Limits
```

```
    Select the objects that contain the filtered objects:None
    Retain outlines of the identified objects?:Yes
    Name the outline image:FilteredNucleiInRange
    Rules file location:Default Input Folder\x7CNone
    Rules file name:rules.txt
    Measurement count:1
    Additional object count:0
    Select the measurement to filter by:Parent_LaminaExpand
    Filter using a minimum measurement value?:Yes
    Minimum value:1
    Filter using a maximum measurement value?:Yes
    Maximum value:100

RelateObjects:[module_num:11|svn_version:\'10300\'|variable_revision_number:2|sh
ow_window:False|notes:\x5B\'Find all \\x7fblue nuclei that have a matching green
nuclei.\'\x5D]
    Select the input child objects:NucleiInLaminaRange
    Select the input parent objects:GreenNuclei
    Calculate distances?:None
    Calculate per-parent means for all child measurements?:No
    Calculate distances to other parents?:No
    Parent name:None

FilterObjects:[module_num:12|svn_version:\'10300\'|variable_revision_number:5|sh
ow_window:False|notes:\x5B\'Filter out the nuclei without matching green
nuclei.\'\x5D]
    Name the output objects:FilteredNuclei
    Select the object to filter:NucleiInLaminaRange
    Filter using classifier rules or measurements?:Measurements
    Select the filtering method:Limits
    Select the objects that contain the filtered objects:None
    Retain outlines of the identified objects?:Yes
    Name the outline image:FilteredNucleiOutline
    Rules file location:Default Input Folder\x7CNone
    Rules file name:rules.txt
    Measurement count:1
    Additional object count:0
    Select the measurement to filter by:Parent_GreenNuclei
    Filter using a minimum measurement value?:Yes
    Minimum value:1
    Filter using a maximum measurement value?:Yes
    Maximum value:1000

ImageMath:[module_num:13|svn_version:\'10718\'|variable_revision_number:3|show_w
indow:False|notes:\x5B\'Invert the Red image to find objects better.\'\x5D]
    Operation:Invert
    Raise the power of the result by:1
    Multiply the result by:1
    Add to result:0
    Set values less than 0 equal to 0?:Yes
    Set values greater than 1 equal to 1?:Yes
    Ignore the image masks?:No
    Name the output image:InvertRed
    Image or measurement?:Image
    Select the first image:CropRed
    Multiply the first image by:1
    Measurement:
```

```
    Image or measurement?:Image
    Select the second image:
    Multiply the second image by:1
    Measurement:

IdentifySecondaryObjects:[module_num:14|svn_version:\'10826\'|variable_revision_
number:7|show_window:False|notes:\x5B\'Use nuclei as seeds, grow out until
membrane is reached.\'\x5D]
    Select the input objects:NucleiInLaminaRange
    Name the objects to be identified:NukeCyto
    Select the method to identify the secondary objects:Distance - B
    Select the input image:InvertRed
    Select the thresholding method:Otsu Global
    Threshold correction factor:1
    Lower and upper bounds on threshold:0,1.0
    Approximate fraction of image covered by objects?:0.01
    Number of pixels by which to expand the primary objects:10
    Regularization factor:0.05
    Name the outline image:SecondaryOutlines
    Manual threshold:0.0
    Select binary image:None
    Retain outlines of the identified secondary objects?:No
    Two-class or three-class thresholding?:Two classes
    Minimize the weighted variance or the entropy?:Weighted variance
    Assign pixels in the middle intensity class to the foreground or the
background?:Foreground
    Discard secondary objects that touch the edge of the image?:No
    Discard the associated primary objects?:No
    Name the new primary objects:FilteredNuclei
    Retain outlines of the new primary objects?:No
    Name the new primary object outlines:FilteredNucleiOutlines
    Select the measurement to threshold with:None
    Fill holes in identified objects?:Yes

RelateObjects:[module_num:15|svn_version:\'10300\'|variable_revision_number:2|sh
ow_window:False|notes:\x5B\'Find nuke cyto that have a stem cell marker in
them.\'\x5D]
    Select the input child objects:NukeCyto
    Select the input parent objects:GreenNuclei
    Calculate distances?:None
    Calculate per-parent means for all child measurements?:No
    Calculate distances to other parents?:No
    Parent name:None

FilterObjects:[module_num:16|svn_version:\'10300\'|variable_revision_number:5|sh
ow_window:False|notes:\x5B\'Remove those that do not have a stem cell
marker.\'\x5D]
    Name the output objects:FilteredNukeCyto
    Select the object to filter:NukeCyto
    Filter using classifier rules or measurements?:Measurements
    Select the filtering method:Limits
    Select the objects that contain the filtered objects:None
    Retain outlines of the identified objects?:No
    Name the outline image:FilteredObjects
    Rules file location:Default Input Folder\x7CNone
    Rules file name:rules.txt
    Measurement count:1
```

```
    Additional object count:0
    Select the measurement to filter by:Parent_GreenNuclei
    Filter using a minimum measurement value?:Yes
    Minimum value:1
    Filter using a maximum measurement value?:Yes
    Maximum value:1000

ExpandOrShrinkObjects:[module_num:17|svn_version:\'10830\'|variable_revision_num
ber:1|show_window:False|notes:\x5B\'Average width of cell membrane is 5 to 13
pixels. Assuming average width of 9 pixels.\'\x5D]
    Select the input objects:NukeCyto
    Name the output objects:ExpandCyto
    Select the operation:Expand objects by a specified number of pixels
    Number of pixels by which to expand or shrink:3
    Fill holes in objects so that all objects shrink to a single point?:No
    Retain the outlines of the identified objects for use later in the pipeline
(for example, in SaveImages)?:Yes
    Name the outline image:ExpandCyto

