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Design of a Wounding Model for Bioengineered Skin Substitutes

A Major Qualifying Project

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In Biomedical Engineering

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1. Introduction

Every year in the United States, over one-million people suffer from burn related injuries. Consequently 45,000 are hospitalized for further treatment, resulting in 4,500 deaths. The severity of the burn wound dictates whether or not the skin will heal naturally or if surgical intervention is required. The latter method can involve transplanting bioengineered skin (BES) onto the wound to assist in the healing. Whether or not BES is required depends on two factors: the depth the burn damage into the layers of skin and the total burn surface area (TBSA). Severe wounds will be incapable of regenerating and regaining native skin function, therefore, surgical intervention involving the application of BES is required. To best understand the application of BES following a wound, it is first necessary to understand how native skin functions and heals.

The major function of skin is to protect the body from the harsh environment. Skin is comprised of two layers, the epidermis and the dermis. The epidermal layer is composed of stratified layers of epithelium cells. These cells consist of keratinocytes that are capable of regenerating following an injury via a process called reepithelialization. This reepithelialization process involves the migration and proliferation of healthy keratinocytes outside the wound margin into the wound area, where a native epidermal layer can be formed and regenerated. The underlying dermis can sometimes exhibit similar regenerative capabilities, depending on the severity of the wound. Hair follicles and glands located in the dermal layer can effectively regenerate the epidermal layer of skin, if they are not completely destroyed during the wounding process. Unlike the epidermal layer that will always regenerate providing that there is a source of keratinocytes at the wound margin, a lack of dermal tissue and glands within the wound will prevent dermal regeneration and result in non-functional scar tissue formation under the new epidermal layer (Ratner BD, 2004). Large, deep burns will not allow the skin to regenerate. Therefore surgical intervention such as the transplantation of BES onto the wound site is required. With limited available of healthy skin, the autografting process takes numerous surgeries causing lengthy periods of pain to the patient. As such, there is a need for an off-the-shelf bioengineered skin substitute that will facilitate the regeneration of native skin function.

Many companies currently produce implantable BES that can be grafted onto burned areas of the patient to promote the restoration of physiological function. Integra® (Integra Life Sciences, Inc.) produces a single layer dermal regeneration template. This template is a

collagen/glycosaminoglycan sponge that promotes dermal ingrowth and regeneration, with a silicone membrane on top of the dermal component to serve as a protective barrier. A second surgery is later required to remove the silicone membrane and to implant an epidermal layer. Organogenesis, Inc. also produces a BES. Their product, Apligraf®, is a dermal-epidermal bilayer skin substitute capable of inducing healing to full-thickness burns; however the small (approximately 4 inch diameter) skin substitute currently costs $1,372 (Organogenesis-Apligraf, 2009) . A major limitation to Apligraf® involves the separation of the epidermal layer from the dermal component during handling/application. Unlike native skin where the epidermis and dermis are interdigitated at the papillary dermis, the dermal-epidermal junction of Apligraf® is relatively flat, allowing for layer separation.

In the Pins Laboratory, Department of Biomedical Engineering, Worcester Polytechnic Institute, a composite bioengineered skin substitute (CBSS) is created. The goal of the CBSS is to overcome the shortcomings of both the Integra® and Apligraf® BES. The Pins' Lab CBSS has both a dermal and epidermal components derived from human neonatal cells with a specialized micro-fabricated pattern template on PDMS (polydimethylsiloxane) that adds an interdigitated interface at the basal lamina analog to prevent layer separation.

While BES has achieved some clinical success and are readily available on the clinical market, these substitutes are very expensive to produce and continue to have several limitations such as the capacity to rapidly regenerate and integrate with the native tissue. Further research is required to study the mechanisms of BES's healing capabilities. This research will lead to better therapies and faster recovery times for patients requiring skin substitutes. One way in which studying the regeneration properties of bioengineered skin could be made possible is through the creation of an in vitro wounding model. Being able to model wound healing in bioengineered skin substitutes will be a valuable tool for understanding the mechanisms of BES healing and for enhancing its rapid regeneration properties. Therefore the objective of this project was to create an in vitro wounding model that creates full thickness, reproducible injuries to bioengineered skin substitutes.

The first part of this project aimed to understand native skin, especially the functions that cause skin to heal following a wound. Secondly, a literature review of currently available BES was completed to identify the functionality and properties of the BES when compared to native

tissue. The end goal of this project is to develop a wounding model for BES. This study will indicate additional requirements needed for the BES to better improve the wound healing rate.

In order to create an injury to engineered skin, various tests were completed. Inducing a burn onto the skin and characterizing the response is a useful aid to predict *in vivo* characteristics and functions of the skin grafts following a wound. A reproducible wound allows for further research to be completed surrounding wound healing and response to injury. Sobral et al. (2007) developed a wounding model for *in vitro* BES by placing heated metallic rods of various sizes on top of the skin to induce a thermal burn. While this model showed promise in inducing a consistent full-thickness wound, some heavier metallic rods removed the epidermal layer as a result of the pressure and temperature when they were removed (Sobral CS, 2007). Other methodologies of inducing a thermal burn include scalding the skin with boiling water. Buth et al. (2007) developed a model for inducing a scratch wound on the backs of mice by applying hot water to their skin, inducing a full-thickness burn. While effective in inducing a reproducible burn, this scratch-wound model could not be applied to our *in vitro* engineered skin which is grown in 6-well plates (Buth H, 2007). Finally, cryoburns were also considered. Researchers from the Leiden University Medical Center were able to generate a model for reproducibly inducing a cryoburn to engineered skin in Petri dishes. By cooling a metallic rod in liquid nitrogen (creating a cryoprobe) and applying to the skin for a period of 10 seconds, a full-thickness burn was generated (Ghalbzouri AE, 2004) Further characterization of this burn was completed by researchers at the University of Minnesota who developed a system of thermocouples that can be placed equidistant from the cryoprobe at the center of the skin substitute and engineered to quantify the degree and consistency of burning that the cryoprobe induces. (Han B, 2005).

The literature review of the current burn model techniques allowed for an extensive evaluation of current burn models to determine if a model already exists that fits the objectives and needs of this particular project. Several were identified and the group used them as a starting point for the creation of their own model. In particular, the group worked to create a model that induced a full-thickness thermal wound with clearly defined wound margins on the BES developed in the Pins lab. The group identified three major conceptual designs based on literature review and a brainstorming session that they believed could accomplish the goals of this project. The three main conceptual designs were a heat burn induced by metallic disks

heated with a laboratory hot plate (called “hot plate discs” design) and two types of cryoburn in the form of a cryoshower, in which liquid nitrogen would be sprayed onto the surface of the test tissue, and finally the cryoprobe which utilized a liquid nitrogen submerged metallic rod as a means of burning. After some initial concept testing using chicken skin as well as the use of several design tools, the team turned their focus toward developing a cryorod injury model. In order to add reproducibility to the cryorod design, the team also developed and manufactured a probe hanger device, comprised of Lexan trays that serve as a guide for applying the cryorod to the surface of the tissue in a consistent and reproducible manner.

Once the team had selected a final design, device and model testing began. Because of the cost and time associated with making the Pins lab CBSS, the team utilized foreskin tissue as a test tissue due to its abundant availability at no cost to the team (the Pins lab had an established relationship with the OB/Gyn Department at UMMS, who provided the tissue). One of the major functions of the device established by the team during the design process was to create a characterizeable wound. In order to accomplish this, the team investigated several different tissue viability methods including a Trypan blue stain, MTT assay, and histology. Although the team hoped they would be able to find a simple bench top assay for determining tissue viability, they experienced inconsistent results with both the Trypan blue and MTT and so they decided to use histology as the primary method for characterizing the induced burns.

In the early phases of testing, the team used a method in which burns were created using the cryorod, which was applied for various time intervals such as 30, 60, 90, and 120 seconds, and then the tissues were immediately fixed and processed for histology. This method however, did not create visible wounds with definitive wound margins, in fact in many samples it was impossible to even distinguish the burned and unburned portions of each sample. Following these inconsistent results, the team returned to the literature and found that several other groups working on similar projects cultured their injured samples for up to 72 hours after inducing a burn. When the team took this approach, coupled with the addition of a histological tissue marking dye which acted as a surface marker for the wound margin, the results were dramatically different. Using this method, the team was able to create reproducible cryoinjuries to foreskin tissue that had visible, full thickness damage and definitive wound margins as marked by the histology paint and the differences in morphology between the burned and unburned tissue. Additionally, the team was able to establish that a 90 second application of the cryorod

was the most appropriate for foreskin tissue as it created the desired burn without obliterating the rest of the tissue as was seen in longer application times.

In moving forward with this project, the team would like to suggest that further work focus on establishing the ideal cryorod application time for CBSS. Investigation of a quick and simple bench top tissue viability assay will also be a helpful next step. The team expects the device and model they established will be a valuable tool for the Pins lab as they continue research in developing their CBSS into a living skin equivalent capable of rapid regeneration. The device will be particularly useful in studies of the paracrine signaling between various cell types following injury.

2. Background

Annually, in the United States, burn wounds affect over one million patients. Burn wounds include thermal, chemical or radiation wounds (such as sunburn). In many cases the scope of the burn injury exceeds the critical depth and size where skin can heal naturally (Ratner BD, 2004). This inability to naturally heal creates a need for bioengineered skin grafts. These burn wounds result in approximately 45,000 hospitalizations and 4,500 deaths annually (Supp DM, 2005). Chronic wounds and diabetes are other types of skin wounds that require skin grafting for treatment. Skin grafting is a costly industry with patient expenses exceeding 1 billion dollars annually (Supp DM, 2005). There is a clinical need to better understand the function and injury response of these skin substitutes. The goal of this project is to develop a novel device capable of creating a reproducible in vitro thermal injury to BES.

2.1 Skin

Skin is the outermost covering of the human body. The major functions of skin including acting as a protective barrier for the body's organs, and providing temperature regulation and sensing. These major functions are accomplished by the two layers that comprise skin: the epidermis and the dermis, and their associated cellular components(Ratner BD, 2004). Figure 1 below shows the layers of skin along with the cellular components that comprise each layer.

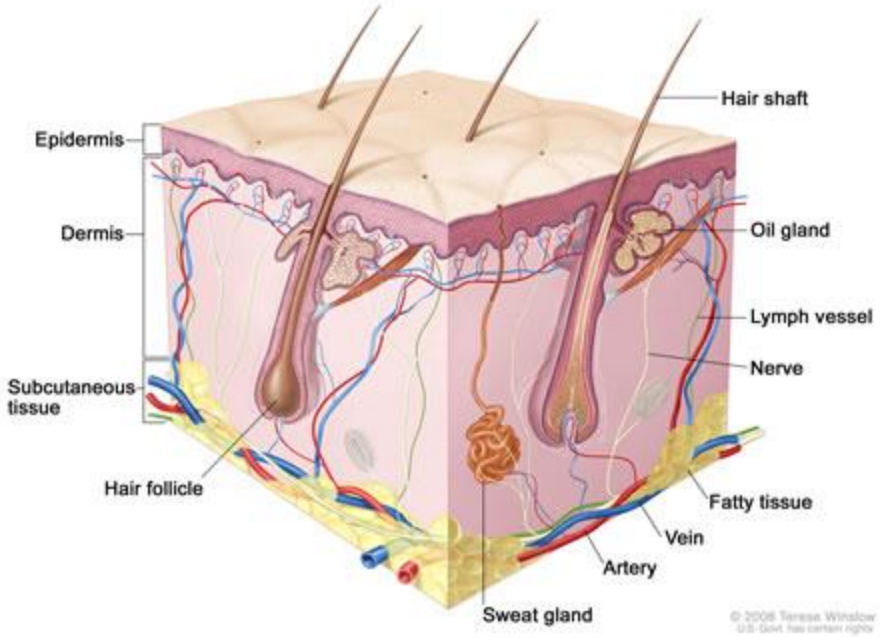

Figure 1: Layers of Skin

The outermost layer of skin is the epidermis. The major functions of the epidermal layer include waterproofing and protecting the body against harmful pathogens and chemicals in the environment. The next layer of skin inward is the dermis which is primarily responsible for providing structure integrity. The role of the dermis is to provide structural support as well as containing nerve endings that register temperature changes. The dermis is made up of connective tissue and it contains deep epidermal appendages such as hair follicles, sweat glands, sebaceous glands, as well as lymphatic vessels and blood vessels which deliver oxygen and nutrients around the body. The dermis also acts as a cushion to protect the body from applied stress and strain. (Ratner BD, 2004)

2.2 Wound Healing

Injuries to the skin pose a large risk because of the resulting loss of barrier function. When skin is wounded it elicits a series of specific responses in order to repair itself and restore normal physiologic function. An overview of the wound healing process is shown in Figure 2 below.

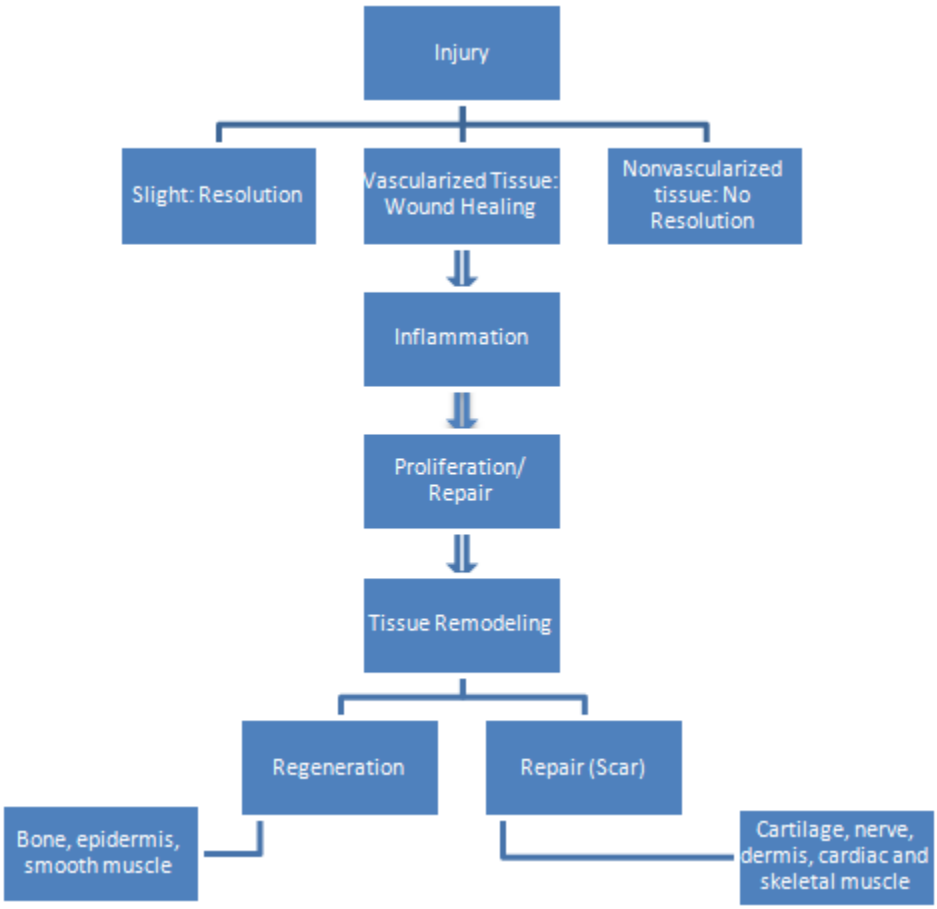

Figure 2: Wound Healing Pathway

Figure 2 outlines the three major phases in the healing of injuries to vascular tissue. The first phase is inflammation which begins almost immediately following injury. The first step in the inflammation phase is the formation of a fibrin clot. This clot acts as a provisional matrix and serves to stop bleeding, provide a temporary wound filler, and begin to recruit the cells necessary for the inflammatory response (Shakespeare, 2001). As the inflammatory phase progresses the wounded area experiences increased blood flow and an influx of various types of leukocytes. These leukocytes are responsible for recognizing healthy tissue, removing necrotic tissue, and protecting the injured area from the colonization of micro-organisms. Once this process is

completed and the tissue has been stabilized, the proliferation and repair phases can begin (Shakespeare, 2001).

During the proliferation and repair phase the body replaces the provisional matrix (clot) with new tissue. This phase can be characterized by three simultaneous processes: angiogenesis, formulation of granulation tissue, and reepithelialization. Angiogenesis is the formation of new blood vessels from existing blood vessels at the wound margin. During angiogenesis endothelial cells migrate, proliferate, and mature into a tubular organization of blood vessels necessary for maintaining the new tissue. The influx of endothelial cells involved with angiogenesis as well as the influx and proliferation of fibroblasts are the start of granulation tissue formation. This specialized tissue is named for its soft granular appearance and is made up of the newly developing blood vessels and proteoglycans and collagen deposited by the fibroblasts (Ratner BD, 2004). This collagen provides the developing tissue with structure in the form of an extracellular matrix. The final process of the proliferation and repair phase is reepithelialization. Reepithelialization is the growth and spreading of keratinocytes from the wound margins and deep epidermal appendages, to bridge the wound and provide closure. The closure of the wound by reepithelialization means that the barrier function of skin has been restored, but wound healing is not completed (Ratner BD, 2004).

The final phase of the wound healing pathway is the tissue remodeling phase. This phase can take years to complete and generally involves a decrease in the number of cells present in the wound coupled with a reorganization of the extra cellular matrix (ECM) (Ratner BD, 2004). In the skin, the extracellular matrix is comprised of the granulation tissue formed during the proliferation and repair stage of the wound healing pathway. Skin is not a regenerative organ so the reorganization of its ECM results in scar tissue, a tissue characterized by dense parallel bundles of collagen. Scar tissue is very effective in filling the voids left by injury, but its mechanical properties are only 70-80% of the original mechanical properties of the area (Ratner BD, 2004).