RelateObjects:[module_num:18|svn_version:\'10300\'|variable_revision_number:2|sh
ow_window:False|notes:\x5B\'Find expanded cytoplasm with a stem cell marker in
them.\'\x5D]
    Select the input child objects:ExpandCyto
    Select the input parent objects:GreenNuclei
    Calculate distances?:None
    Calculate per-parent means for all child measurements?:No
    Calculate distances to other parents?:No
    Parent name:None

FilterObjects:[module_num:19|svn_version:\'10300\'|variable_revision_number:5|sh
ow_window:False|notes:\x5B\'Remove those that do not have a cell marker.\'\x5D]
    Name the output objects:FilteredExpandCyto
    Select the object to filter:ExpandCyto
    Filter using classifier rules or measurements?:Measurements
    Select the filtering method:Limits
    Select the objects that contain the filtered objects:None
    Retain outlines of the identified objects?:No
    Name the outline image:FilteredObjects
    Rules file location:Default Input Folder\x7CNone
    Rules file name:rules.txt
    Measurement count:1
    Additional object count:0
    Select the measurement to filter by:Parent_GreenNuclei
    Filter using a minimum measurement value?:Yes
    Minimum value:1
    Filter using a maximum measurement value?:Yes
    Maximum value:1000

IdentifyTertiaryObjects:[module_num:20|svn_version:\'10300\'|variable_revision_n
umber:1|show_window:False|notes:\x5B\'Find cell membrane by subtracting NukeCyto
from the expanded NukeCyto.\'\x5D]
    Select the larger identified objects:ExpandCyto
    Select the smaller identified objects:NukeCyto
    Name the tertiary objects to be identified:CellMembrane
    Name the outline image:CellMembraneOutlines
    Retain outlines of the tertiary objects?:Yes
```

```
IdentifyTertiaryObjects:[module_num:21|svn_version:\'10300\'|variable_revision_n
umber:1|show_window:False|notes:\x5B\'Find filtered cell membrane by subtracting
FilteredNukeCyto from the expanded FilteredNukeCyto.\'\x5D]
    Select the larger identified objects:FilteredExpandCyto
    Select the smaller identified objects:FilteredNukeCyto
    Name the tertiary objects to be identified:FilteredCellMembrane
    Name the outline image:CytoplasmOutlines
    Retain outlines of the tertiary objects?:No

OverlayOutlines:[module_num:22|svn_version:\'10672\'|variable_revision_number:2|
show_window:False|notes:\x5B\'Display cell membrane outlines on CropRed.\'\x5D]
    Display outlines on a blank image?:No
    Select image on which to display outlines:CropRed
    Name the output image:RedCellMembranes
    Select outline display mode:Color
    Select method to determine brightness of outlines:Max of image
    Width of outlines:1
    Select outlines to display:CellMembraneOutlines
    Select outline color:White

ExpandOrShrinkObjects:[module_num:23|svn_version:\'10830\'|variable_revision_num
ber:1|show_window:False|notes:\x5B\'Prepare stem cell marker seeds, for MATLAB
purposes.\'\x5D]
    Select the input objects:FilteredNuclei
    Name the output objects:FilteredNuclei2
    Select the operation:Shrink objects to a point
    Number of pixels by which to expand or shrink:3
    Fill holes in objects so that all objects shrink to a single point?:Yes
    Retain the outlines of the identified objects for use later in the pipeline
(for example, in SaveImages)?:No
    Name the outline image:ShrunkenNucleiOutlines

ExpandOrShrinkObjects:[module_num:24|svn_version:\'10830\'|variable_revision_num
ber:1|show_window:False|notes:\x5B\x5D]
    Select the input objects:FilteredNuclei2
    Name the output objects:FilteredNucleiSmall
    Select the operation:Expand objects by a specified number of pixels
    Number of pixels by which to expand or shrink:4
    Fill holes in objects so that all objects shrink to a single point?:Yes
    Retain the outlines of the identified objects for use later in the pipeline
(for example, in SaveImages)?:No
    Name the outline image:ShrunkenNucleiOutlines

SaveImages:[module_num:25|svn_version:\'10822\'|variable_revision_number:7|show_
window:False|notes:\x5B"Save the overlay image as an 8-bit TIF, appending the
text \'outline\' to the original filename of the nuclei image."\x5D]
    Select the type of image to save:Image
    Select the image to save:CropRed
    Select the objects to save:None
    Select the module display window to save:OutlinedNuc
    Select method for constructing file names:From image filename
    Select image name for file prefix:OrigRed
    Enter single file name:RedImage
    Do you want to add a suffix to the image file name?:Yes
    Text to append to the image name:Outlines
    Select file format to use:tif
    Output file location:Default Output Folder sub-folder\x7CCropped Red Images
```

```
    Image bit depth:8
    Overwrite existing files without warning?:Yes
    Select how often to save:Every cycle
    Rescale the images? :No
    Save as grayscale or color image?:Grayscale
    Select colormap:gray
    Store file and path information to the saved image?:No
    Create subfolders in the output folder?:No