In some cases of wounding, the skin may not be capable of fully regenerating both the dermal and epidermal layers. Skin will always reepithelialize the epidermal layer if a source of keratinocytes is available at the wound margins. However, large wounds may not reepithelialize in a timely manner and therefore require surgical intervention. Dermal regeneration may not occur with large, severe wounds. Deep burn wounds are common examples of instances where

dermal regeneration is not possible. To better understand dermal regeneration of bioengineered skin it is necessary to first understand burn characterization.

2.3 Burns

In this project, there will be a specific focus on burn injuries. A burn injury is damage to the epidermis and dermis that is caused by heat, cold, chemicals, electricity, radiation or friction. Burn injuries are characterized by a number of factors including the severity of tissue damage, the means of burning, the contact time with the burning agent, the strength of the burning agent, and the total body surface area affected. The severity of tissue damage in a burn is further characterized using a scale of degrees of burning shown in Figure 3 below.

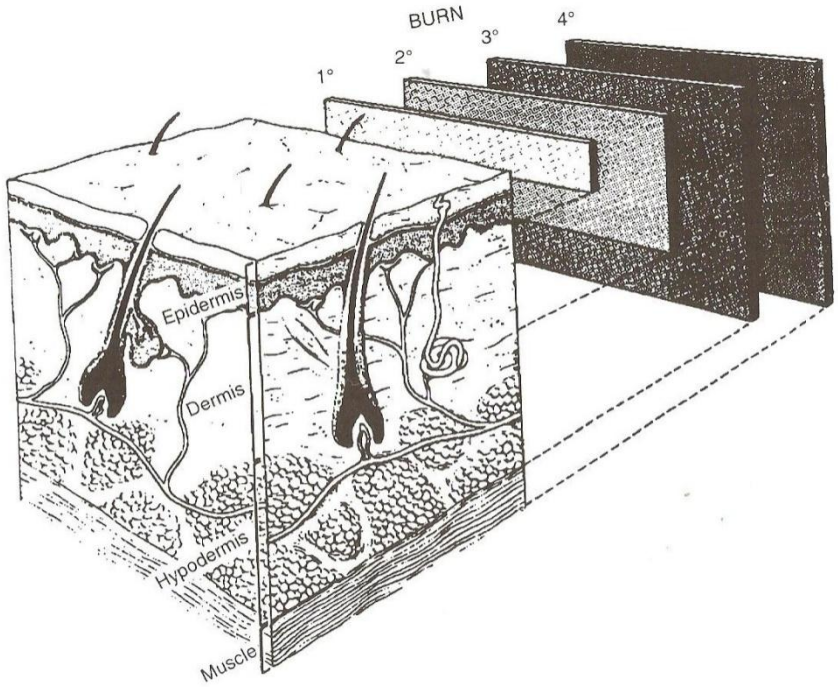

Figure 3: Degrees of Burning (Ratner BD, 2004)

As shown in Figure 3, the severity of a burn ranges from a first degree burning to fourth degree burning. All burns follow a similar wound healing pathway with only slight deviations depending on the burn's severity. A first degree burn involves damage only to the skin's epidermal layer. First degree burns are generally capable of healing by themselves through the process of reepithelialization in about ten to fourteen days following injury. Second degree burns are characterized by loss of the epidermal layer and partial loss of the dermis. Second degree burns can be further broken down into superficial and deep second degree burns. In a superficial

second degree burn some of the deep epidermal and superficial dermal layers remain intact. Following the wound healing pathway superficial second degree burns are able to spontaneously reepithelialize and show only minor dermal scarring. Deep second degree burns are characterized by full epidermal loss and damage into the deep dermal layers. Healing in deep second degree burns can result in scarring of the dermis accompanied by non-optimal function and cosmetic distortion of the affected area. Some reepithelialization is able to occur over the scarred dermal tissue in a deep second degree burn from the wound margins and the deep epidermal appendages (Ratner BD, 2004).

Third and fourth degree burns are both considered full thickness burns as they involve complete loss of the epidermal and dermal layers including the deep epidermal appendages. Fourth degree burns also involve damage to the subcutaneous tissue such as muscle, tendon, ligament, and bone. Third and fourth degree burns are major medical emergencies which completely expose the injured area to fluid loss, viruses, and bacterial growth. In these full thickness burns the dermal layer must be replaced in order to restore any functionality to the skin. Methods of replacement include autografts and more recently the use of a bioengineered skin substitute (Ratner BD, 2004).

2.4 Bioengineered Skin Substitutes

In third and fourth degree burns, the complete loss of the epidermal and dermal layers means that the body is completely exposed to bacteria and other dangerous pathogens that cause infection. The goal of a skin graft is to fill the void of tissue, restore the barrier function of the skin, and act as a scaffold for native tissue ingrowth, promote neo-dermal formation and prevent scarring. Autografts generally work well for this purpose but in the case of large or chronic wounds or when another factor limits the possibility for autografts such as disease, bioengineered skin substitutes become a very attractive option (Supp & Boyce, 2006).

There are many bioengineered skin substitutes (BES) available in today's market and their intended applications range from acting as a temporary wound dressing to a permanent skin replacement. Some BES also incorporate autologous or allogenic cells to speed up the wound healing process. The following is a review of currently available BES on the market.

Epicel® is a cultured epidermal autograft produced by Genzyme, Inc., that is commonly used to treat dermal or full thickness burns. A sheet of the patient's own keratinocytes are extracted and cultured to be used for the treatment. An important advantage of using Epicel® is

that there will be no immune rejection by the patient's body. Furthermore Epicel® carries a minimal risk of disease transmission because it is autologous (Lee, 2000). Epicel® is also commonly used for the treatment of burns that cover more than 30% of the TBSA, which means that large burn areas can be treated efficiently, using Epicel®. Unfortunately, it takes three weeks for the cultured keratinocytes to proliferate, meaning that patients must wait for the 2nd transplant. In practice, Epicel® is costly (with BES cost alone exceeding $400,000 to cover 30% of the total body surface) and difficult to handle because of its fragile nature(Lee, 2000).

Apligraf®, shown in Figure 4 below, is a dual layer composite graft produced by Organogenesis, Inc. This graft is commonly used for the treatment of 2nd and 3rd degree wounds (wounds that penetrate the dermal layer), specifically diabetic foot and venous leg ulcers that do not heal in 3-4 weeks independently (Organogenesis-Apligraf, 2009). Apligraf® is derived from neonatal foreskin and consists of an outer layer of keratinocytes and an inner layer of dermal fibroblasts in a collagen matrix that make Apligraf® structurally similar to native skin(Lee, 2000). Apligraf® also delivers essential growth factors, cells, nutrients and proteins directly to the wound site, decreasing the probability of major side effects. The dual layer graft has also been prone to layer separation, rendering the expensive therapy useless. Apligraf® also has a short shelf life; approximately five days requiring hospitals to specially order Apligraf® on a patient by patient basis(Lee, 2000).

Figure 4: Apligraf Produced by Organogenesis Inc. (Organogenesis-Apligraf, 2009)

Other commonly used skin substitutes are Dermagraft and the INTEGRA Dermal Regeneration Template produced by Advanced BioHealing Inc. and Integra Life Sciences Corporation, respectively. Dermagraft is a human fibroblast-derived dermal substitute. This substitute is created by seeding neonatal foreskin fibroblast cells onto a biocompatible polyglactin mesh scaffold (which is capable of resorbing over time in the body). Once implanted, the scaffold promotes dermal proliferation and regeneration at the wound site. The Dermagraft application is a common treatment for diabetic ulcers as the scaffold promotes fibroblast regeneration (Advanced BioHealing, 2009).

INTEGRA has a top layer of silicone (important as a provisional barrier and to prevent the loss of fluid) and a bottom dermal layer of a collagen-GAG matrix (degrades within the body). The INTEGRA scaffold is a collagen sponge which allows for dermal ingrowth and promotes dermal proliferation within the matrix. Over time, the dermal tissue grows within the INTEGRA scaffold. INTEGRA is a temporary solution as a second surgical procedure that is required to remove the silicone barrier from the matrix and add an autograft component capable of regrowing the epidermal layer of the wound. (Integra, 2009).

Some major drawbacks of many bioengineered skin substitutes are their slow reepithelialization rates. Reepithelialization is vital following a skin graft because of the need for normal skin function to be restored. For BES research, because of the immediate need for skin substitutes, it is important to study the mechanisms by which cells from the wound margin can reepithelialize and replicate these mechanisms in BES.

2.5 Reepithelialization of Natural Tissue

Reepithelialization is an important part of the wound healing process, which is required to restore an intact epidermal barrier that is lost due to various types of injuries. These injuries can vary from minor abrasions to severe skin burns that leave the epidermal layer destroyed. When skin reepithelializes, the epithelial cells, also known as keratinocytes, migrate from the margins of the wound towards the center of the wound to form a barrier between the wound and the environment. In an intact epidermis, the keratinocytes are attached to each other via desosomes, and attached extracellular matrix of the basement membrane via hemidesosomes. When a wound penetrates the epidermis, these attachments are released to allow the keratinocytes to migrate to the wound site to begin reepithelialization (Sivamani, 2007). The

keratinocytes start migrating from the wound margin within approximately 24 to 48 hours after wounding (Clark, 1996). As the keratinocytes migrate towards the center of the wound, they form a migrating epithelial tongue (MET), as shown in Figure 5 below (Laplante A, 2001).

Next, a set of keratinocytes adjacent to the wound bed proliferate by undergoing mitosis and feed the advancing epithelial tongue (Sivamani, 2007). The multi-layering and differentiation of the newly formed epidermis then can take place, followed by the regeneration of an intact basement membrane from the margin of the wound towards the center in a zipper-like fashion. The severity of the injury affects the regeneration of the basement membrane. If the injury destroys the basement membrane, the epidermal cells migrate over a temporary matrix that consists of type-I collagen, fibrin, and fibronectin. Finally, the wound area is repopulated with specialized cells that are essential in the restoration of sensory functions, pigmentation and important immune functionality (Clark, 1996).

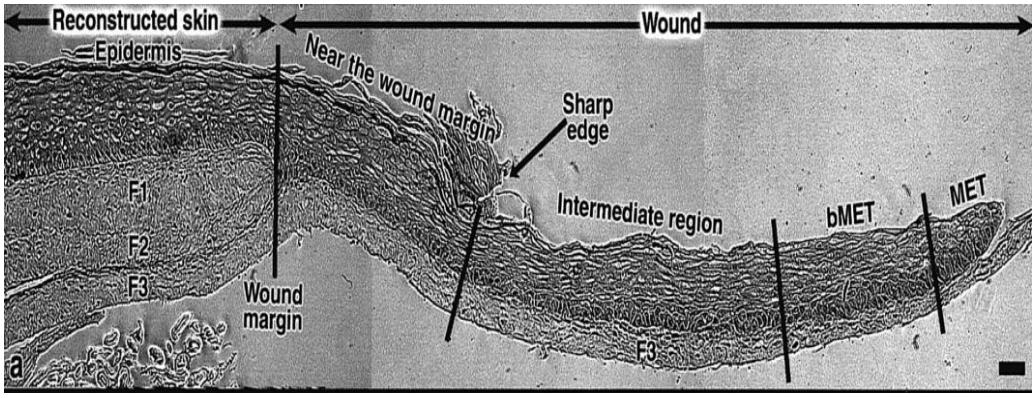

Figure 5: Keratinocytes Forming a Differentiated Epidermis(Laplante A, 2001)

Through medical research, mechanisms have been hypothesized regarding the migration of keratinocytes over the wound bed of a disrupted epidermis. In a passive sliding mechanism, the superficial layers near the wound margin (Figure 5 above) have been observed to passively advance over the wound. In a study carried out by Laplante et al (2001), it was noted that the keratinocytes of the cornified layer of the epidermis moved further towards the center of the wound than the keratinocytes of the granular layer. This demonstrated that the sliding of these layers occurred due to a pushing force, possibly due to the increase in mitotic activity (proliferation) of keratinocytes that were adjacent to the wound. This passive displacement over

the wound is possible because of the absence of resistance at the wound site due to a missing epidermis, allowing the proliferating keratinocytes to cover the empty space(Laplante A, 2001).

Another mechanism, known as the leap-frog model, involves the "crawling" of individual keratinocytes over each other at the tip of the MET (Figure 5 above). It was observed in the mentioned study that the keratinocytes at the tip of the MET were extended beyond the basal cells at the tip to reach the basal layer. The keratinocytes continue to extend towards the middle in this leap frog fashion, until cells from the wound edges meet at the center, covering the wound. Finally, it is believed that there is a possibility of the local release of growth factors such as epithelial growth factors (EGF), fibrin, collagen and fibronectin, which induce epidermal migration and proliferation(Clark, 1996)(Wearing H J, 2000) .

2.6 Reepithelialization in Amphibians

Certain amphibians such as salamanders and frogs have been known to have the capability to regenerate entire limbs and tails. Understanding the mechanisms by which these animals regenerate their body parts can aid scientists in developing mechanisms for mammalian limb regeneration and more efficient reepithelialization. Morrison et al. (2006) observed that the integral step in amphibian limb regeneration was the formation of a blastema. The blastema is a clump of mesenchymal stem cells that form at the injury site and cause rapid closure of the wound. During blastema formation, the dedifferentiation, migration, proliferation and re-differentiation of the stem cells occurs at the wound site (Morrison J I, 2006).

Mammals lack this dedifferentiation and re-differentiation of the mesenchymal stem cells after wounding and are therefore unable to regenerate limbs. Furthermore, blastema formation is not seen in mammals and is pinpointed as the key reason for their incapability to reproduce limbs (Morrison J I, 2006). Another important difference between mammal and amphibian reepithelialization is that amphibians do not form scars, whereas mammals go through scar formation depending on the severity of the wound (Morrison J I, 2006).

2.7 The Pins Lab Approach to Bioengineered Skin

The Pins lab has developed its own approach to bioengineered skin that incorporates both a dermal and epidermal layer into a skin substitute, as well as a microfabricated basal lamina analog at the interface between the layers. Motivation for this skin substitute involves overcoming the shortcomings of the Apligraf® skin substitute by adding an inter-digitated interface between the dermal and epidermal layers to increase cellular proliferation and to prevent layer separation during application (an observed problem with Apligraf®). The creation of this substitute begins with a specialized microfabricated pattern template on PDMS (polydimethylsiloxane) that creates ridges and channels. A collagen gel is then cast onto this template and allowed to polymerize. This collagen gel, patterned with a precise micro topography from the PDMS template is the basal lamina analog for the bioengineered skin substitute. This analog also provides a surface for the keratinocytes that are seeded on top of the topographic features. Depending on how the topography of the analog is changed, the topography will be identical to the initial topography of the seeded keratinocytes on top of the analog. Seen below in Figure 6, the ridges topography provides an increased surface area for epithelial cell adhesion and proliferation, allowing from more cellular growth and less chance of the layers separating during application. A collagen sponge is "glued" to the basal lamina analog using collagen gel as glue. This sponge, together with the basal analog forms the dermis of the bioengineered skin substitute. Keratinocytes are seeded onto this dermal layer and cultured. This forms an epidermal layer of the skin substitutes. The Pins' lab bioengineered substitute is shown in Figure 6 below. The micro-pattern decreases the chance that the epidermal layer will detach from the dermal layer during handling as well as increasing the surface area for the keratinocytes to adhere and proliferate onto the matrix.

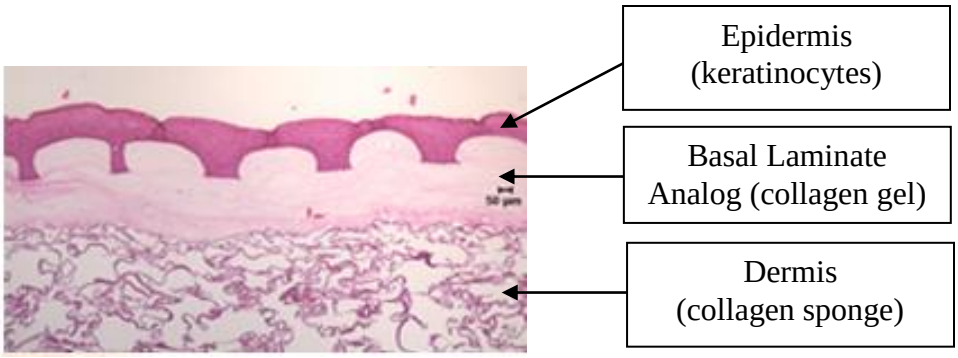

Figure 6: Bioengineered Skin Substitute (Bush, KA. Pins Lab, WPI)

The Pins lab creates consistent skin substitutes as described in the above section. To better understand the functions and to enhance rapid regeneration of these skin substitutes, it is necessary to study how these BESs heal after being wounded. To mimic a common skin wound, the BES can be thermally burned to create a full-thickness burn. This burn can then be characterized and the resulting healing mechanisms of the BES studied. Therefore it is necessary to study methods of inducing a thermal wound onto the BES in the Pins lab.

2.8 Skin Wound Models

Currently used methods of thermally burning skin in a reproducible manner were researched. Some criteria for a reproducible burn include creating a burn of consistent depth (i.e. 2^{nd} degree) as well as creating definitive wound margins around the burn so the burned area can be visually recognized. The five (5) following burn models were reviewed: metallic rod, scald-burn, chemical burn, scratch, and cryoburn. Each model is explained in depth in this section, highlighting the method used to wound the skin, as well as the feasibility of adapting the model to the project.

2.8.1 Metallic Rod Burn Model

This metallic rod model, demonstrates how to thermally injure engineered skin with hot circular metallic tubes. This study used human keratinocytes cultured on collagen matrix as the BES for in vitro reproducible thermal wound. The collagen matrices were seeded with 2×10^6 keratinocytes derived from neonatal foreskins and were fed and allowed to grow (Sobral CS, 2007).

Once the engineered skin was ready to be burned, stainless-steel rods were heated using a hot plate to 170°C (Sobral CS, 2007). Five differently sized rods were used such that the

researchers could determine if differently sized rods induced a more controlled full-thickness burn. Cylindrical rods with base diameters of 1.25, 2.5, 3.75, and 5mm wide and with a 5mm height were used to induce a controlled burn (Sobral CS, 2007). The rods were placed onto the scaffold and kept there for 30 seconds, see below in Figure 7. Only the weight of the rod supplied the pressure to the ES; therefore the smaller rod provided less pressure than the larger ones (Sobral CS, 2007).

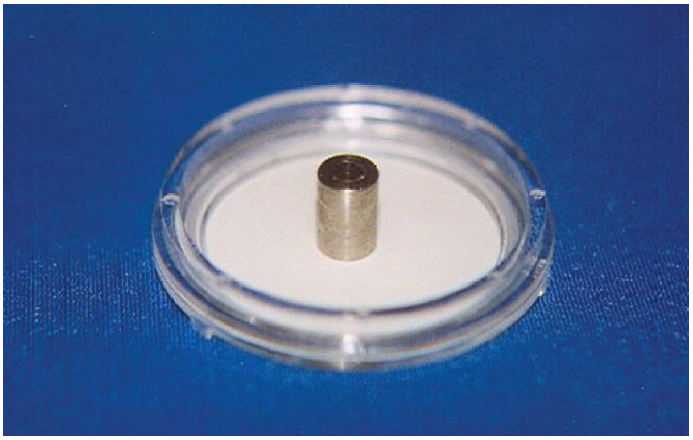

Figure 7: Metallic Rod on BES (Sobral CS, 2007)

The thermal wounds resulting from the rods were different depending on the size rod that was used. The largest (5mm) rods proved to be too heavy because the added pressure and detached part of the epithelial when the rod was removed. The smallest rod (1.25mm) proved to be vertically unstable, and fell during the burn, resulting in irregular burn patterns. The two rods left, the 2.5 and 3.75mm seemed to promote proper burning (Sobral CS, 2007). The 2.5mm rod was not heavy enough to disrupt the collagen matrix (the dermal layer equivalent) while the 3.75mm rod was capable of this disruption but, similar to the 5mm rod, accidently removed the epithelial layer when the rod was removed from the BES (Sobral CS, 2007). To analyze the burn, the authors visually examined both sides of the skin via hematoxylin and eosin histological staining and analysis. These assay stains clearly show live vs. dead cells by staining the nuclei of the cells a deep purple color while staining connective tissue surrounding the nuclei pink. The staining results of burned and control (unburned) samples can be compared to determine if the burn method kills the cells in the burn area.

The results of the study indicate that various sized rods must be utilized to determine which size properly produces a full thickness burn without wounding the matrix or removing the epithelial layer. The proper size of rod can be determined by testing and evaluating the rods on the BES.

This method appears to be useful and promising to aid in the development of the wound device. The metallic wound model is easily reproducible as the same rod can be used multiple times. Furthermore, modifying the procedure by changing the temperature or the size of the rod can easily be done, should the burn not be completed correctly. The metallic rods that were used in this study were small enough to fit the BES scaffolds that are created in the lab. Therefore, many tests can be completed with the metallic rods, i.e. different sizes, temperatures, durations to determine with best mimics a full-thickness burn of the BES.

2.8.2 Scald Burn Model

The scald-burn model by researchers at The Ohio State University involves using hot water to induce a thermal burn on the dorsal side of live mice. The mice were anesthetized prior to the procedure. The shaved dorsal side of the mouse was placed against a specially modified syringe. The syringe had a 2 x 3 cm hole cut in its side and was filled with hot water capable of scalding the mice. The mouse was held against the syringe/water for a period of 45 seconds (Cribbs RK, 1998). An image of the syringe used in this experiment is shown below in Figure 8.

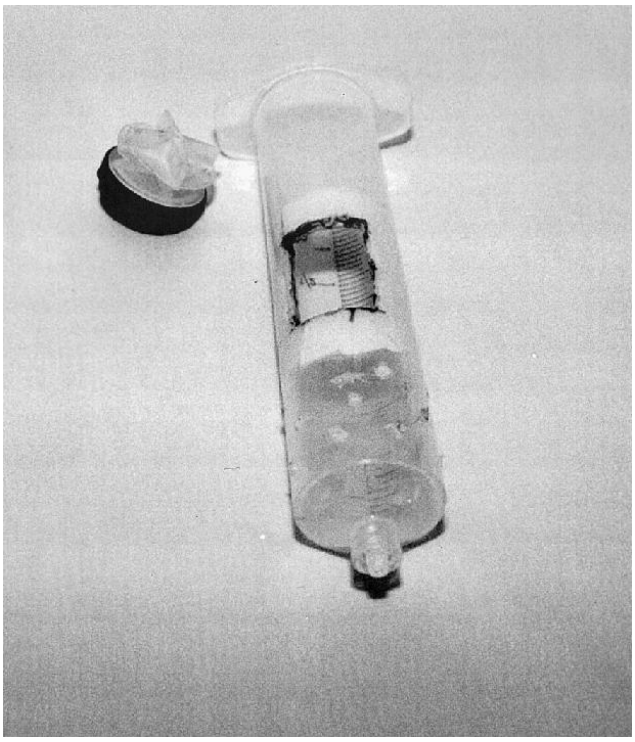

Figure 8: Scald-Burn Syringe (Cribbs RK, 1998)

The variable in this experiment was the water temperature. The water temperatures used in this experiment were 90, 80, 70, 65, and 60°C (Cribbs RK, 1998). Following the scalding of mice, researchers conclude that the 90, 80 and 70°C water induced a full thickness burn resulting in dermal necrosis 3 to 5 days post-burn. The burns were analyzed by carrying out hematoxylin and eosin histological staining and analysis on the burned portion of the mouse as well as unharmed tissue to determine relative differences between burned and native tissue. The results of the histology staining demonstrated that the burned area had fewer live nuclei (and therefore live cells) than the unburned area of the skin. A full-thickness burn was confirmed when no live cells are seen on the skin following histological staining. The 65 and 60°C water temperatures were capable of inducing a partial to full-thickness burn that did not result in dermal necrosis (Cribbs RK, 1998).

This experiment validated the ability to induce partial and full-thickness burns to live tissue with the use of hot water. Certain variables could be adjusted such as the temperature of the water along with the size of the burn created and the duration that the skin is wounded.

This application of thermal wound is difficult to apply to engineered skin substitutes in 6-well plates, especially since moving and repositioning the scaffolds may prove difficult while

maintaining the integrity of the product. While this method of burning is effective and accurate, as shown in the above study, scalding is not a viable method of solving the problem, due to the inability to apply the burn method to the BES in the 6-well plate with reasonable reproducibility.

2.8.3 Scratch Wound Model

Many type of scratch wound models exist that are capable of inducing a surface abrasion (simulating a wound) on the surface of BES. Researchers at The State University of New York at Buffalo wound BES on the bench top via this scratch wound technique. This wounding method does not involve burning the skin in any way; instead, the scientists scratched the BES with a pipette tip, inducing wound margins and depth injuries that coincide with a thermal burn (Geer DJ, 2003).

By drawing pipette tips across a piece of engineered skin, scientists were able to create a wound approximately 1.4mm in width. The goal of this wound was to replicate the damage that is created when skin is superficially wounded via a scratch or cut (Geer DJ, 2003).

In a similar research study, Buth et al. also wounded skin by designing a scratching device that consisted of forceps, capable of reproducibly scratching the engineered skin within a 6-well plate, mimicking the effects of a burn (See Figure 9 below) (Buth H, 2007). This device can be placed on multiple wells in succession to create a reproducible wound.

The method of which this model wounds skin does not apply to this project because it does not involve a thermal burn, but merely an incision that mimics cellular responses of a thermal wound. However, the reproducibility of this experiment, namely the black device made of commodity plastic i.e. polyethylene teraphthalate or polystyrene (although not specified in the paper), that rests atop the 6-well plate seen below in Figure 9, is a concept that could be applied to the project (Buth H, 2007).

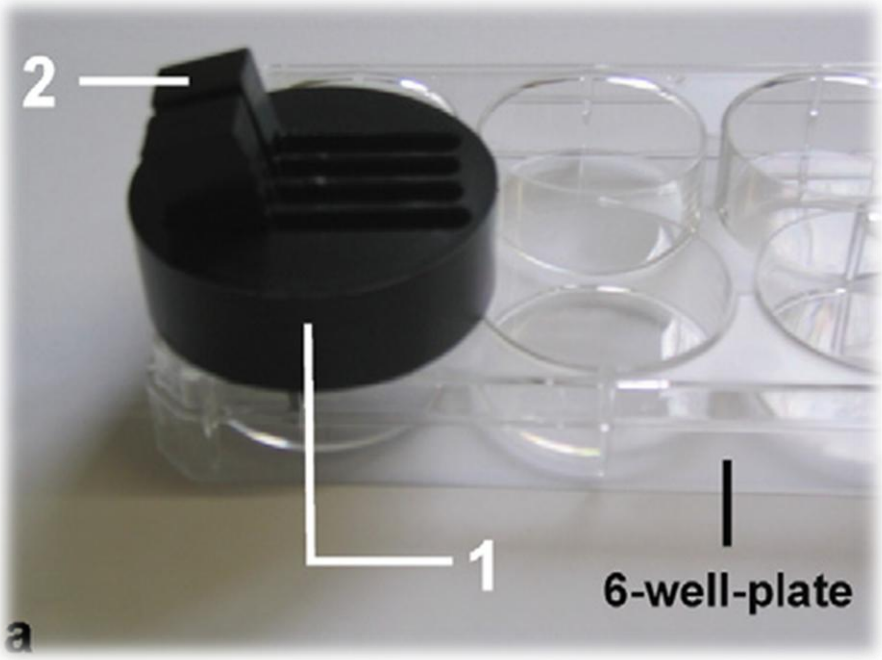

Figure 9: Scratch Wound Model Device (Buth H, 2007)

2.8.4 Chemical Burn Model

The use of chemicals to induce a burn to the skin is another method of wounding skin that has been researched at the Battelle Memorial Institute Biomedical Research Center (Price JA, 2008). This study investigated the application of bromide, a common industrial chemical, to porcine skin.

Chemicals have the ability to induce severe full-thickness burns to the skin; however, many variables prevent chemical burns from being reproducible. Because there is often no immediate reaction, chemical burns are difficult to characterize initially. Following burn presentation, it is still difficult to analyze the depth and severity of the burn because of the lack of controllable burn area that chemical burns present (Garlick J, 2006). Therefore, ensuring that a reaction will take place can be difficult to confirm with chemical burns. Furthermore, instances of inflammatory reactions may not become apparent for 24 hours post-burn. This delay-onset response of the skin further limits the reproducibility of chemical burns. Finally, many chemicals burn the skin by penetrating the skin (Garlick J, 2006). When this happens, there could be increased difficulty in stopping the chemical burn following application (Price JA, 2008). If the chemical is unreachable by a catalyst intended to stop the reaction, the ability to control and

make the burn reproducible is once again limited. As previously mentioned, the chemicals ability to penetrate and interact with the skin on a cellular level eliminated the possibility of creating, (or more importantly, controlling the creation of a defined wound margin (Thomas D, 2009). Chemicals can also affect and even contaminate the sterile environment that the BES is grown in, therefore decreasing the feasibility of this method for inducing burning.

Many of these aforementioned shortcomings resulting from chemical burns were seen in the porcine skin wounding model. There were different chemical reactions 7 days following identical treatment with bromide (Thomas D, 2009). There was also substantial overlap with groups that were treated differently with chemicals. Therefore, the use of chemical burns to injure skin is poor model for this project.

2.8.5 Analyzing Burns with Histology

Garlick et al. (2006) used histological analysis and hematoxylin and eosin staining to analyze their chemically burned skin samples for cell viability. In their work, they showed that healthy skin has an intact epidermal and dermal layer with an abundance of nuclei present in both layers. Seen below in Figure 10 is a microscopic image of healthy tissue. An abundance of nuclei (dark dots) can be seen in both the epidermal and dermal layers along with no disruption of the layers or the basal lamina (Garlick J, 2006).

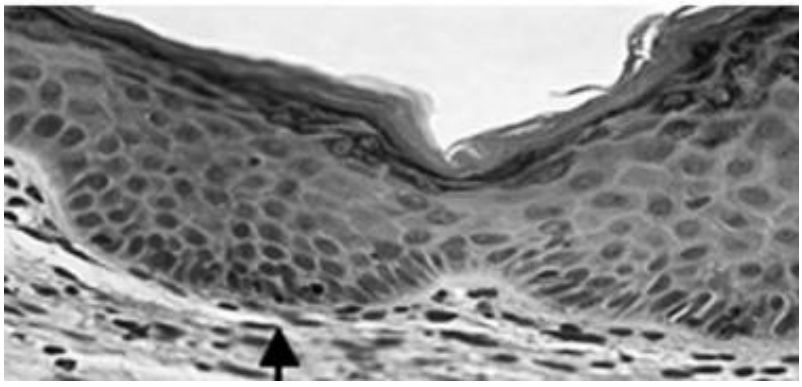

Figure 10: Healthy Skin

Garlick et al (2006) also characterize skin that has been wounded via a chemical burn. Seen below in Figure 11 is a microscopic image of burned skin. There is visible flaking-off of epidermal layers along with basal lamina disruption. Furthermore, there is a lack of nuclei (dark dots) present when compared to the unburned sample above. This lack of nuclei indicates that the

tissue is less viable then the healthy tissue. Observing the lack of nuclei into the dermal layer of the tissue can indicate a full-thickness burn (Garlick J, 2006).

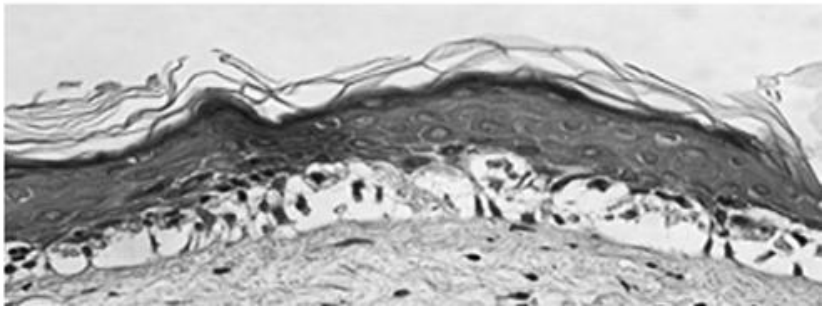

Figure 11: Burned Skin

The team will use the markers characterized by Garlick et al in their project to confirm the presence of a burn on bioengineered skin substitutes.

2.8.6 Cryoburn Model

Inducing a thermal wound on skin substitutes can also be completed by using a cryoburn application. To accomplish a thermal cryoburn of the skin, the skin must rapidly be cooled between -5˚C to -50˚C. To accomplish a cryoburn of the skin a super-cooled elements, often a cotton swab or a metallic rod (also known as a cryoprobe) is placed on the skin and held there for a period of time. Liquid nitrogen can also be sprayed onto the skin, inducing a thermal burn (MD, 2004).

A cryo-wounding model presented by researchers from the Leiden University Medical Center accomplished a full-thickness cryoburn of engineered skin substitute. A metallic rod of 2.5mm in diameter was super-cooled in liquid nitrogen and then applied to engineered skin for a period of 10 seconds (Ghalbzouri AE, 2004). As a result, the engineered skin was burned in a consistent, precise manner as expected. To measure and characterize the burn created by the cryoprobe, the researchers used a 5% Trypan blue stain to assess fibroblast cell viability for the burned sections as well as the control sections of the skin (Ghalbzouri AE, 2004).

The above research confirms that controlled liquid nitrogen-based burns are possible; however, it is necessary in this project to have the ability to characterize the burns, to determine whether or not a full-thickness, uniform burn took place. Researchers from the University of Minnesota developed a method of continuously monitoring a cryoburn in engineered skin as a

result from a cryoprobe. This system, seen below in Figure 12 uses a series of thermocouples places equidistant from the center of the skin (the location of the cryoprobe) (Han B, 2005). These thermocouples can measure and determine a complete, full-thickness burn that was burned in a consistent manner on the entire surface of the skin. The full-thickness burn was further characterized and analyzed via histological analysis of the samples.

Benefits to this method of wounding include the ability to continually characterize the burn as well as create a reproducible burn using cryology (Han B, 2005). The cryoprobe and thermocouples together prove to be excellent tools to measure and quantify a full-thickness wound to engineered skin. This method will be investigated further to better develop a cryoburn-based.

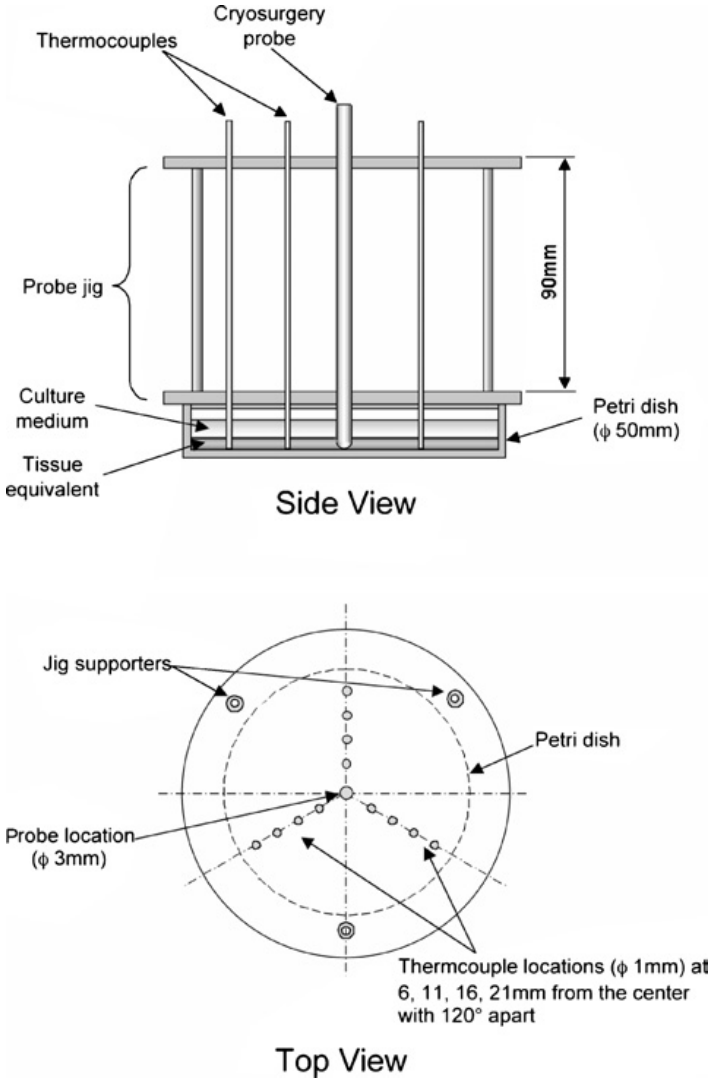

Figure 12: Schematic of Measuring Cryoburn(Han B, 2005)

2.8.7 Other Injury Models

Beyond the models discussed in the previous sections, the team investigated several other wounding models as well as the cell/tissue viability techniques employed by the scientists who created them. Table 1 shows the results of this investigation and provides a brief summary of injury and techniques used. The techniques outlined were considered when the team was creating its own wound model.