SaveImages:[module_num:26|svn_version:\'10822\'|variable_revision_number:7|show_
window:False|notes:\x5B\'Save the objects for use with MATLAB.\'\x5D]
    Select the type of image to save:Objects
    Select the image to save:RedCellMembranes
    Select the objects to save:CellMembrane
    Select the module display window to save:OutlinedNuc
    Select method for constructing file names:From image filename
    Select image name for file prefix:OrigRed
    Enter single file name:RedObjects
    Do you want to add a suffix to the image file name?:Yes
    Text to append to the image name:Objects
    Select file format to use:tif
    Output file location:Default Output Folder sub-folder\x7CRed Objects
    Image bit depth:8
    Overwrite existing files without warning?:Yes
    Select how often to save:Every cycle
    Rescale the images? :No
    Save as grayscale or color image?:Grayscale
    Select colormap:gray
    Store file and path information to the saved image?:No
    Create subfolders in the output folder?:No

SaveImages:[module_num:27|svn_version:\'10822\'|variable_revision_number:7|show_
window:False|notes:\x5B\'Save the cell membranes that have stem cell markers in
them.\'\x5D]
    Select the type of image to save:Objects
    Select the image to save:RedCellMembranes
    Select the objects to save:FilteredCellMembrane
    Select the module display window to save:OutlinedNuc
    Select method for constructing file names:From image filename
    Select image name for file prefix:OrigRed
    Enter single file name:RedObjects
    Do you want to add a suffix to the image file name?:Yes
    Text to append to the image name:Filtered
    Select file format to use:tif
    Output file location:Default Output Folder sub-folder\x7CRed Filtered
Objects
    Image bit depth:8
    Overwrite existing files without warning?:Yes
    Select how often to save:Every cycle
    Rescale the images? :No
    Save as grayscale or color image?:Grayscale
    Select colormap:gray
    Store file and path information to the saved image?:No
    Create subfolders in the output folder?:No
```