Table 1: Wound Model Matrix

Authors	Tissue Used	Injury Type	Injury Media	Cell Viability Techniques
Han et al, 2005	Engineered Tissue Equivalents (cell seeded type I collagen matrix)	Cryoinjury	Surgical probes – argon driven cryosurgical probe (CRYO-40, Endocare, Irvine, CA)	• Membrane integrity assay (Hoechst) • Histology
Bischof et al., 1997	Rat Prostate Tumor – single cell, tissue slice, in vivo	Cryoinjury	• LN_2 immersion • Copper block slam-freezing • controlled cooling on a cryomicroscope or a directional solidification stage	• Post freeze culture • Histology
Falanga et al, 2002	Human bilayered skin substitute (HBSS) = Apligraf, Organogenesis	• Meshing • Fenestration	• Skin mesher • #15 scalpel blade	• Histology • Immunohistochemistry (Ki67 to show reepithelialization) • Flow Cytometry (to show reepithelialization)
Garlick et al, 1994	Organotypic cocultures (previously	Cut	Scalpel incision	• Histolochemical staining • Immunohistochemical

	described by Organogenesis)			staining
Kandyba et al, 2010	Three-dimensional organotypic model	Scratch	Epidermal layer scratched with pipette tip	Imaging to show reepithelialization
Harrison et al, 2006	Tissue engineered human skin	Meshing	Skin mesher	• MTT to determine initial viability of composite and epidermal integrity after meshing • Histology
Ghalbzouri et al, 2004	Human skin equivalents	• Full thickness (cryoburn) wound • Superficial incision	• 2.5 mm metal rod cooled in LN and applied to HSE for 10s • Scalpel blade to create 3mm incision	• Trypan blue – incubate tissue in 5% TB for 2min then rinsed with PBS • Histology

3 Project Strategy

Using current methods and models that have been extensively studied by researchers, the design team will create a functional wounding device. They will use the design tools (outlined in the next chapter) to determine the appropriate method or model for each component of the device. In creating this device the team begin defining project hypotheses, assumptions and aims. Complete understanding of these initial topics by all involved in the design of this device will be critical to ensure the delivery of a well thought out and successful device.

3.1 Project Expectations

In order to further the development of BES, a system must be created that will deliver full thickness burns to the BES. In creating this device, the group has researched current burn models including thermal applications (hot and cold), electricity, chemical, and radiation. It is believed that treatment with extreme cold applications to the BES will produce the following desired burn wound characteristics; a full thickness burn with definitive wound margins, and no denaturation of the matrix.

To mimic the behavior of skin during a burn injury, a full-thickness burn that wounds the entire epidermal and dermal layers will be created. This burn will create definitive wound margins such that the regeneration of the skin at these margins can be studied. Even with exposure to a burn wound, the regeneration process is the primary objective for the researchers using the device. Therefore, damage to the matrix or cellular re-growth cannot happen within the burn.

3.2 Project Assumption

As stated above, the design team will treat the BES with a technique to create a reproducible burn. It is expected that this method will be able to achieve the following objectives; create a full thickness burn (on the level of second to third degree burns) with definitive wound margins without damage to the matrix. In creating this device, the design team has made certain assumption about the bioengineered skin substitutes that with serve as the final model:

- The layers of the bioengineered skin fully and correctly represent that of human skin; including a dermal, epidermal, and basal layer.
- The mechanical properties of the BES will represent that of human skin.

- The formation of these BES implements is reproducible in that the products have uniform dimensions of area and each layer of the skin.
- The histological techniques are accurate representations of the skin wound.

3.3 Project Aims and Initial Client Statement

"The goal of this project is to design and develop a strategy for reproducibly creating *in vitro* thermal injuries to bioengineered skin substitutes." (George D. Pins, PhD)

More specifically, the design team aims to design a device that will create full thickness burn wounds to BES. The design team will need to create a device that generates wounds with definitive wound margins without denaturing the matrix. These specifications are considered in the following section.

A consistent burn will be created each time by a system capable of measuring and controlling the burn time at the same temperature or concentration. Finally this device must create a burn that can be characterized and analyzed following the injury. Many live/dead stains such as Trypan blue qualitatively measure cell viability. Chemical stains, such as MTT (3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide) recognize metabolic activity on a cellular level. Although still producing quantitative results, chemical stains made be faster and easier than histological analysis. Hematoxylin and eosin histological staining and analysis on the BES is a better solution to analyze the burn because the BES in the Pins' lab is already the proper size for completing histological analysis.

4. Design

This section will focus on the process of designing the team's device. The design process is a vital component to properly engineering an acceptable device. The following sections illustrate the design process that the group followed to produce each component of the product.

4.1 Clarification of Design Goals

The design of wounding model device that the team develops will follow the specific procedures and protocols in the design process defined by the WPI BME MQP design book Engineering Design (Dym C L, 2004), as seen in Figure 13. This five-stage model will be used as a template for the remainder of the design process for the team. At each level, numbered tasks that need to be completed are listed in order to achieve the goal.

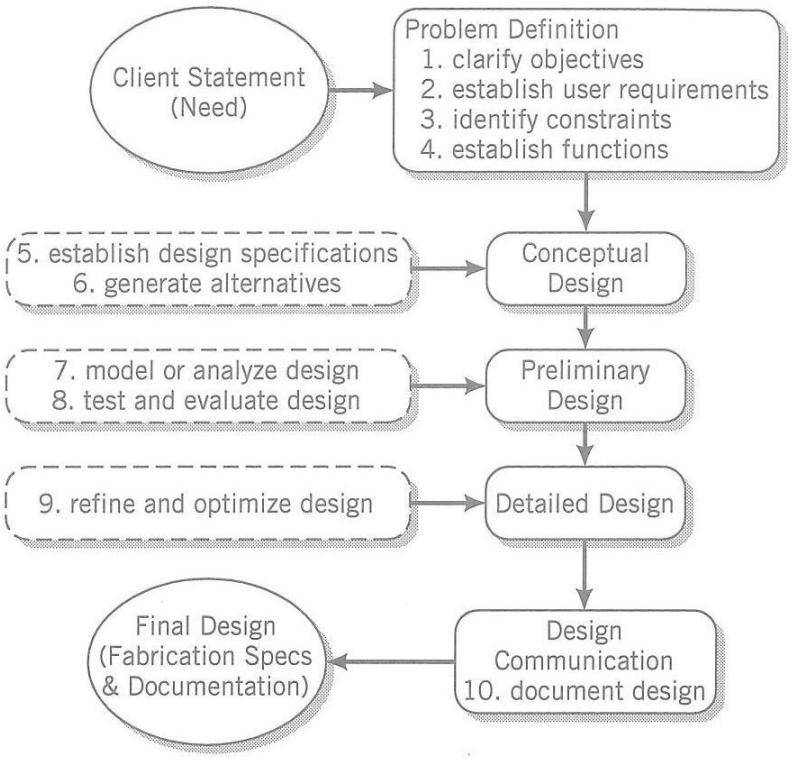

Figure 13: Five-Stage Prescriptive Model of the Design Process (Dym C L, 2004)

4.2 Objectives

In this model, it states that the first step in designing is to clarify the objectives of the project. Objectives are defined as goals that the design strives to achieve (Dym C L, 2004). After further development of the design process, the design team needed to separate the objectives of the project from the constraints, functions, and specifications. The remaining objectives are listed below in an Objectives List. There are six (6) main objectives of this project. They are listed below in section 4.2.1 Objectives List along with sub-objectives of each of the main objectives.

4.2.1 Objectives List

The following list of objectives and sub-objectives seen in Figure 14 was generated during a brainstorming session, where the team met with the project advisors Dr. George Pins and graduate candidate Amanda Clement to discuss what they each needed and wanted the device to be able to do. Based on the goals that the design team was given at the initiation of the project, the group could determine which requests from the advisors needed to be achieved. The following objectives are placed to ensure that the group meets the needs of the advisors and of the project.

Sub-objectives are identified by the lighter blue rows and are listed directly under each of the main objectives that they fall under, with the exception of "Device Should be Accurate". Many main objectives, such as safety and ease of use relate to many devices, sub-objectives further specify these broad statements into specific goals for this project.

Figure 14: Objective and Sub-objective List

4.2.2 Objectives Tree

A more effective way to clarify the objectives of the design project is to implement an objectives tree where the higher order objectives are located further to the left of the tree, and the sub-objectives of each category move out towards the right. The full objective tree is shown below in Figure 15.

The device will be considered successful only when it satisfies these objectives, but more specifically the objectives of the three stakeholders. These stakeholders have specific interests in

the device. Therefore, the design team met with the stakeholders and outlined the primary goals of the project. The stakeholders in this design project are: the client, the designer and the user. The role of the client is to present the project team with a specific problem that they would like to have the designer(s) solve. The designer(s) formulate ideas and solutions to the problem, in this project a device will result, and use various methods to validate their ideas. In testing the device it is important to tailor the device appropriately for the user of the device. Each of their objectives needs to be clearly defined before the design team begins the rigorous process of prototyping and testing.

For the wounding model device, Professor George Pins and Ph.D. candidate Amanda Clement, the advisor and co-advisor respectively, are the clients. The designers are the project group, including Jason Forte, Kathleen Most, Jennifer Sansom, and Ishita Tyagi. The user will be Amanda Blackwood and the other lab technicians who will use the device for BES analysis.

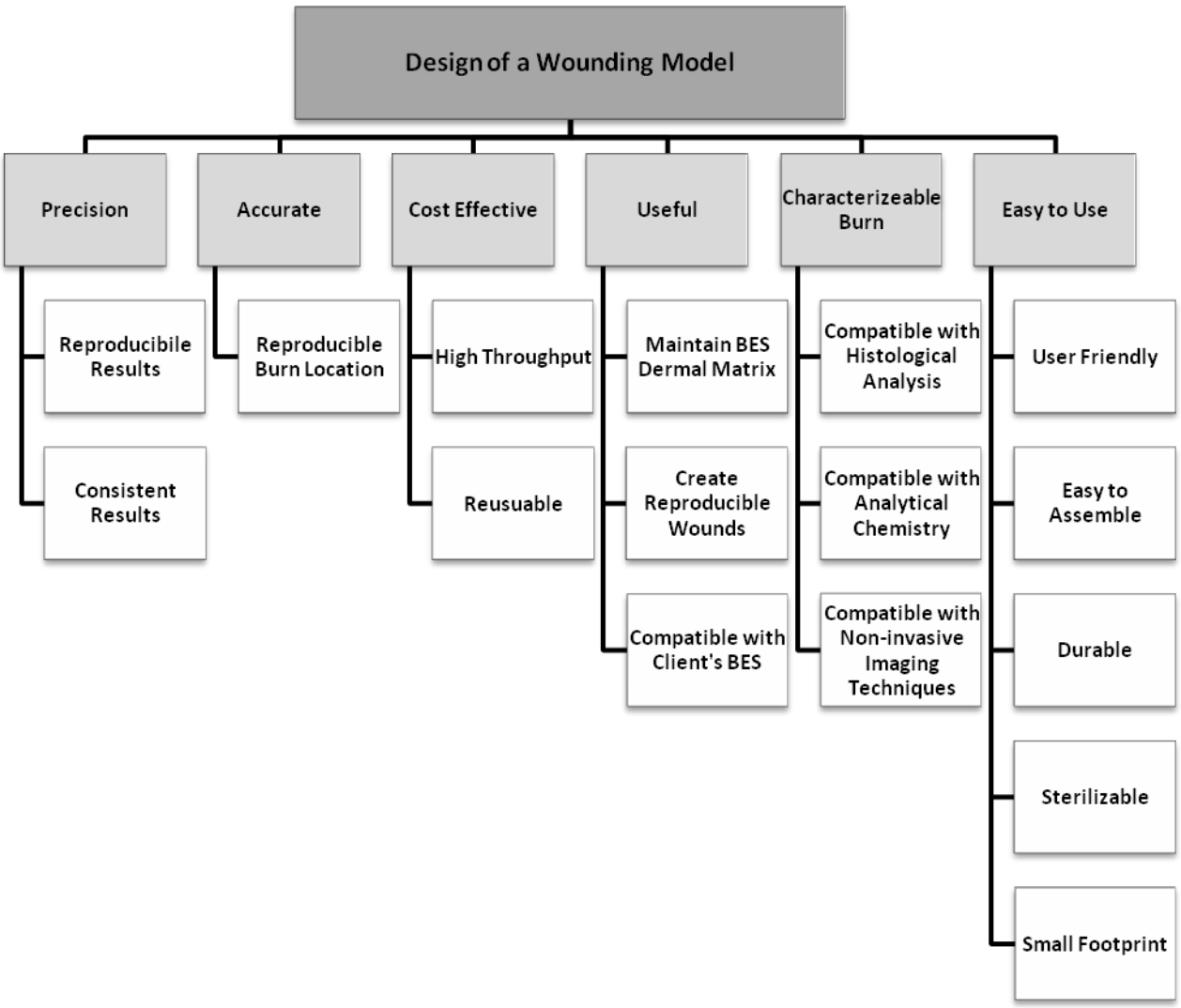

Figure 15: Objectives Tree for Wounding Model Design

4.2.3 Pairwise Comparison Charts

To understand which objectives are most important to each of the stakeholders, the design team used Pairwise Comparison Charts (PCC) as a mean to rank the objectives for the client, the designer, and the user. In each of the tables, the design team used the list of objectives in the first column and ranked it against the objectives in the top row. Specifically, one objective from the first column was compared to one object from the topmost row. If the objectives from the column and the row are the same, then the box was assigned an "X". If the objective in the first column was more important when compared to the objective in the top row, then the box was assigned a 1. If the objective in the first column was less important when compared to the objective in the top row, then the box was assigned a 0.

Finally, if both the objectives being compared shared an equal amount of importance then the box was assigned a 0.5. Once the scoring was complete, the values in each row were added up and recorded in the column labeled "Total". To complete the pair-wise comparison chart, the total value of each row was divided by the sum of the values in the "Total" column and recorded in the "Weight" column. The general equation for determining the weight of an objective is as follows:

(Objective total value) / (Sum of the "Total" column) = Weight of the objective

For instance, the device's capability to produce precise burn wounds was ranked by the client and it was weighted at 0.27 or 27% against the other objectives. The calculation follows:

(4) / (1+.5+1+.5+1)

= (4) / (15)

= 0.27

or

0.27 x 100 = 27%

From Table 2 it can be seen that, to the client, the device should be useful in creating precise, full-thickness burns that can be characterized analytically. These three objectives are equally ranked at 27%, and the remaining objectives are ranked by their importance when compared to the each other.

Table 2: Client Pairwise Comparison Chart

Top Level Objectives	Cost Effective	Device Should be Useful	Device should be accurate	Burn Model should be able to be characterized	Ease of Use	Device should be precise	Total	Weight
Cost Effective	**X**	0	1	0	1	0	2	0.13
Device Should be Useful	1	**X**	1	0.5	1	0.5	4	0.27
Device should be accurate	0	0	**X**	0	0	0	0	0
Burn Model should be able to be characterized	1	0.5	1	**X**	1	0.5	4	0.27
Ease of Use	0	0	1	0	**X**	0	1	0.07
Device should be precise	1	0.5	1	0.5	1	**X**	4	0.27

In Table 3, it can be seen that the most important objective for the designer is to create a device that is useful. When compared to the other objectives, the usefulness of the device has the highest weight of 33%. Secondly, the precision and accuracy of the device that the design team develops are weighted at 20%. Therefore, these three objectives will be highly considered during the design process.

Table 3: Designer Pairwise Comparison Chart

Top Level Objectives	Cost Effective	Device Should be Useful	Device should be accurate	Burn Model should be able to be characterized	Ease of Use	Device should be precise	Total	Weight
Cost Effective	X	0	0	0	0	0	0	0
Device Should be Useful	1	X	1	1	1	1	5	0.33
Device should be accurate	1	0	X	1	.5	.5	3	0.20
Burn Model should be able to be characterized	1	0	0	X	1	.5	2.5	0.17
Ease of Use	1	0	.5	0	X	0	1.5	0.10
Device should be precise	1	0	.5	.5	1	X	3	0.20

Additional pairwise comparison charts, including those for sub-objectives of the main objectives, completed by the client, designers, and user can be found in Appendices B, C, and D respectively.

4.2.4 Weighted Objectives Tree

From the pairwise comparison charts, the weighted objectives tree seen in Figure 13 below was created in order to assign varying degrees of importance to the main objectives and the sub-objectives. The higher numeric value a certain objective has, the more important it is to the effectiveness of the project. The entire project is assigned a score out of 1.0, which can also be represented as percentages (100%). Of the 100%, the six main objectives were assigned a percentage of that total depending on their relative importance. For example “cost effectiveness” was assigned a score of 0.13 (13%) while “easy to use” was assigned a value of 0.07 (7%) implying that when comparing cost effectiveness to ease of use, it is more important that the device is cost effective. The sub-objectives off each main objective are assigned a percent value of the score that the main objective achieves. For example, the “precise” main objective was given a score of 27%. The two sub-objectives of the precision objective were deemed to be equally important. Therefore each of them received 50% of the score awarded to the “precise” objective.