```
SaveImages:[module_num:28|svn_version:\'10822\'|variable_revision_number:7|show_
window:False|notes:\x5B\'Save the objects for cells that both fall within range
and have a matching green nuclei.\'\x5D]
    Select the type of image to save:Objects
    Select the image to save:None
    Select the objects to save:FilteredNucleiSmall
    Select the module display window to save:None
    Select method for constructing file names:From image filename
    Select image name for file prefix:OrigRed
    Enter single file name:OrigBlue
    Do you want to add a suffix to the image file name?:Yes
    Text to append to the image name:Seeds
    Select file format to use:tif
    Output file location:Default Output Folder sub-folder\x7CRed Stem Seeds
    Image bit depth:8
    Overwrite existing files without warning?:Yes
    Select how often to save:Every cycle
    Rescale the images? :No
    Save as grayscale or color image?:Grayscale
    Select colormap:gray
    Store file and path information to the saved image?:No
    Create subfolders in the output folder?:No
```

Appendix I: User Guide

Automated Image Analysis Standard Operating Procedure

Preparation

Install CellProfiler

CellProfiler is a free, open-source cytometry and cell segmentation program.
In order to perform analysis, this program must be installed.
If this program has already been installed, you may skip this step.

1. Browse to http://www.cellprofiler.org/
2. Mouse over the “Download” heading and choose “CellProfiler”.
3. Click the image link representing your operating system.
    a. Macintosh, Linux, and Windows 32 and 64 bit versions are available.
4. Download the file at the file saving prompt.
5. Follow onscreen instructions to install the program.

Install MATLAB

MATLAB installation is required for this analysis system.
Installation of this program is the charge of your institution or workplace.
Please verify that the system you are working with has:

Basic MATLAB program; and
Image Processing Toolbox
installed. Image Processing Toolbox is necessary for this analysis system.
If this program has already been installed, you may skip this step.

Organize Images and File Structure

This system requires a specific file structure in order to run.
Create a series of folders in this manner:
Main folder: (desired title)
Subfolder: "Input"
Subfolder: "Output"
Within the main folder, place
quantest.m; and
Pipeline.cp
which will be used in analysis.

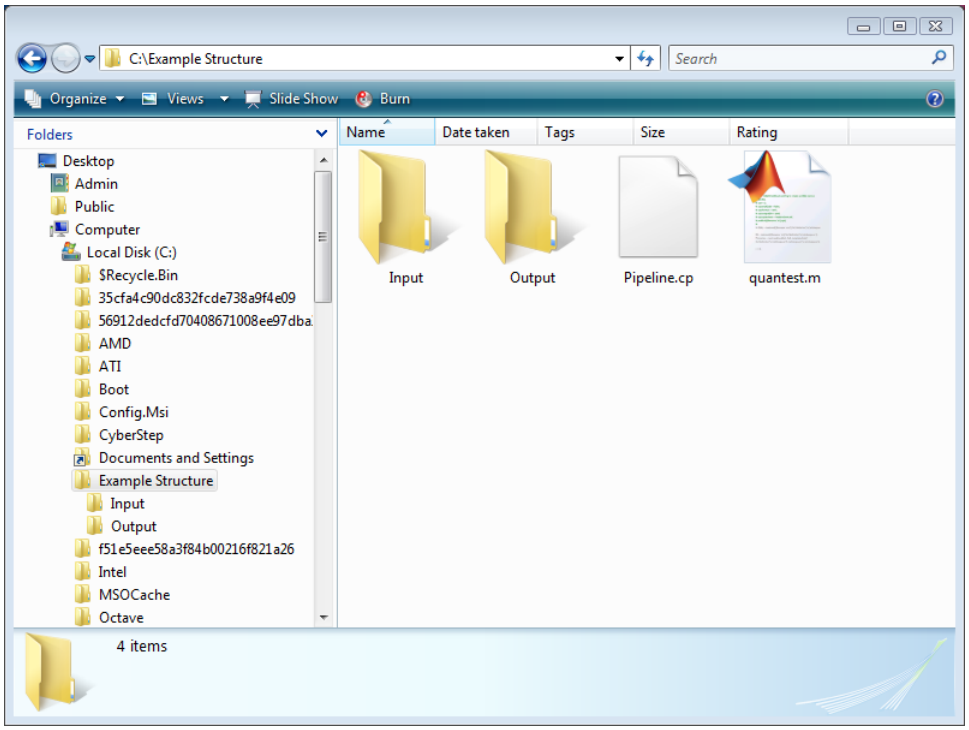

Figure I: Diagram of initial folder placement.

Place the images to be analyzed in the Input Folder.

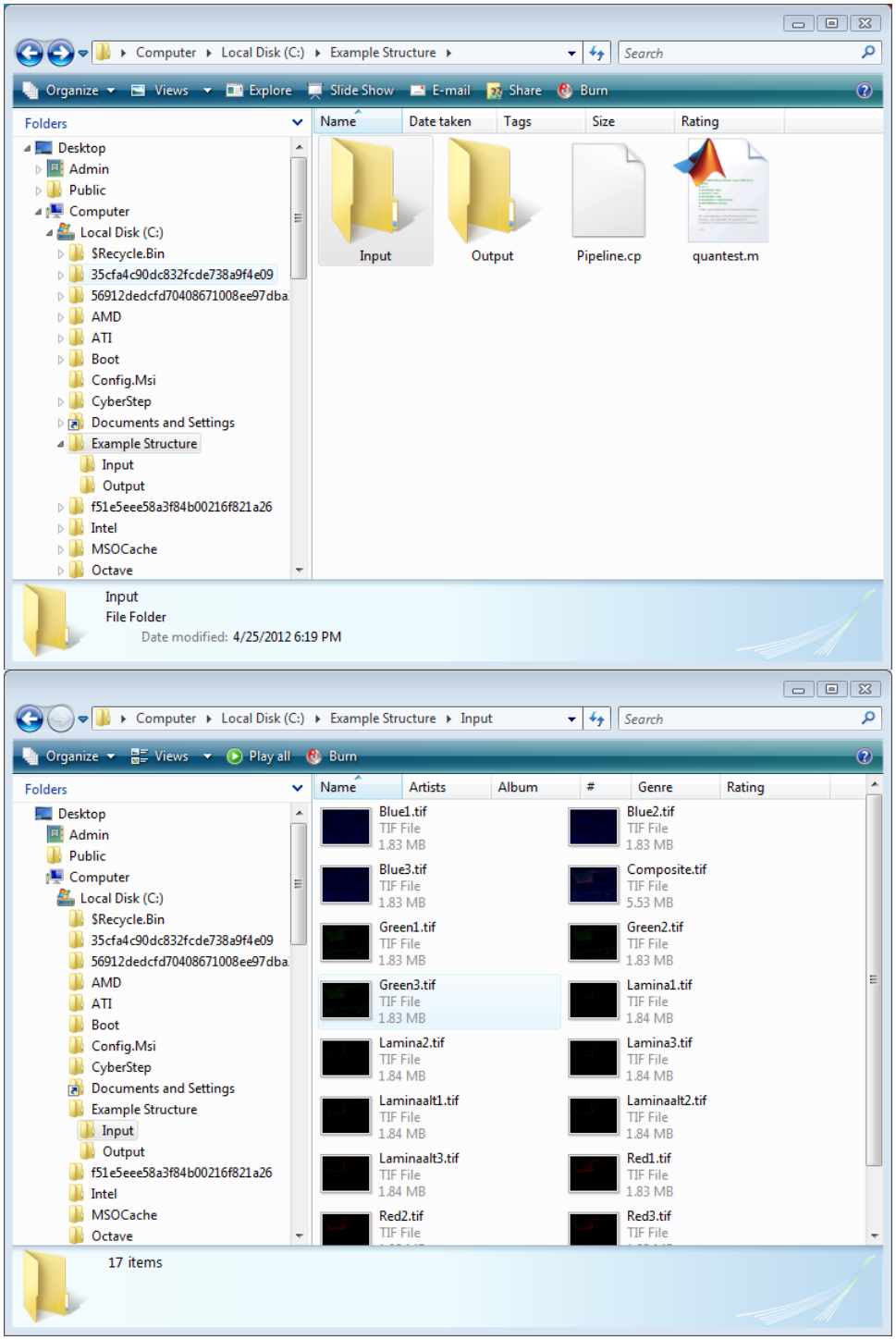

Figure II: Structure and organization of input folder.

These images include all red, blue, and green channel images for each section to be analyzed. A composite image is not necessary.

Isolating Basal Lamina

The system currently requires an image of only the dermal-epidermal junction to function. This can be accomplished by using Photoshop, or a similar image editing program. Instructions for isolating the lamina in Photoshop are as follows.

1. Open the file in Photoshop.
2. Press the “Q” key to go into Quick Mask mode.
    a. This will allow you to select the lamina using a brush.
3. Press the “D” key to switch to the default foreground/background colors.
4. Using the Brush tool, trace the basal lamina in the image. The basal lamina should be outlined opaquely in red.

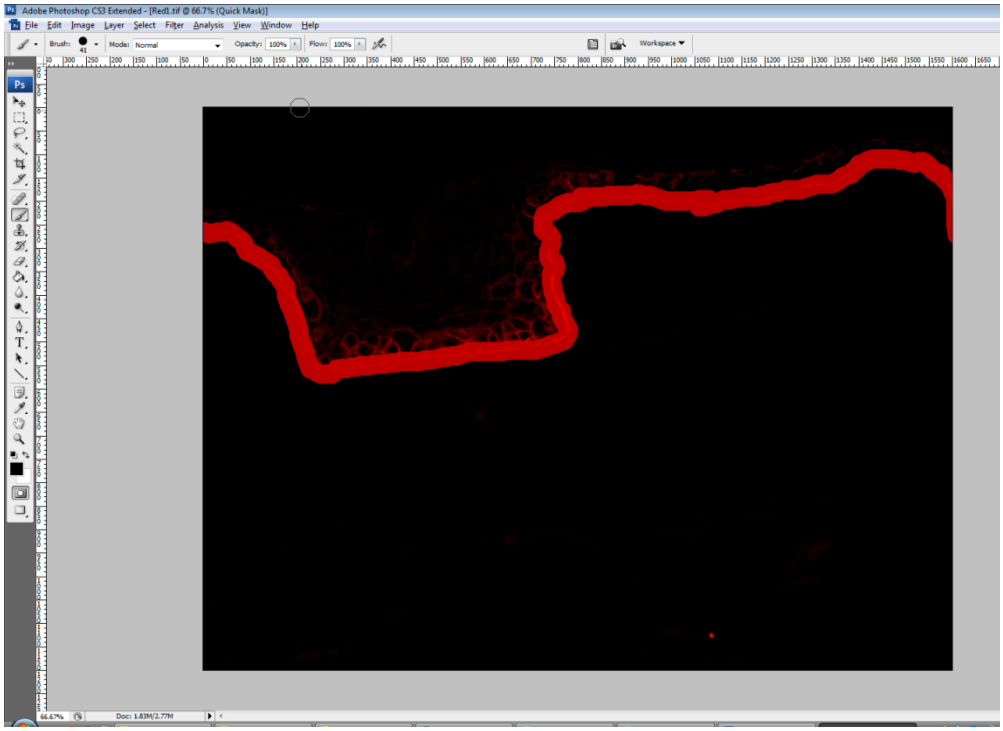

Figure III: Basal Lamina outlined using Quick Mask.

5. Press “Q” to leave Quick Mask mode, and “X” to switch the foreground/background colors. You should now have selected everything but the basal lamina.

6. Press Delete to remove the parts of the image that are not the basal lamina.

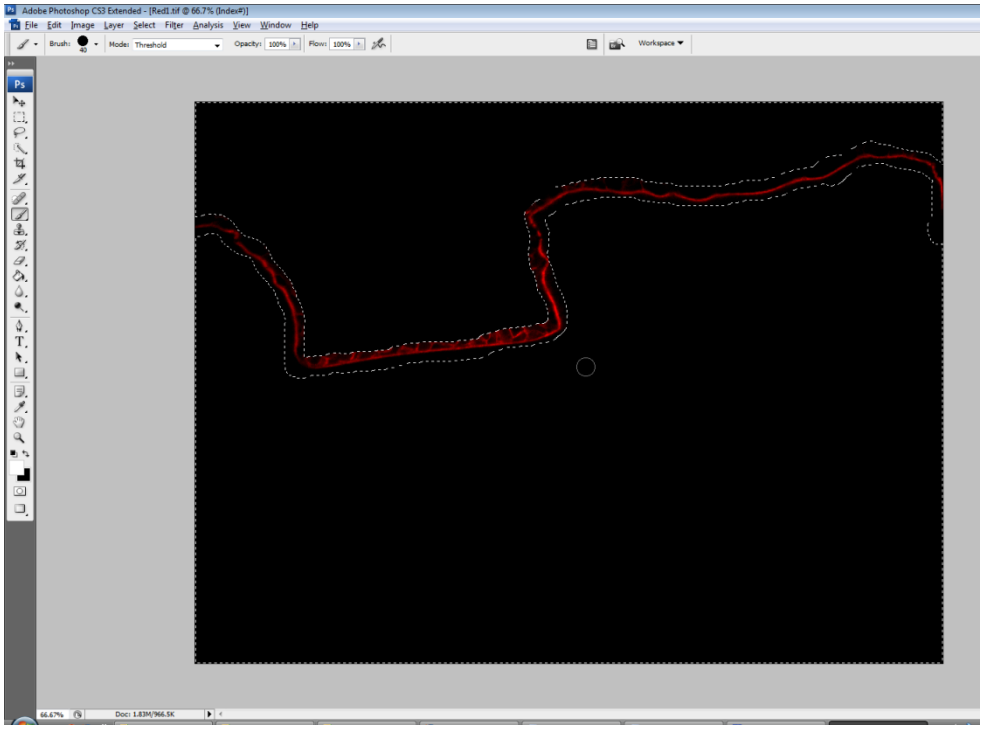

Figure IV: All parts of the image besides the lamina deleted.