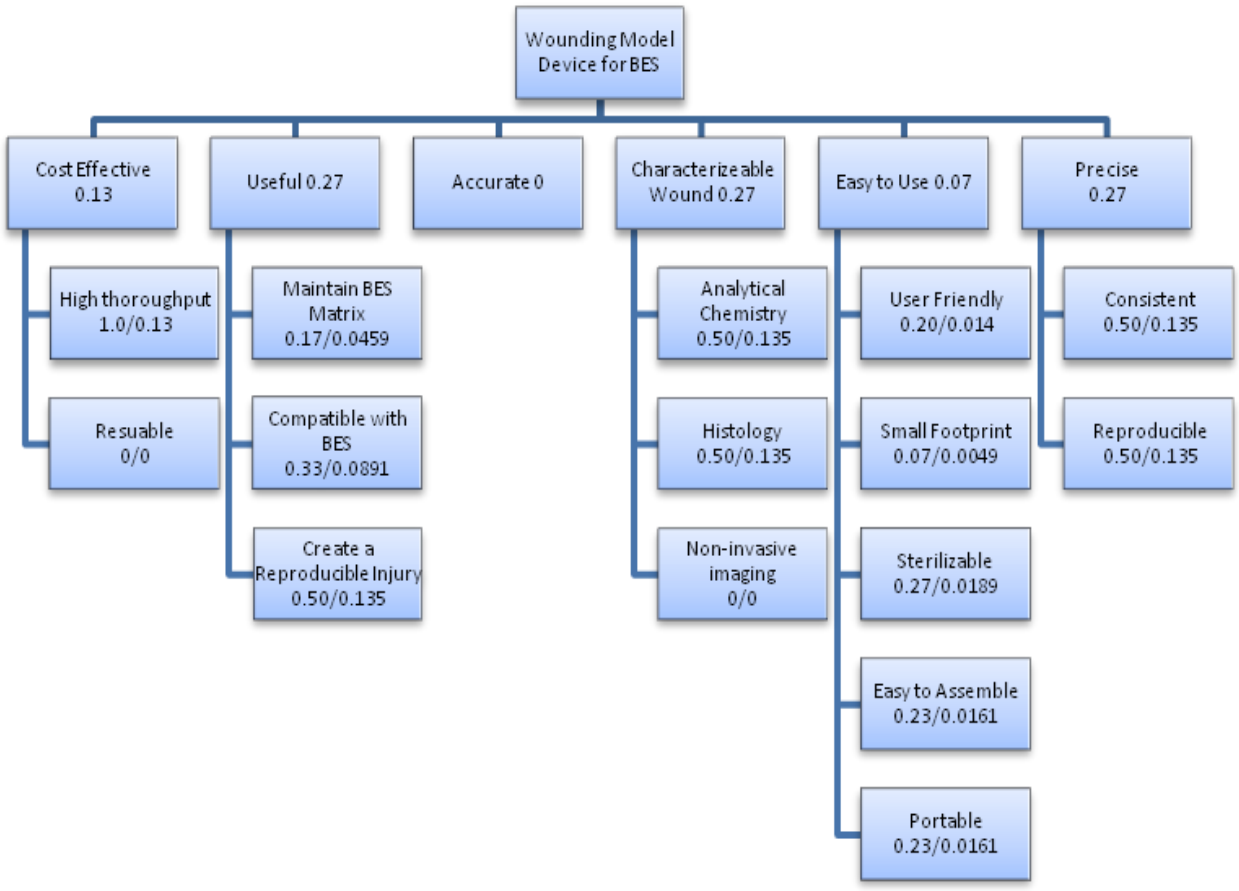

Figure 16: Weighted Objectives Tree

4.3 Identify Constraints

In designing a device that achieves the objectives and goals of all the stakeholders, there are constraints or strict limits that the design team must consider in order for the device to be acceptable (Dym C L, 2004). These constraints are derived both from the project description developed by the clients and from the weekly meetings between the advisors and design team.

4.3.1 List of Constraints

In these meetings, the group started by asking the simplest of questions like, "How should this wounding model be used?" and "What is the most that the client is willing to spend on this device?", to the more specific questions like, "How much weight should be exposed to the skin to allow for contact pressure without damaging the skin or BES?" and "Should the rod used to deliver the burn be made of aluminum, stainless steel, ceramic, or other metals?". The resulting list of project constraints is shown below:

- Time-completed by the end of 2009-2010 school year
- Design Budget –approximately $1000
- Device must fit the specific size and shape of the Pins Lab BES
- Safe-must be safe for user and non-cytotoxic
    - Burn component must include non-toxic materials that could be ingested by user
    - May not have sharp edges
    - Must not pinch or clamp user or skin during operation
- Must fit Dr. Pins' BES dimensions (approximately 1cm by 2cm surface)
- Burn component must be made of a material that does not corrode (Medical Grade)
- Testing must be done *in vitro*
- Device must be sterilizable

4.3.2 Design Specifications

In addition to identifying the constraints that will govern the designed device, the answers to these questions helped to establish design specifications. More specifically, if the device must be safe to the user, then how high should the device be to allow for the user to reach and monitor the device and burn? Also how much pressure is required to provide the optimal burn?

These specifications are supposed to assist the team in making necessary design choices for the device. The team will keep in mind the following specification while developing preliminary conceptual and future alternative designs that will lead them to their final design for the wounding model.

- Height of device should not exceed 16" to allow for clearance on a laminar flow hood auto and functionality for user accessibility
- Must produce a full-thickness burn that damages the entire thickness of the epidermis and dermis
- The burn experienced by the skin must fit the dimensions of the burn device to ensure reproducibility (a burn of approximately 1/2cm^2 in the center of the BES)
- The exterior width and length dimensions of the device are limited to 2" beyond the dimensions of a six-well plate, to limit the use of unnecessary material

4.4 Establish Functions List

There are also specific functions that the design must accomplish during the use of the device. The functions are shown below:

- Able to produce a full-thickness burn through the Pins Lab BES
- Able to control burn time on the BES
- Pressure experienced by BES must result in full evenly distributed contact
- Able to control burn rate
- Able to create a reproducible burn on different BES samples
- Monitor burning of BES

4.4.1Stakeholder's Constraints and Functions

The stakeholders, (Designer, Client, and User) often has different views on constraints and function. For the constraints outlined above, some constraints, such as time and cost are irrelevant to the user, as it does not affect the device and/or how it functions. Safety, on the other hand, is essential for the user. These differences in the importance of the constraints are shown in Table 4.

Table 4: Stakeholder Constraints

Constraint	Importance to the Designer	Importance to the Client	Importance to the User
Time	X	X	
Cost/budget	X	X	
Device must interact with BES in a controlled manner	X	X	X
Safe	X		X
Must fit client's BES dimensions	X	X	X
In vitro testing	X		X

Stakeholders can also differ in their relative importance of the functions of the device. For example, the client does not care whether or not the burn can be monitored, since the end results are the important goal for the client. However, the user would like the ability to monitor the burn, as this could increase the likelihood of positive results. A table of the Stakeholders Functions is shown in Table 5.

Table 5: Stakeholder Functions

Function	Importance to the Designer	Importance to the Client	Importance to the User
Produce a full-thickness burn	X	X	X
Control burn time	X	X	X
Control burn rate	X	X	X
Create a reproducible burn	X	X	X
Monitor burning of BES	X	X	X

The design team concluded that functions such as the device's ability to produce a full-thickness burn, control the burn time, control the burn rate and create a reproducible burn are essential to the designer, client and user.

4.5 Development of Revised Client Statement

After the design team clarified the objectives, constraints, and functions, a revised client statement was written. It encapsulates the top-level objectives, the necessary information, and the initial problem statement set by the client.

> The design team will design, build, and test a device that will create reproducible burns on Bioengineered Skin Substitutes (BES) from Professor Pins' laboratory. The device should generate a consistent, full thickness burn with definitive wound margins without damaging the underlying matrix. The burn device must create a burn roughly 5 mm (+/-1mm) in diameter. All tests will be completed *in vitro* on the Pins laboratory BES which has approximate dimensions of 25mm x 15mm. The device will produce an accurate burn that can be characterized through hematoxylin and eosin histological

staining and analysis. The device should also be easy to use, sterilizable, durable, and reusable. The construction of the device must be completed within the budget specified by the client.

5. Alternative Designs

In order to progress with the design process and move toward the final development of a device, the design team needed to identify a variety of different solutions to the problem. The solutions that the team generated are listed below as design alternatives. Using a variety of testing and evaluation methods the team was able to identify the alternative designs in terms of feasibility towards accomplishing this project.

5.1 Conceptual Designs

The following sections describe the process for developing design alternatives, descriptions of each alternative, methods for evaluation, and an overview of the chosen design.

5.1.1. Development of Conceptual Design Alternatives

To develop a comprehensive list of alternative designs and methods for creating a wounding model, the team and stakeholders held a brainstorming session. During this session six primary design alternatives were developed. These primary design alternatives focused on the creation of a full thickness, reproducible wound with definitive wound margins. Ideas were also established for several accessory type items that would help to control the burn time, rate, shape and size, monitor burning, and create several simultaneous burns. Following the brainstorming session, the team performed further investigation of each primary design alternatives and accessory alternatives. Pictures, descriptions, and list of pros and cons can be found below for each design.

5.1.2 Primary Conceptual Design Alternatives

Cryoprobe

The cryoprobe design consists of a metallic rod that is held with tongs and dipped in liquid nitrogen to cool. The cooled cryoprobe is then applied to the bioengineered skin substitute for a specific amount of time. Following removal of the cryoprobe, the skin can be analyzed to determine the severity of the damage to the tissue. An illustration of the cryoprobe can be seen in Figure 17.

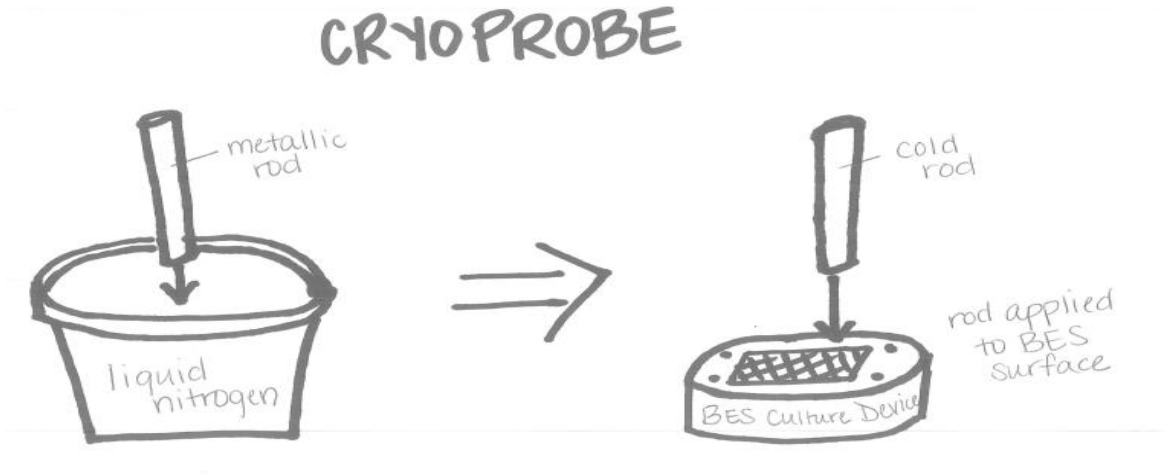

Figure 17: Cryoprobe Conceptual Design

Seen below in Table 6 are the pros and cons associated with the Cryoprobe device method of burning BES.

Table 6: Pros and Cons-Cryoprobe

Cryoprobe	
Pros	**Cons**
• Controlled shape and size burn • Reproducible • Easy to manufacture • Portable • Easy to sterilize	• Potential for rapid heat loss • Potentially difficult to handle

Hot Plate Disks

The hot plate disk method involves placing small disks on a hot plate set at a range of temperatures. After the disks have been brought to a particular temperature, they are removed with forceps and placed onto the BES for a controlled period of time. Once appropriate burn conformation is achieved, the discs can be removed from the BES with forceps and then the burn can be analyzed. An illustration of the hot plate disks primary design alternative can be seen in Figure 18.

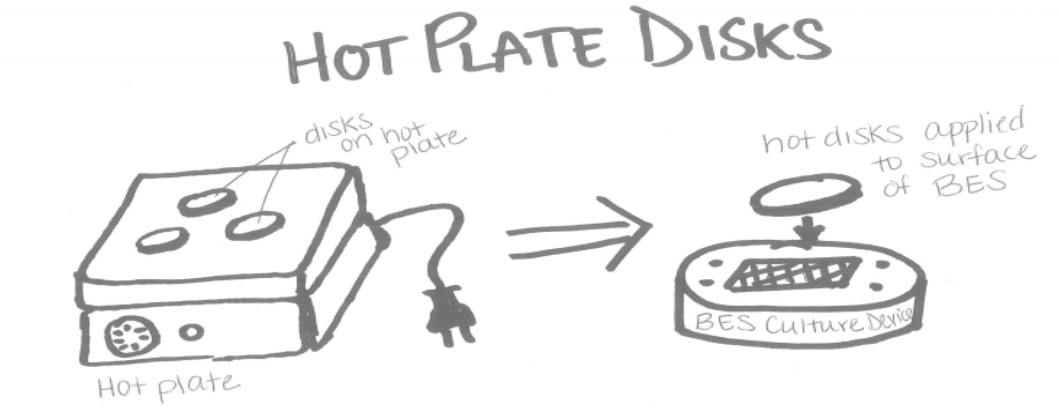

Figure 18: Hot Plate Disks Conceptual Design

Seen below in Table 7 are the pros and cons associated with the Hot Plate Disk method of burning the BES.

Table 7: Pros and Cons- Hot Plate Disks

Hot Plate Disks	
Pros	**Cons**
• Controlled shape and size burn • Easy to manufacture • Portable	• Difficult to move disks from hot plate to skin • Difficult to remove disks from skin • Difficult to sterilize • Potential for rapid heat loss during

	application • Difficult to control temperature of disk

Toaster Oven

The toaster oven primary design alternative utilizes a separate heating element that delivers forced hot air down through the shaft of the cylinder to a heating coil located at the tip of the cylinder. To burn the BES, the heating probe will be suspended over the covered BES samples resting in a 6-well plate. The appropriate burn time and temperature will be determined by testing and application on model heat transfer equations. This design alternative also incorporates a "mask" with single or multiple perforated "sunspots" that will rest over the BES samples in order to create defined wound margins. An illustration of the toaster oven primary design alternative can be seen in Figure 19.

Figure 19: Toaster Oven Conceptual Design

Seen below in Table 8 are the pros and cons of the Toaster Oven method for burning BES.

Table 8: Pros and Cons - Toaster Oven

Toaster Oven	
Pros	**Cons**
• Applicable heat transfer models and equations • Portable	• Expensive • Hard to control burn areas without masks • Variations in temperature during delivery • Requires a heat source • Difficult to sterilize • Potentially dangerous to user • Difficult to manufacture

Cryoshower

The cryoshower design would consist of a hollow cryoprobe that would be able to hold liquid nitrogen cartridges. The cartridges would be placed in the hollow cryoshower tube which has holes in the tip to allow for a shower-like application of the liquid nitrogen onto the surface of the bioengineered skin substitute (BES). The liquid nitrogen would be sprayed onto the BES for a certain amount of time by holding down the spring-loaded "pen" top for the desired time. The tip of the cryoshower pen would be held a certain distance away from the BES and this distance, along with the time and pressure, can be determined through trial and error. An illustration of the cryoshower primary design alternative can be seen in Figure 20.

Figure 20: Cryoshower Conceptual Design

Seen below in Table 9 are the pros and cons of the Cryoshower BES burning method.

Table 9: Pros and Cons-Cryoshower

Cryoshower	
Pros	**Cons**
• Easy to use • Portable • Prefabricated cartridges • Minimal temperature loss	• Expensive • Hard to control burn area • Difficult to determine appropriate time and pressure • Difficult to sterilize • Potentially dangerous to user • Difficult to manufacture

	• Requires user assembly

Chemical Filter Paper

The chemical filter paper design has a multi step application process. The first step is to cut numerous identical sized pieces of filter paper (approximately 5mm in diameter) which are capable of fitting in the center of the BES. Second, the paper will be coated in a controlled amount of chemical agent capable of generating a reproducible burn. Finally, the chemical coated paper will be applied to the surface of the BES for a set period of time and then removed with forceps. The burn can then be characterized by a variety of cell viability assays. An illustration of the chemical filter paper primary design alternative can be seen in Figure 21.

Figure 21: Chemical Filter Paper Conceptual Design

Seen below in Table 10 are the pros and cons of the Chemical Filter Paper BES burning method.

Table 10: Pros and Cons-Chemical Filter Paper

Chemical Filter Paper	
Pros	**Cons**
• Reproducible size of paper and amount of chemical	• Hard to control burn area • Potentially dangerous to user

• Affordable • Portable • Sterilizable	• No definitive wound margins • Possibility for adverse reactions with cell viability assays

Laser

The laser system would introduce the use of light emission (radiation). Like the toaster oven design, the laser would utilize masks to control the burn shape and size. To ensure that the radiation only passes through the holes in the masks, the mask material needs to be able block the light emitted by the laser. The UV-light induces a characterizeable thermal wound onto the surface of the BES. An illustration of the chemical filter paper primary design alternative can be seen in Figure 22.

Figure 22: Laser Conceptual Design

Seen below in Table 11 are the pros and cons of the Laser method for burning BES

Table 11: Pros and Cons- Laser

Laser	
Pros	**Cons**
• Can control heat at specific wavelengths	• Expensive

• No Direct contact with BES • Controlled burn area • Definitive wound margins	• High loss of energy and heat • Requires assembly • Requires additional user training/knowledge

5.1.3. Mechanisms for Controlling and Monitoring Burning in Design Alternatives

Masks

The use of masks in this device could be useful as a means of controlling the size and shape of the created burn. The masks are placed over the bioengineered skin substitute so a specific portion is exposed to the burning agent. In some cases masks could also provide vertical placement and stability to the burning device. Masks could be made out of a variety of materials depending on the desired burning method chosen ranging from ceramic to metal to plastic. An illustration of masks with different sizes and hole shapes can be seen in Figure 23. The masks in the illustration are intended to integrate with the six-well plates that the current BES culture device utilizes.