7. **Use the "Save as" function** to save the image as a .TIF file named "Lamina#.tif", where # is the number associated with the original image. Do not use the Save function, or it will overwrite the original image.
8. Make sure the lamina image is in the same location as the original red channel image.

Running CellProfiler

Open CellProfiler from the start menu or wherever it is installed.
You will see a screen similar to this.

Figure V: Initial CellProfiler screen.

Click the folder icon next to the Default Input Folder line at the bottom, and navigate to the folder you wish to use as your Input folder. Repeat this process for the Output folder.

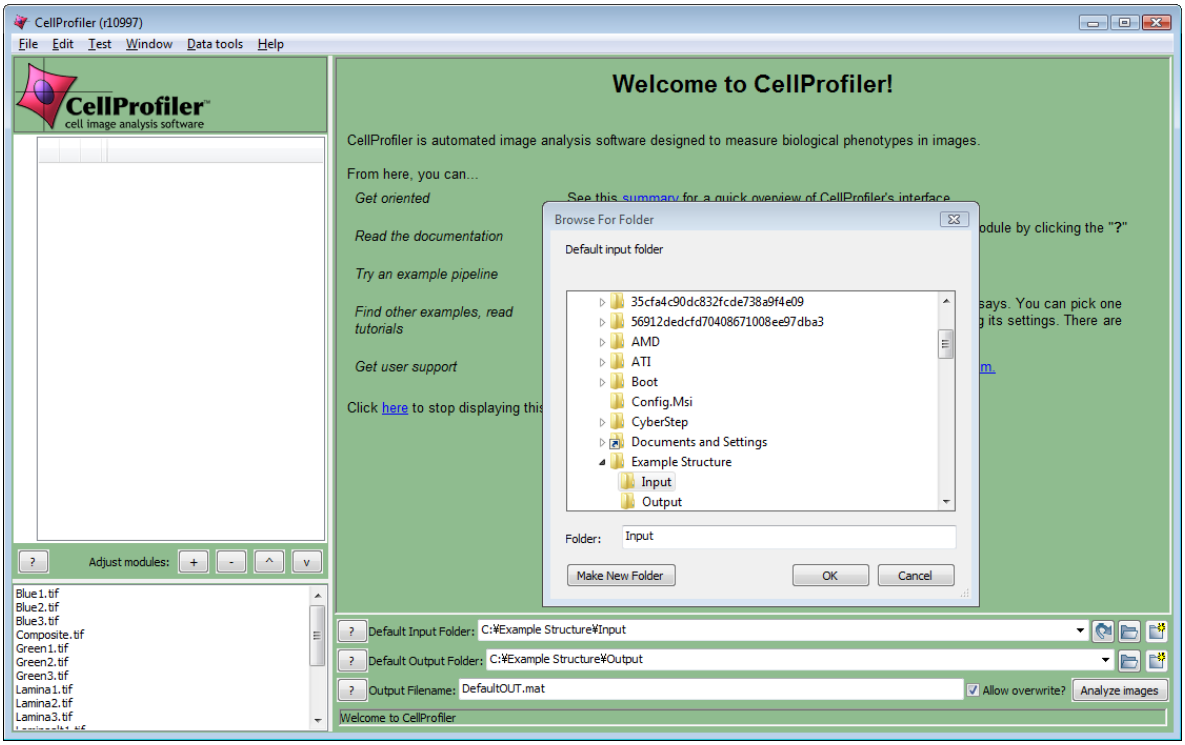

Figure VI: Selecting the Input folder.

When both the Input and Output folders are found, click File, then Load Pipeline. Open “Pipeline.cp” in the main folder when prompted to.

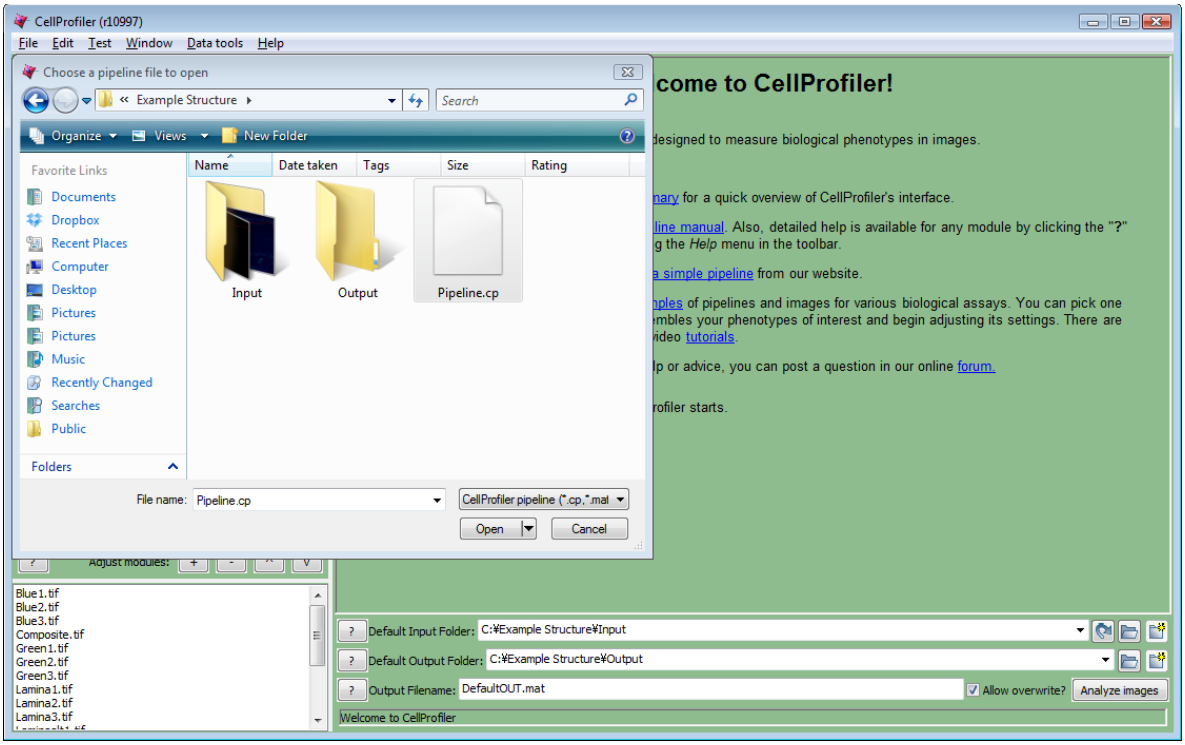

Figure VII: Select Pipeline.cp when prompted.

Once selected, press Ctrl+N to start the analysis. Depending on the number of images, it may take 10 or more minutes to complete. Each image takes roughly 30-45 seconds to complete. You will be prompted to select the area to be analyzed for each image. You may select the whole picture if you wish to analyze the entire image. The selected region will be saved for each subsequent cropping prompt, so simply click OK if you wish to use the same region for each image.