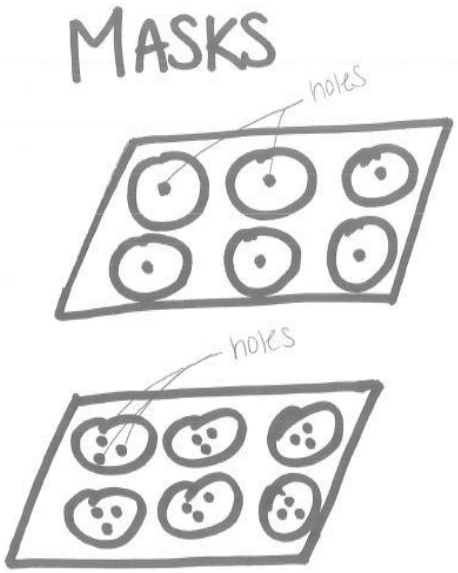

Figure 23: Masks Conceptual Design

Seen below in Table 12 are the pros and cons of the mask mechanisms that can potentially control the burning of the BES.

Table 12: Pros and Cons- Masks

Masks	
Pros	**Cons**
• Control shape and size of burning • Provide physical stability for burning device • Help to create definitive wound margins • Easy to sterilize (material dependent) • Inexpensive (material dependent)	• Additional parts • Does not work with all proposed burning methods

• Easy to manufacture	

Probe Hanger

The probe hanger is a support mechanism and stabilizing device that can potentially minimize human error during the burning procedure. The probe hanger will be able to support one or several of the burning devices (i.e. cryoprobe, cryoshower, toaster oven, and laser) as well as a six well plate containing several BES culture devices. The probe hanger could have several layers useful for support. Similarly, the probe hanger could have a collar-like system for stabilizing vertical probes. The probe hanger will allow for controlled size, shape, and placement of burns on the bioengineered skin substitute. An illustration of the probe hanger can be seen in Figure 24. The probe hanger in the illustration is intended to work with the six-well plates utilized by the current BES culture system.

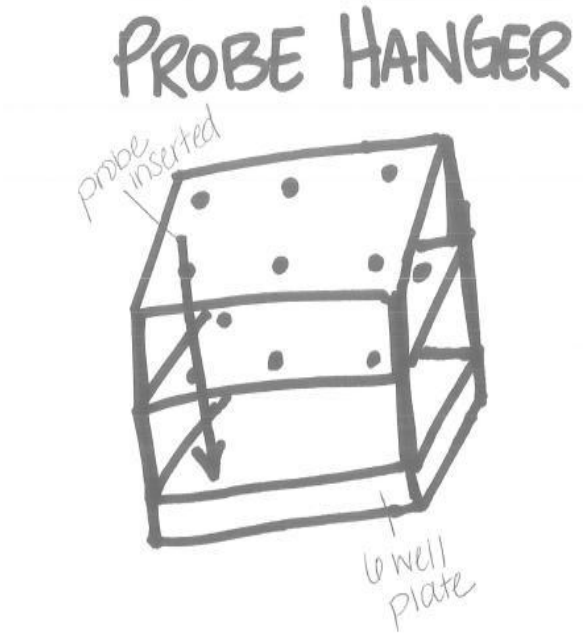

Figure 24: Probe Hanger Conceptual Design

Seen below in Table 13 are the pros and cons of the probe hanger device that can potentially support and stabilize the device during the burning process of the BES.

Table 13: Pros and Cons- Probe Hanger

Probe Hanger	
Pros	**Cons**
• Control shape and size of burning • Provide physical stability for burning device • Help to create definitive wound margins • Works with currently used BES culture device system	• Additional parts • Does not work with all proposed burning methods • Bulky design • Potentially difficult to manufacture • Potentially difficult to sterilize

Pulley System

The pulley system design would use a pulley to lower the burning device to the surface of the BES and then retract it from the surface after a specific amount of time. The entire pulley system could be attached to a timer to minimize human error. Instead of using a thread or a rope as the extension for the burning device, a thin sheet of metal could be wrapped around the pulley. This might help in stabilizing the device and avoid any dangling or swinging while it is being lowered. An illustration of the pulley system can be seen in Figure 25.

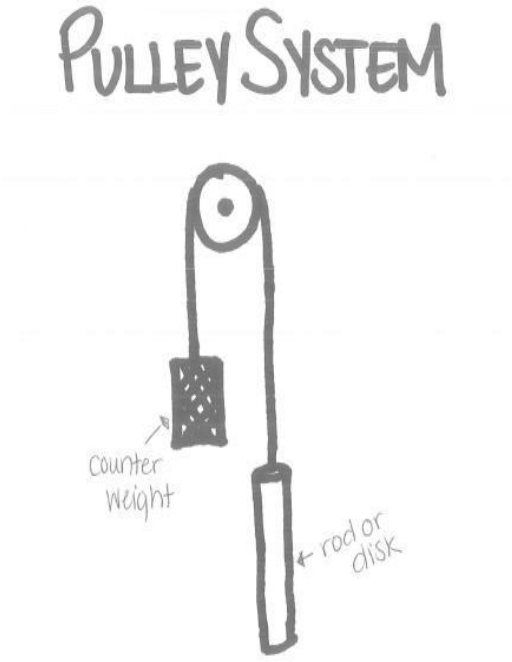

Figure 25: Pulley System Conceptual Design

Seen below in Table 14 are the pros and cons associated with the pulley system.

Table 14: Pros and Cons-Pulley System

Pulley System	
Pros	**Cons**
• Easy to assemble • Controlled delivery • Automated • Cost effective • Can be used with multiple burn devices • Works with currently used BES culture device system	• Additional parts • Does not work with all proposed burning methods • Bulky design • Potentially difficult to sterilize • Temperature loss during movement • Potential for mechanical failure

Automated Spring System

To control the time of burning an automated spring system could be employed. Similar to the mechanism used by a retractable pen, this system would allow the device to be "engaged" (spring compressed) to burn the skin, and then "released" (spring relaxed) after a desired amount of time. An illustration of the automated spring system can be seen in Figure 26.

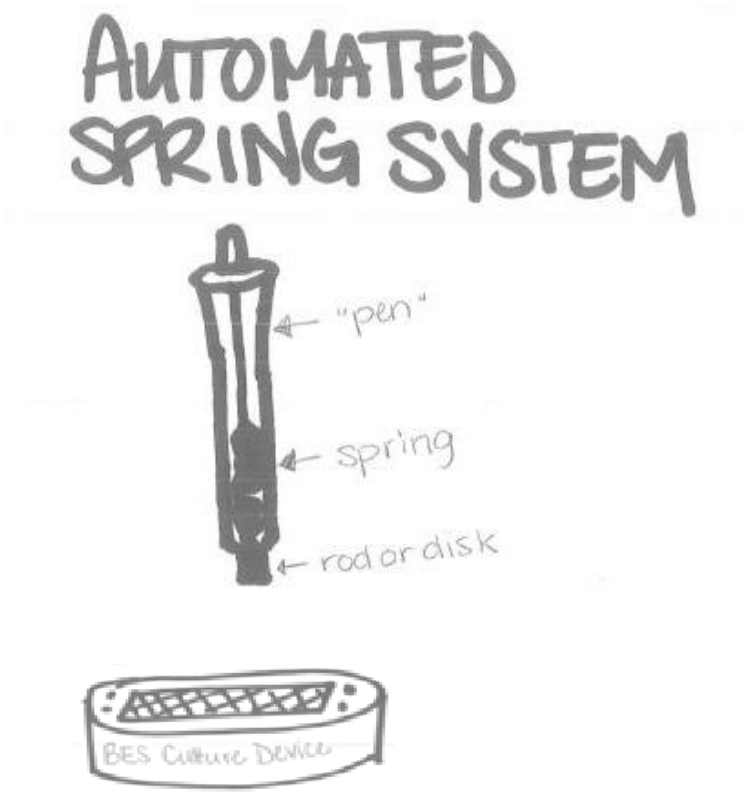

Figure 26: Automated Spring System Conceptual Design

Seen below in Table 15 are the pros and cons associated with the automated spring system.

Table 15: Pros and Cons- Automated Spring System

Automated Spring System	
Pros	**Cons**
• Easy to use • Controlled delivery • Automated • Can be used with multiple burn devices • Works with currently used BES culture device system	• Additional parts • Does not work with all proposed burning methods • Potentially difficult to sterilize • Potential for mechanical failure

Dwell Mechanism

The dwell mechanism can be used to control the burn time in any of the probe-like design alternatives. A dwell (or commonly a "double-dwell" mechanism) can be used to move the burning element up and down at a constant rate, with intermittent pauses. Therefore, the burning tool, for example the cryoprobe can be lowered to the BES surface, held there for a pre-determined period of time, and then pulled up away from the BES. This mechanism incorporates a motor rotating around a rod in a 360° motion. An illustration of the dwell mechanism can be seen in Figure 27.

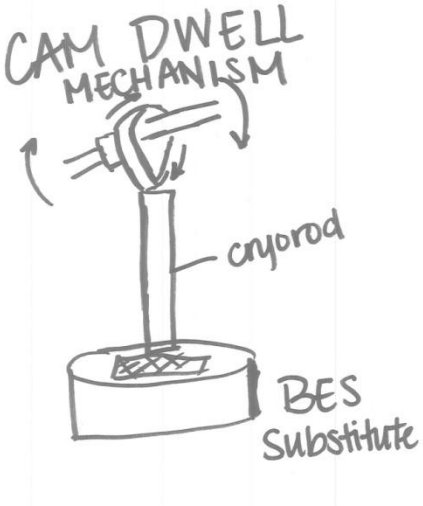

Figure 27: Dwell Mechanism Conceptual Design

Seen below in Table 16 are the pros and cons of the Dwell Mechanism.

Table 16: Pros and Cons- Dwell Mechanism

Dwell Mechanism	
Pros	**Cons**
• Easy to use • Controlled delivery • Automated • Can be used with multiple burn devices • Works with currently used BES culture device system • Adjustable for different desired burn times	• Additional parts • Does not work with all proposed burning methods • Potentially difficult to sterilize • Potential for mechanical failure • Does not integrate with thermal applications well

Attachable Weights

The main purpose of using attachable weights on a particular system is to control the pressure of the probe on the BES surface. Probes can use the weights as an attachment to control pressure. A desired weight would be placed onto the probe to apply a constant pressure for the desired amount of time and would then be removed from the block to release the pressure. An illustration of the attachable weights can be seen in Figure 28.

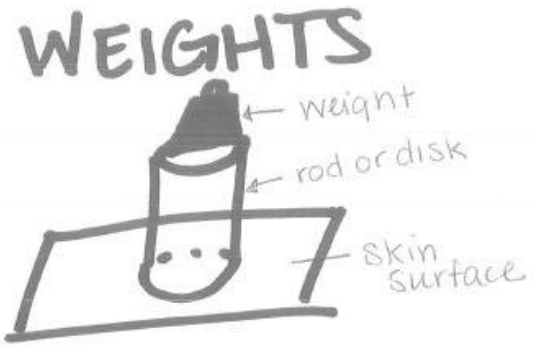

Figure 28: Attachable Weights Conceptual Design

Seen below in Table 17 are the pros and cons of the Attachable Weights system.

Table 17: Pros and Cons- Attachable Weights

Attachable Weights	
Pros	**Cons**
• Easy to use • Controlled delivery • Easy to monitor • Can be used with multiple burn devices • Works with currently used BES culture device system • Adjustable for different desired burn times	• Difficult to determine weight needed to achieve desired results • Does not work with all proposed burning methods • Potentially difficult to sterilize

Pressure Sensors

Pressure sensors can be used along with most of the design alternatives to ensure that the pressure exerted on the BES is constant. This could be accomplished by either placing pressure sensors at the tip of the design or at the suspension interface in the probe hanger device. An illustration of the use of pressure sensors can be seen in Figure 29.

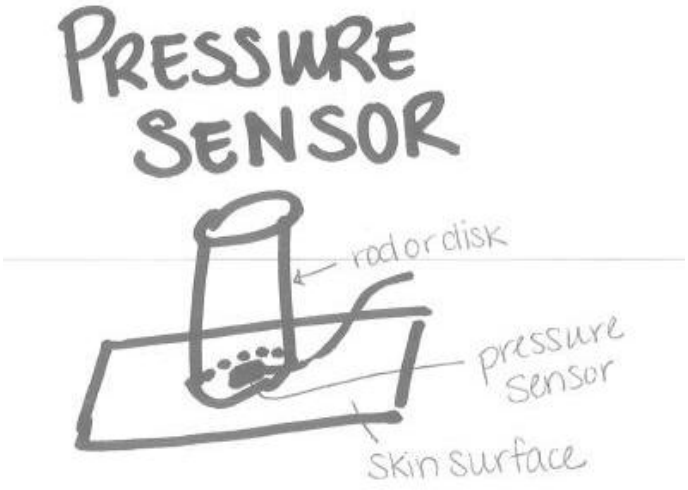

Figure 29: Pressure Sensors Conceptual Design

Seen below in Table 18 are the pros and cons of the pressure sensors method.

Table 18: Pros and Cons - Pressure Sensors

Pressure Sensors	
Pros	**Cons**
• Easy to acquire • Accurate pressure reading	• At BES/device interface could interfere with burning pattern • Added expense • Require computer programming

Thermostat Switch

A thermostat switch can be used to accurately control the temperature delivered to the BES samples. By creating a set point for the switch, the group can ensure that a specific temperature is reached. With the implementation of the thermostat switch, the heat and cold systems can be made to turn off once a temperature is reached. An illustration of the use of a thermostat switch can be seen in Figure 30.

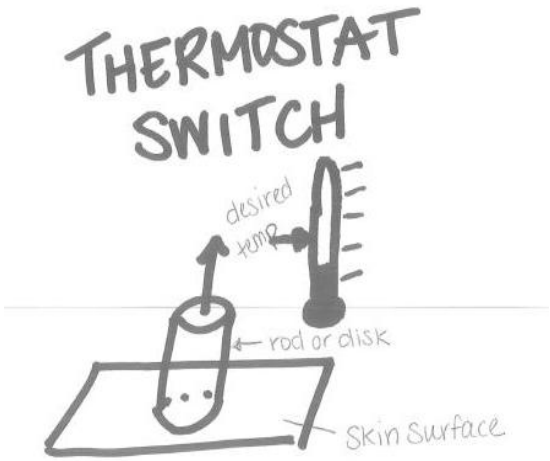

Figure 30: Thermostat Switch Conceptual Design

Seen below in Table 19 are the pros and cons of the thermostat switch method.

Table 19: Pros and Cons- Thermostat Switch

Thermostat Switch	
Pros	**Cons**
• Easy to use • Controlled delivery • Easy to monitor • Can be used with multiple burn devices • Works with currently used BES culture device system	• No way to maintain temperature • Potential for reading errors • Potentially difficult to sterilize

5.1.4 Design Tools Used for Eliminating Unsuitable Designs

In order to make an educated and justified decision on which design alternatives would accomplish the functions, specifications, and objectives of this project, numerous charts and tables were generated to highlight the best design alternative.

5.1.4.1Functions Means Tree

The brainstorming session provided the design team with alternative designs that could potentially accomplish the project functions. These functions are listed vertically in

Table 20 below, and the specific conceptual designs or means are listed horizontally. This table provides the team with a clearer picture of the direction that they could move in while continuing in the design process.

Table 20: Functions-Means Chart

Function	Possible Means					
Produce a Full-Thickness Burn	Cryoprobe	Hot plate disk	Cryoshower pen	Toaster oven	Laser	Chemical filter paper
Control Burn Time and Rate	Timer with manual release	Timer with spring release	Dwell mechanism	Thermostat switch	Capacitive heating	
Create a Reproducible Burn	Pressure sensor	Pulley system	Probe hanger	Attachable weights		
Control Burn Shape and Size	Masks	Probe tips	Hot plate disk	Chemical filter paper		
Monitor Burning of BES	Cell viability assay	Histology	Imaging tools			
Create Multiple Burn to BES Simultaneously	Masks with multiple holes	Multiple Probe Tips	Probe hanger			

5.1.4.2 Constraint Matrix

Each stakeholder has a list of constraints that they need to keep in mind when choosing among a list of design alternatives. Below in Table 21 is the list of constraints from the client. The design team attempted to decide whether or not the design idea met the standards of the constraints.

Table 21: Constraint Matrix

	Cryoprobe	Hot Plate Disk	Cryoshower	Toaster Oven	Laser	Chemical Filter Paper
C: Time	Y	Y	Y	N	N	Y
C: Budget	Y	Y	Y	N	N	Y
C: Controlled Burning	Y	Y	N	Y	Y	N
C: Safe	Y	Y	N	Y	N	N
C: Fits BES Dimensions	Y	Y	Y	Y	Y	Y
C: In Vitro	Y	Y	Y	Y	Y	Y
C: Fit in T.C. Hood	Y	Y	Y	Y	N	Y
C: Durable	Y	Y	Y	Y	Y	N
C: Sterilizable	Y	Y/N	Y	N	N	Y

This constraints matrix indicated only two designs met necessary criteria. These designs were the cryoprobe and the hot plate disk. While these two designs best fulfilled the constraints, the other alternative designs all achieved a portion of the constraint list and could therefore be modified to address the remaining constraints.

5.2 Selection Process for Wounding Model

Throughout the design process for the creation of a burn wounding model, the design team worked to compile ideas for conceptual designs to achieve the three stakeholder's objectives. After the brainstorming session, the design team had a better understanding of what routes could be taken to achieve functions criteria designated by the client and user. The team made the pros/cons lists found in the previous section of this chapter. After compiling these lists, the team used them to decide which means would be beneficial in achieving the best final design.