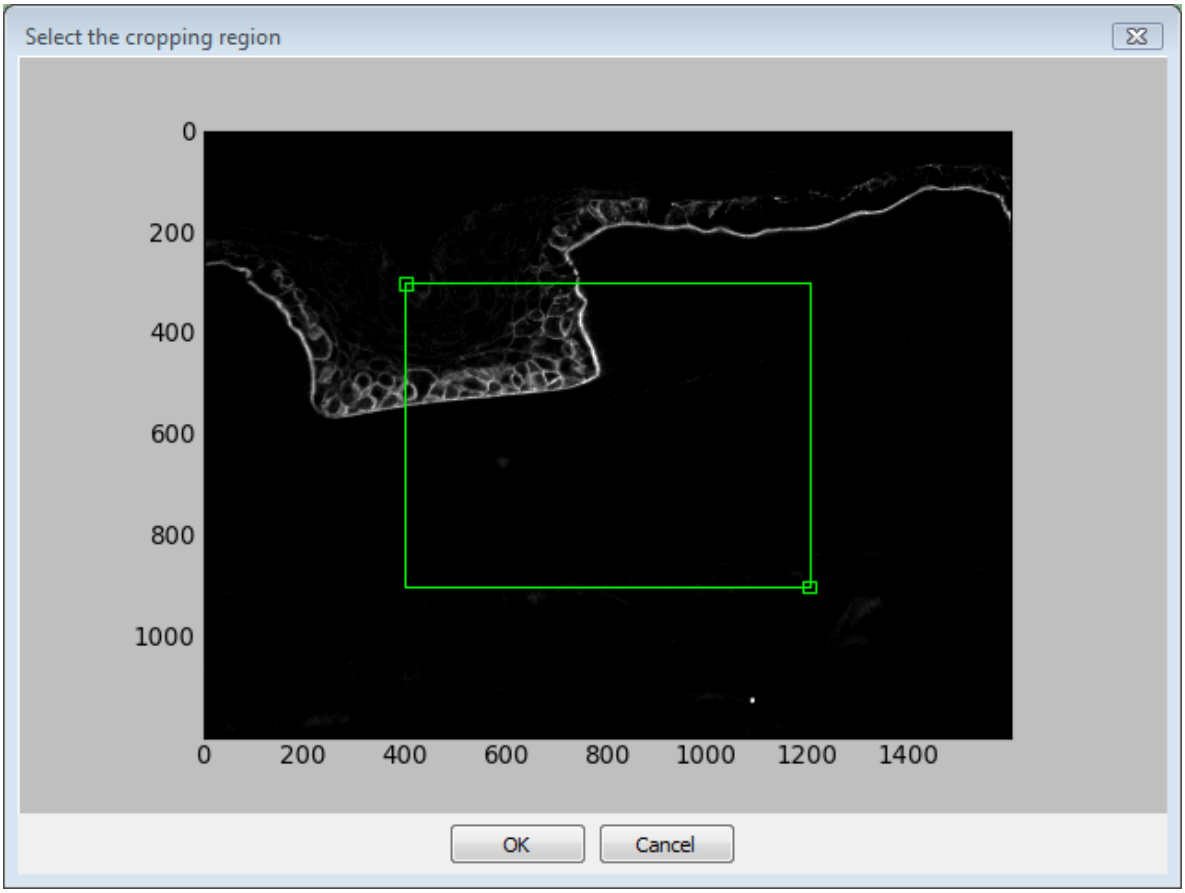

Figure VIII: Cropping region prompt.

Once each image has been analyzed, the resultant objects will output to the selected Output folder.
Output images are sorted into folders:

Cropped Red Images: the regions of the original red channel image that were analyzed.
Red Objects: object data for the cell membranes.
Red Filtered Objects: object data for cell membranes with stem cell markers.
Red Stem Seeds: object data for stem cell markers.

MATLAB will use these files for its analysis.

Running MATLAB

Open the main image analysis folder, and locate quantest.m.
Right click the file, and select "Run".
MATLAB will automatically open and perform the image analysis.

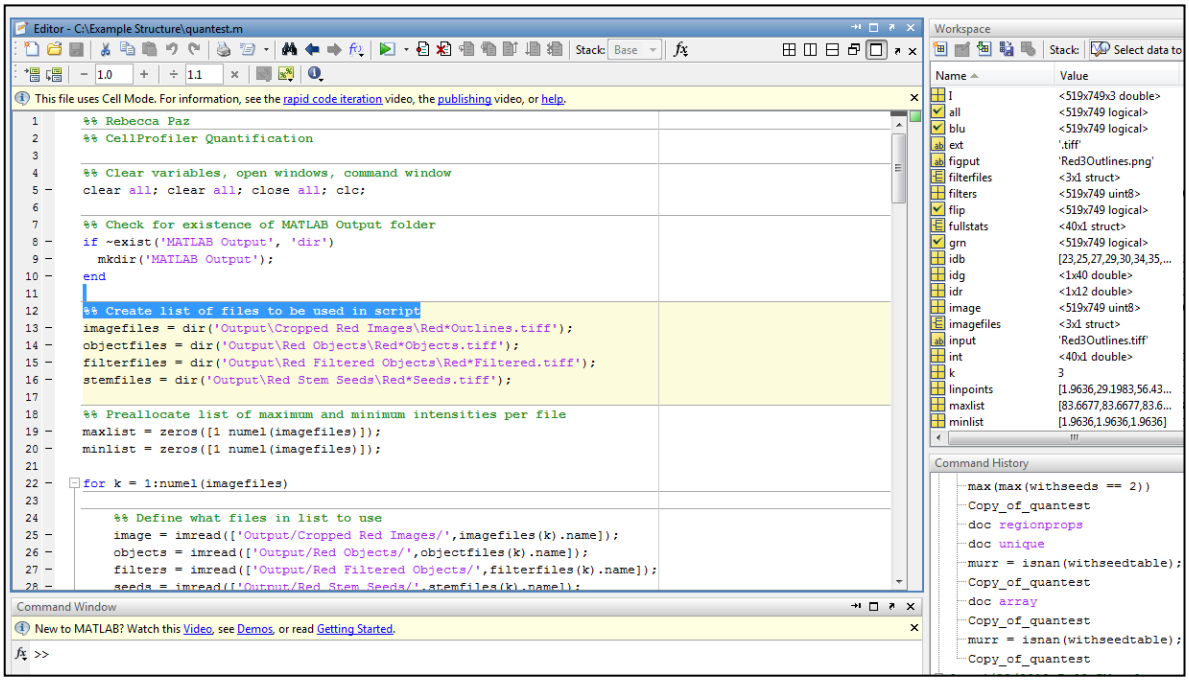

Figure IX: MATLAB image analysis in progress.

The images will be output to the MATLAB Output folder created by the script.
Each image will have a matching Excel file with the intensity, centroid, and stem cell status of each cell found in the images.
The images should look similar to the one shown below.

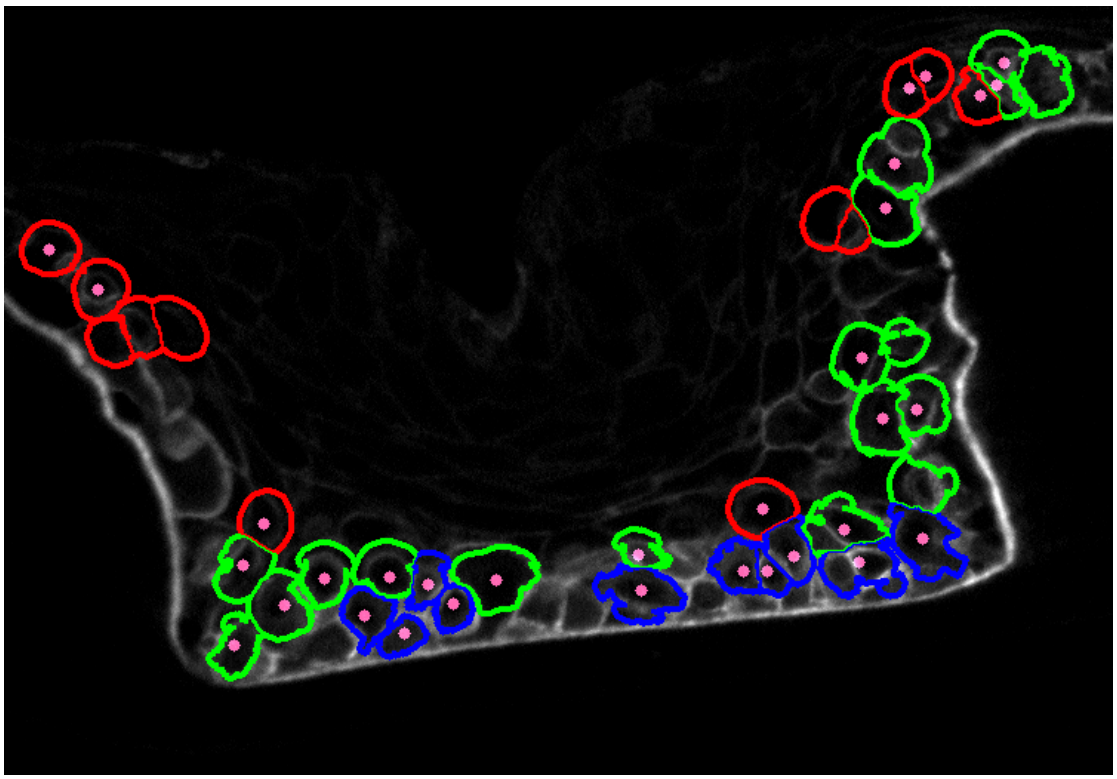

Figure 59: Sample resultant image.