5.2.1 Feasibility Study of Conceptual Designs

Prior to any preliminary testing of conceptual designs, the team was able to eliminate several designs based on their feasibility.

The toaster oven design requires the use of radiation to deliver thermal heat to the BES and because of this the design team decided that it would be difficult to control the delivery of the heat. This led the team to conceptualize a series of masks, ensuring that the thermal heat would only pass through to the BES in the desired exposed areas. Another drawback of the

toaster over design, however, was that it required a great deal of materials that could be potentially difficult to assemble and sterilize, which are two of the most important design objectives. Because of these factors, the toaster oven design was eliminated from the possible conceptual design list.

In addition to the toaster oven design, the laser system was also eliminated due to difficulties with sterilization as well as the intricateness involved in controlling the heat delivery. The team believed that these factors would significantly hinder objectives such as user friendliness and ease of use, which were both very important to the user.

The final design eliminated by the team due to feasibility issues was the chemical filter paper design. The design team believed that this design posed too much risk to the user and also did not allow for the creation of a burn with definitive wound margins, because of the nature of chemical burning.

5.2.2 Objective Ranking Matrix

Following the completion of the feasibility study, the remaining designs were the cryoshower, the cryoprobe, and the hot plate disks. In order to determine which design would be best in accomplishing the objectives set forth by all three stakeholders, the design team used a ranking matrix. In this matrix, whose results are shown in Table 22 below (for full matrix see Appendix E: Weighted Objectives Ranking Matrix), the three designs were listed across the top and were assigned a score based on the team's idea of which design would best meet the objectives, listed in rows. A score of .33 was assigned to the design that best achieved the objective, a score of .66 was assigned to the second best option, and a score of 1 was assigned to the design that seemed to be the worst of the three options to accomplish the objective. In the case of tie for the best option, a score of .5 was split amongst the two comparing designs. Therefore the lowest weighted score represents the design that would best achieve the objectives of the project. The score totals were calculated to each of the three designs. The weighted total for the entire list of objectives was then divided by each of the score totals for the three objectives and the resulting percentages were inserted into the bottom row of the table.

As seen in the bottom row of Table 22 below, the percentages for the three designs range from 81.8% to 40.3%. Because it received the lowest score in this matrix, the design team was

able to conclude that the cryoprobe was the best design alternative. Therefore, the design team moved to produce a system that could use the cryoprobe component in addition to other components to achieve optimal results.

Table 22: Objective Ranking Matrix

		Cryoshower	Cryoprobe	Hot Plate Disk
	Weight	Weighted Score	Weighted Score	Weighted Score
Total	301	246.14	121.31	169.83
Percentage		0.818	**0.403**	0.564

5.2.3 Preliminary Validation Testing of Remaining Conceptual Designs

In order to confirm the results of the objective ranking matrix, the team performed a series of validation testing of each of the top three conceptual designs. This testing involved the application of the broad concepts of each of the three designs to a preliminary test tissue: chicken skin from grocery store bought chicken thighs. By testing the designs on chicken skin and not a more expensive material such as BES, the group was able to collect initial data regarding the feasibility of each of the final conceptual designs. Unfortunately, in the preliminary validation testing, the group could not feasibly test the cryoshower concept given the available resources and therefore, only the cryoprobe and hot plate disk designs were included.

The first step in the preliminary design validation testing was to prepare the test tissue. The preparation of the chicken skin samples involved careful removal of the skin layer from the uncooked chicken breast seen below in Figure 31.

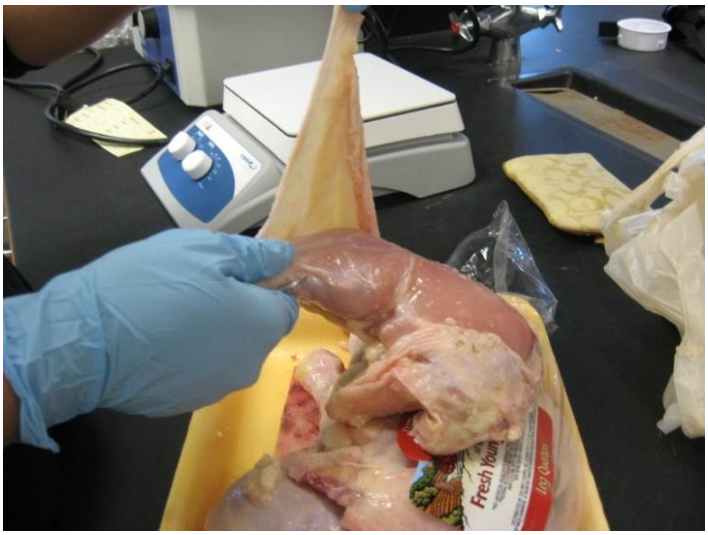

Figure 31: Removal of the Skin Layer

For the hot plate disks design, stainless steel disks (washers purchased at a hardware store) were heated to temperatures ranging between 200-400° using a laboratory hot plate and subsequently placed onto the cut samples of chicken skin for five (5) seconds. The results of this testing did not produce definitive wound margins (as seen in Figure 32 below) which led the team to believe that this conceptual design may not be the best option (confirming the results of the objective ranking matrix).

Figure 32: Hot Plate Disk Burn on Chicken Skin

In order to test the cryoprobe conceptual design, the design team used protective gloves and forceps to submerge stainless steel rods (standard drill bits, purchased at a hardware store) into an insulated bucket containing liquid nitrogen as shown in Figure 33 below. In the first

attempt, where the rod was only submerged in the liquid nitrogen for five seconds and applied to the skin samples, the rod stuck to the sample, as denoted by the arrow in Figure 34 below.

Figure 33: Submersion of Rod in Liquid Nitrogen

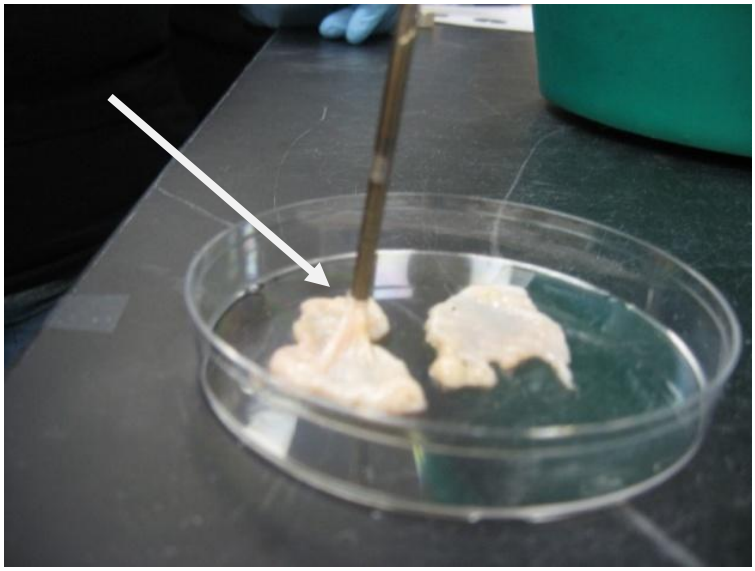

Figure 34: Cryorod Stuck to Sample

With increased exposure time of the stainless steel rod to liquid nitrogen, the team found that the rod produced a better burn on the skin sample. In using this conceptual design, the wound margin was clearly visible and well defined. As shown in Figure 35 and Figure 36 below, the chicken skin experienced a cryoburn from the rod. Once the sample returned to room temperature, the skin still had a slight discoloration at the wound site. The cryorod conceptual design testing showed the design team that it would be the best option for creating a full-

thickness burn with a definitive wound margin, which was consistent with the results of the objective ranking matrix.

Figure 35: Cryoburn with Rod on Chicken Skin

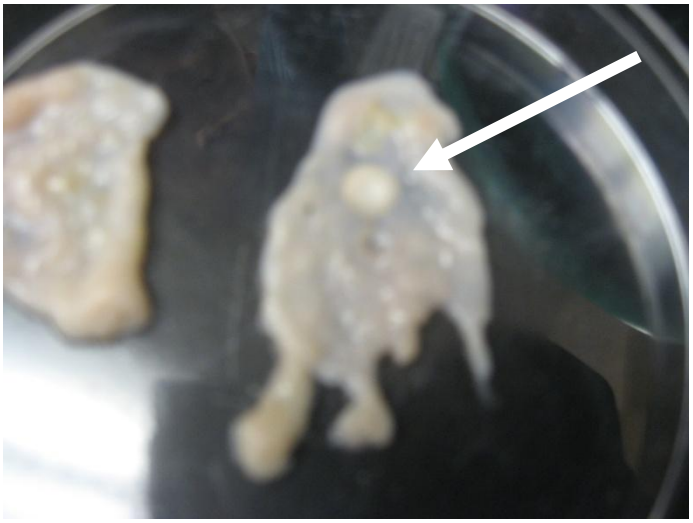

Figure 36: Wound Margin of Cryoburn on Chicken Skin

The full, detailed procedures for this preliminary validation testing can be found in Appendix F: Testing Procedures

5.3 Preliminary Wounding Model Design

As previously discussed, the design team used a series of design tools to choose the optimal design capable of achieving the functions and objectives of this project. The results of

these design tools as well as preliminary validation testing, led the team to choose the cryoprobe assembly, along with the probe hanger accessory, complete removable and interchangeable Lexan® masks, and two stabilizing trays as the most suitable option.

The cryoprobe design was chosen for producing full-thickness cryoburns on the BES due to its many advantages over the other design alternatives. It was shown that the cryoprobe design fulfills most of the important objectives. The cryoprobe is useful, easy to use, and precise, is capable of holding the BES matrix in place, and is compatible with the client's BES substitutes.

The cryorod shown below in Figure 37 is 3/8" in diameter. One setback of using the cryoprobe was the potential rapid heat loss that would occur during the transfer from the liquid nitrogen bucket to the BES substitute. In order to ensure minimal or controlled heat loss, the team plans on using materials such as stainless steel 316L which has improved heat transfer properties over some other commonly available metals. Stainless steel has a low thermal conductivity, and the material itself is easily sterilizable, which is an important factor of the design team's project.

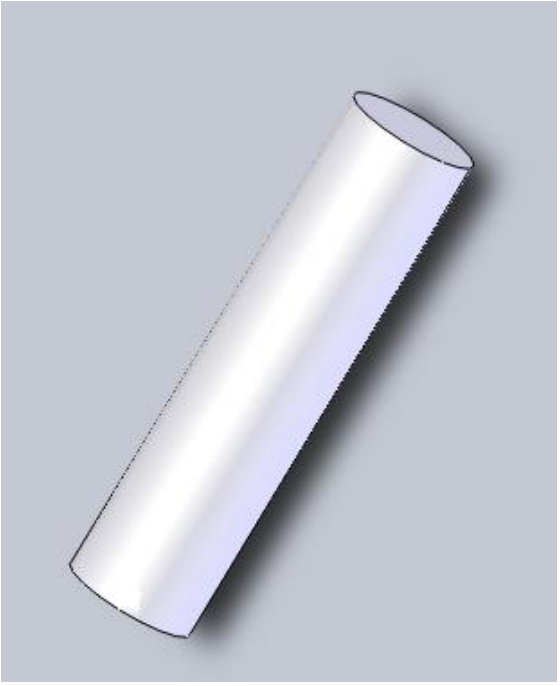

Figure 37: Stainless Steel Cryorod

The team suggests the design of a grip for the cryoprobe in order to make the probe easier to handle. Furthermore, having a grip with insulating properties will minimize user injury while using the device.

An alternative for controlling the shape and size of the burn would be to design detachable tips of several shapes and sizes that would be used with the cryoprobe. These tips would mimic the attachments used in screwdrivers as seen below in Figure 38 and would be easily attached and removed from the end of the cryoprobe. Depending on the material costs, the tips could also be disposable, which would significantly reduce the need for the sterilization of the tips.

Figure 38: An example of the detachable tip mechanism (Shack)

The probe hanger design was formulated in order to ensure stability and accuracy of the cryoprobe during the wounding process. Another integral role of the probe hanger was to use interchangeable masks to allow the user to create simultaneous burns on multiple BES samples, or multiple burns on each BES sample. The probe hanger setup includes a set of two trays that are fixed using stainless steel spaces within a few inches of each other vertically. The bottom-most tray will hold the 6-well plate in place, as seen in Figure 39. The top tray will hold a Lexan® mask which will provide guidance and stability for the probe. This tray and mask will serve as the insertion point of the cryoprobe and help to keep the rod stable during insertion of the rod. The entire assembly can be seen in Figure 40 below. CAD drawings of all other components can be seen in Appendix G: Components of Tray Assemblies.

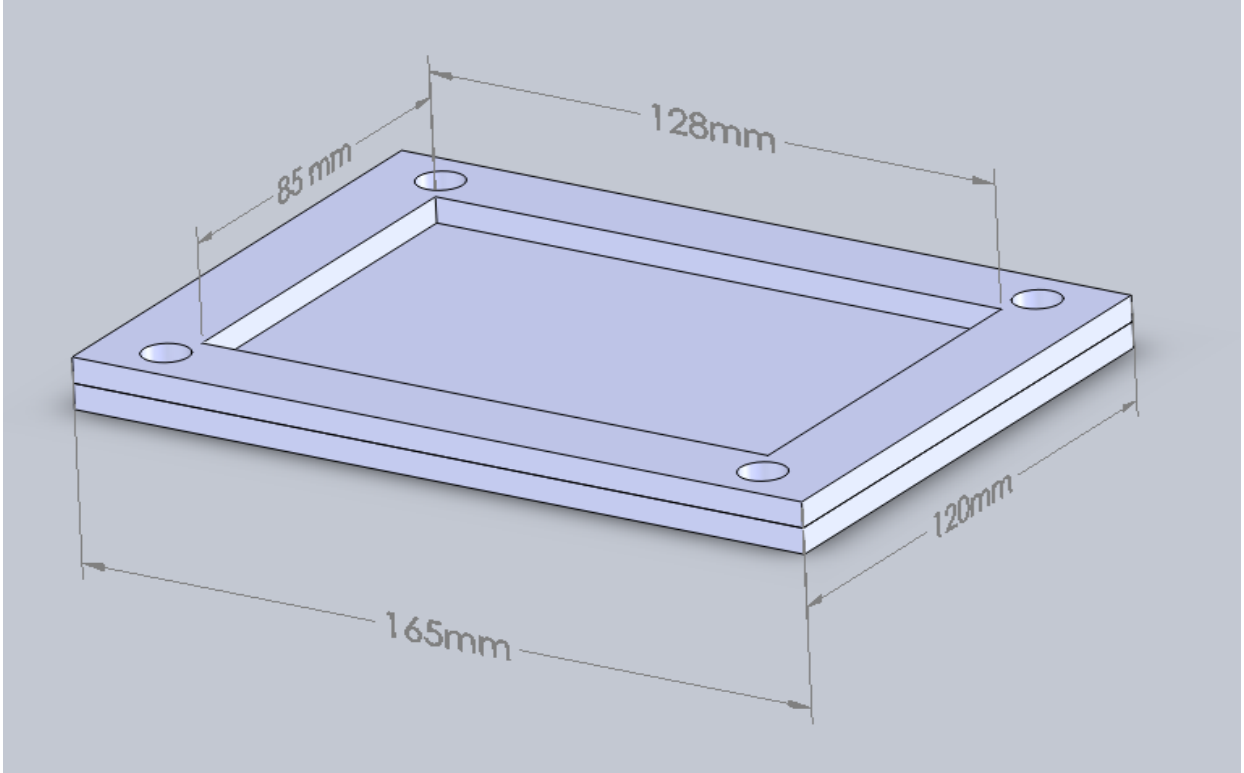

Figure 39: Base Plate CAD Drawing

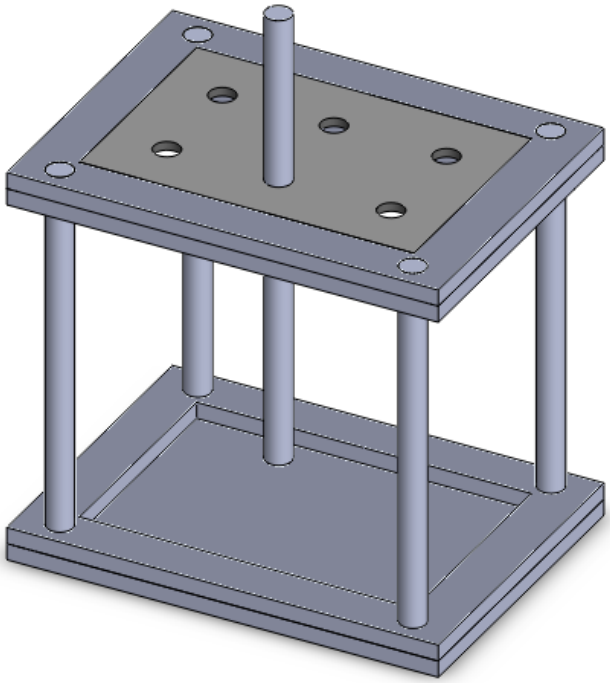

Figure 40:Preliminary Wounding Model Design

Lexan® masks with a range of shapes and sizes (Figure 41) can be produced to fit the trays of the device, giving the user the freedom to study a variety of burns without losing controllability. The Lexan® masks will also be cost effective since Lexan® is a readily available and affordable material that is easy to machine. The dimensions of the entire assembly, including the trays and masks, would be that of a six-well plate, approximately 127.5 mm in length and 88.2 mm in width.

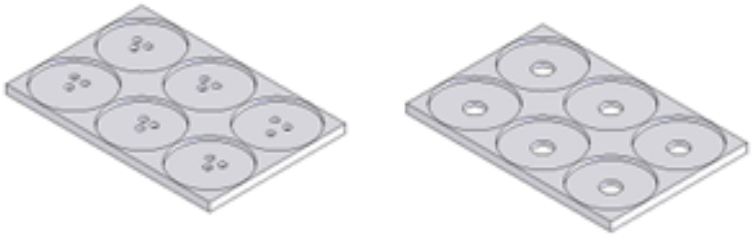

Figure 41: CAD drawings of potential Lexan® masks.

5.4 Prototyping the Hanger Assembly

The hanger design developed by the design team was broken down into separate components for further alterations and specifications. The separate components of the device are as follows; trays, masks, spacers, and fixtures.

5.4.1 Tray and Mask

At the base level of the design, the team needs to choose a material that the trays and masks can be formed with. One of the specifications that the team addressed from the specification list, found in Section 4.3.2 Design Specifications, was to choose a material that would not corrode. The material will need to be made from a plastic that can withstand the high temperatures of autoclave sterilization. The advisors, also desire a clear or white material, to allow for visibility during delivery of the burn and to confirm sterility. Using these specifications, the design team focused on two plastics, Delrin and Lexan, which could potentially fit the needs of the design.

5.4.1.1 Tray and Mask Design and Manufacturing Feasibility and Logistics

The design team met with the manufacturing lab coordinator to discuss the options for cutting either of the two plastics into trays for the hanger assembly. Due to logistical concerns with the available cutting machines, the team was told that Lexan would not be able to be automatically cut by the laser machine because the plastic would melt under the laser. If the group were to use Delrin with the laser cutter, then the thickness would have to be kept to less than a ¼". Since the preliminary design included the use of ½" sheets of plastic, the group needed to work out a secondary design where each tray would be broken into two ¼" pieces of white colored Delrin. The two pieces would be clamped in place between two fixation devices so that they would act together as a ½" tray.

In splitting up the trays to two separate pieces, this design allowed for the creation of a lip by simply cutting the inner rectangles to two different sizes. The top half of the upper tray would be cut to the size of the masks, and the bottom half of the upper tray would but cut with a smaller opening. This allows the masks to rest in place and fit snugly which increases the chances of creating reproducible placement of the rod through the masks. As for the lower tray, the upper half was cut to the dimension of a standard BD 6-well plate where the skin samples will be suspended, and the lower half was left as a solid tray to also serve as the base of the hanger.

In total the Delrin would be four trays with equal outer and varying inner dimensions that would be programmed into the laser software for cutting. After deliberating with the project advisors, the team decided that Delrin would not be suitable for the construction of the trays and masks because it would limit user visibility of the burn process. Lexan would be a better plastic for bench top proposes and it was favorable amongst the advisors. The group then met with another manufacturing lab coordinator to determine whether or not the ¼" Lexan would be able to be cut with the available machines. After the design was approved, the design group worked on generating real dimensioned CAD drawings of the four trays and the masks. The detailed drawings can be found in Appendix H.

The fifth tray, called the mask, was designed to be removable so the user could interchange masks to best fit the burning dimension requirements. Since the group wanted to create a full-thickness burn that could be characterized with histological analysis by taking slices of the burned samples, the group decided to create two different burn shapes. The round burn

would be created from a medical grade stainless steel rod. Six round holes would be drilled into the masks to act as a collars and stabilizers during placement for burning.

5.4.1.2 Trays and Masks Machining

Manual manufacturing of the trays included cutting the sheet of the plastic into four sections with the band saw, Figure 42 Part C below. The band saw only cuts a rough line into the Lexan, so the edges needed to be squared with the milling machine, Figure 42 Part A below. This machine was also used to remove the middle section of each piece. Hand tools, including a file were used to round the edges of each piece to lower the risk of injury to the user. The trays were then held into place with clamps on the drill press, seen in Figure 42 part B before the corner holes were removed using a 3/16" bit. By stacking the squared trays, the group ensured that the holes were all aligned correctly. These holes were left untapped, so the group would not have to commit to a specific type of spacer.

Figure 42: Manual Machines Used for Manufacturing

5.4.2 Spacers and Fixtures

For assembly of the trays into a hanger, holes were drilled into the four corners of each tray allowing for positioning of the spacers. The options for spacers made from medical grade stainless steel parts included male-to-female and male-to-male hex ends. However, the length was limited to only 2" segments, which raises a design issue. Technically, numerous spacers could be added to each other to increase the length between the two trays, but that mean that the user would have to disassemble more parts, making it more involved and difficult for autoclaving.

To keep the amount of material used to a minimum, the group decided to use a 3/16" threaded rod with a length of 12" for the first prototype that was machined. Each rod was held in place between fixation hex nuts and washers. Once the hanger was assembled, the total number of fixation pieces was 32. It was also noted after assembly that they large distance between the two trays made the device seem unstable and it did not sit square on the bench top. Their next choice was to use a 3/16" threaded bolt that was now 6" in length and that already had one end capped, this bolt lowered the number of fixation pieces by a factor of 8. Therefore, the most recent prototype achieves the objective to make the device easy to use, and easy to assemble.

5.4.3 Wounding Instrument

For the prototype design, a 12" stainless steel rod was used because the pressure exposed on the suspended skin samples was enough that the user would not have to hold the rod while burning. The rod was cleaned using a lathe, pictured in Figure 42 Part D above, where the belt was set to the slowest rotational speed. While the rod rotated, the group passed steel wool and sand paper along the rod until it shined. The edges of the rod were squared and cleaned; this would ensure that the contact point between the skin samples and the rod was consistent and even.

Figure 43 below is the wounding model device manufactured. The rod is passed through the holes of the mask positioned on the top tray to the six well plate resting in the tray below.

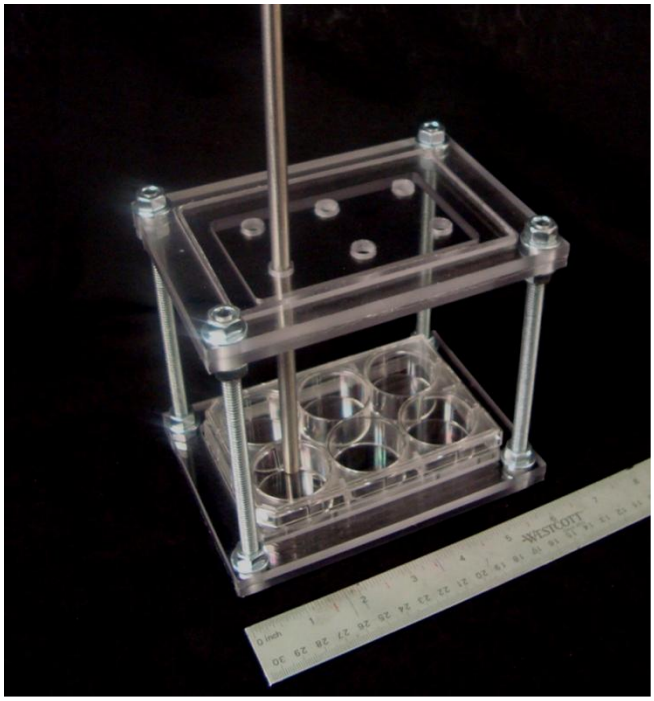

Figure 43: Manufactured Wound Model Device

6. Design Validation

In order to validate the prototype device and model design, the team conducted a series of laboratory tests. As shown in the previous chapter, the cryoprobe design was chosen as the design that best accomplishes the objectives of the project. Therefore the goal of this section is to test the ability of the cryoprobe design to induce a full-thickness thermal wound on skin samples. The skin samples used in this section are neonatal foreskins obtained from UMASS Memorial Hospital (Worcester, MA) via an IRB exempt protocol between Professor Pins and the OB/GYN department at UMASS Memorial Hospital. Foreskins are provided to the group as anonymous discarded tissue. Therefore, the group can use these foreskins to complete various tests to validate the design without destroying expensive, and difficult to fabricate BES samples from the Pins Lab.

6.1 Preliminary Foreskin Testing

After promising results with the preliminary validation testing using chicken skin, the group began testing on neonatal foreskins in order to create a proof of concept. The foreskins were graciously obtained by graduate advisor, Amanda Clement. The foreskins were less than one week old and were considered to still have viable cells in them. The foreskins were brought to the project lab, removed from the media and placed on a culture plate inside the biological safety cabinet. The fat layer on the dermal side of each skin sample was removed with a scalpel as shown below in Figure 44.

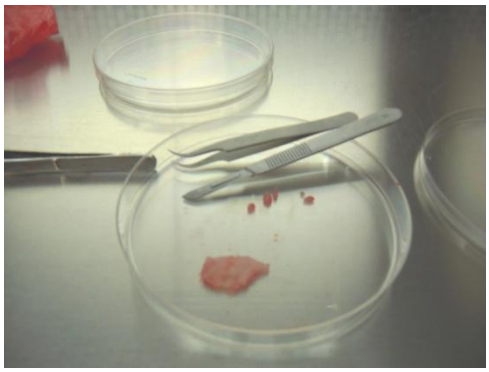

Figure 44: Fatty tissue removed from dermal layer of foreskin

After the fatty tissue was removed from the foreskin, the team induced a thermal burn starting on the epidermal layer of the skin using a liquid nitrogen cooled, stainless steel rod. The stainless-steel rod was cooled in the liquid nitrogen until the rod and liquid nitrogen were in thermal equilibrium (Marked by the subsiding of "boiling" of the rod) and then immediately applied to the foreskin as shown below in Figure 45.

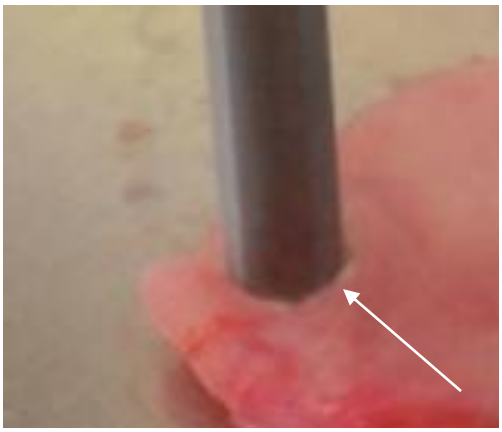

Figure 45: Stainless-steel rod applied to foreskin

Figure 46 below shows a foreskin that has undergone multiple thermal cryoprobe wounds all less than 5 minutes prior to imaging. There are definitive wound margins that can be seen for each of the burns as well as depressions in the skin as a result of the pressure that was applied to the skin during the burning process (the arrows indicate three areas of the foreskin sample that were burned).

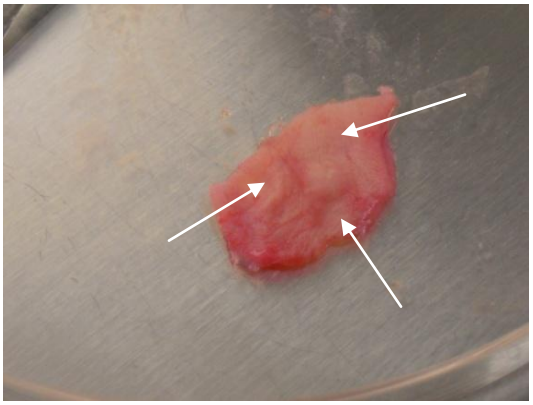

Figure 46: Burned foreskin depicting multiple burns/wound margins

Following the burning, it was necessary to test for tissue viability in the burned region compared to the unburned region. To test for this cell viability, Trypan blue cell viability stain

was applied to the surface of the foreskin tissue as seen below in Figure 47. The Trypan blue was allowed to sit on the skin for 10 seconds before being rinsed with the original foreskin media.

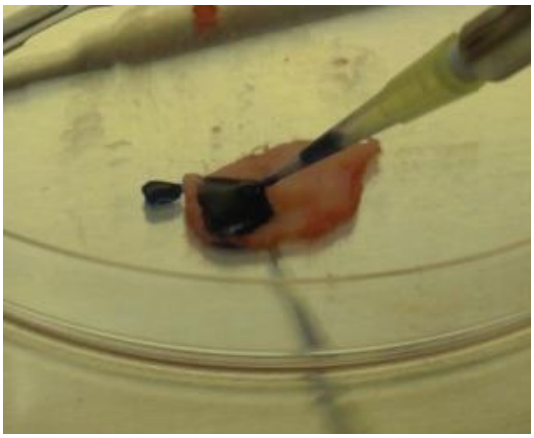

Figure 47: Trypan blue added onto foreskin

After the Trypan blue was washed from the foreskin, the team examined each sample and visually compared the burned vs. unburned areas as seen below in Figure 48 (circle indicates where the cryorod was applied. It proved impossible to discern any differences between burned and unburned areas of the skin with the human eye. The foreskin was then moved under a light microscope to further examine the wound margins. Unfortunately, it was not possible to focus on the foreskin under the microscope due to the thickness of the skin. Future tests and evaluations will require either a different cell viability assay or thinner sections of the skin to be cut to allow for evaluation and observation under the microscope. Histological analysis can also be completed to determine whether or not the cryoprobe is inducing a thermal wound on the foreskin.

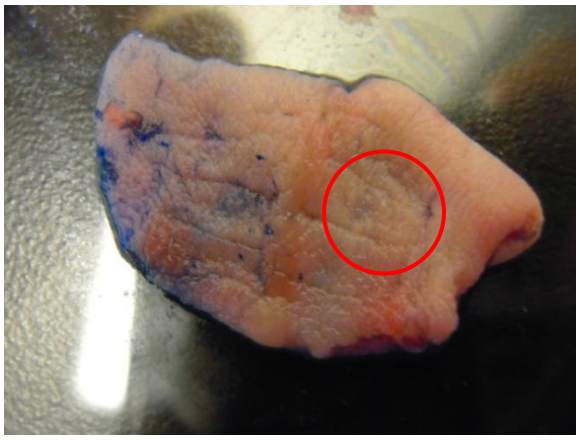

Figure 48: Foreskin post-Trypan blue staining

Following this initial proof of concept testing, it was clear to the team that it would be necessary to create protocols for preparing the tissue, using the cryorod, and determining the viability of the tissue before and after burning.

6.2 Cell Viability Using Trypan Blue Stain

In order to determine efficacy of the Trypan blue stain as a viability assay for foreskin tissue as well as the difference in viability between older and newer foreskin samples, the team performed several important cell viability tests. Human neonatal foreskin tissues collected from UMass Memorial Hospital were characterized as “old” or “new”, depending on when they were collected. For this particular test, the foreskins that were more than two days old were considered “old”, whereas foreskins that were collected on the day of or less than two days before testing were considered “new”. This differentiation between foreskins was made in order to evaluate whether the cell viability in the foreskins depended on how old they were. Older foreskins are expected to have fewer viable cells than newer ones, which should be indicated in the results of the Trypan blue staining.

In this test, three samples were used: two old samples (Old1 and Old2) and one new sample (New1). Prior to testing each foreskin was removed from its cell media and placed on a polystyrene tissue culture dish. A #10 scalpel blade was used to cut each foreskin such that it lay flat on the dish and to remove excess fat from the dermal (under) side of the tissue. Following tissue preparation, each foreskin sample was thoroughly coated in approximately 200µL of 0.4% (w/v) Trypan blue stain (Cellgro – Mediatech Inc., Masassas, VA) and allowed to sit for

approximately 30 seconds. Each sample was then rinsed in non-sterile phosphate buffered saline (PBS) and photographed as seen in Figure 49.

Figure 49: Trypan blue staining on three foreskin samples to see the difference in cell viability of old and new samples. Arrows indicate areas of possible cell death in both the old and new samples.

The results of these tests suggest that there is no significant visible difference in tissue viability between the old and new samples. Figure 49 above, shows the Trypan blue staining of each of sample with blue areas (arrows) showing areas where there may be regions of dead tissue, though not enough to indicate that the tissue is not viable.

6.3 Cryorod Burns with Trypan Blue Stain

The team's first set of tests involved applying liquid nitrogen cooled stainless steel rods to the foreskin and staining the samples for cell viability with Trypan blue. During this test, different time intervals were used to evaluate the duration of cryorod application time necessary to create useful full-thickness burns. Foreskins were prepared as in previous tests before burning. To create a cryoburn on the foreskin samples, a stainless steel rod with a diameter of 3/16 in (cryorod) was immersed in the liquid nitrogen until boiling subsided, indicating equilibrium cooling of the rod. The rod was then removed from the liquid nitrogen and immediately applied to the epidermal layer at the center of the foreskin samples used in this test. Each was held for a different amount of time to understand whether applying the rod to samples for longer periods of time produced a more promising burn than the shorter periods of time.

Six samples were used for the tests. Immediately after being removed from the liquid nitrogen, the cryorod was held against the epidermal layer of sample 1 for approximately 10 seconds. It was also observed that transfer time of the cryorod from the liquid nitrogen to the foreskin sample was approximately 5 seconds. After each burn the cryorod was re-submerged into the liquid nitrogen until boiling stopped for one minute. The cryorod was then applied to samples 2, 3 and 6 for 30, 60 or 120 seconds respectively. To create a control one foreskin

sample was cut in half. One half of this sample (sample 5) was treated as the positive control and was left in the Petri dish unburned. The other half (sample 4) was placed in the liquid nitrogen for 10 minutes in hopes of creating a completely burned negative control.

The burns were visible on the skin as round white regions immediately after the cryorod application. Unfortunately, the burn margin visually disappeared as the foreskin thawed. To characterize the burned area, Trypan blue was applied to the skin. Since earlier staining with 0.4% w/v Trypan blue did not yield expected results, the second batch of cryoburned foreskins were washed with a diluted solution of Trypan blue. This solution was formulated using 1:3 ratio of Trypan blue to phosphate buffered saline (PBS). Each burned foreskin was washed with 200 µL 0.1% w/v Trypan blue solution. After approximately 10 seconds, the foreskins were placed in a 20 ml solution of PBS and shaken vigorously to wash off excess Trypan blue solution. Finally, all the samples were stained with Trypan blue and examined as mentioned earlier. Samples 1-6 were stained with diluted Trypan blue (1:3 Trypan blue to PBS).

A final sample was obtained and cut in half. One half of the sample was stained with 0.4% w/v Trypan blue and the other half was placed in 100% bleach (a drastic pH change for the foreskin aimed at creating a dead control) for approximately 10 minutes. The first half of the sample stained blue at random areas of the foreskin, possibly indicating that the cells were dying off even without producing a burn on the foreskin. The bleached sample retained residue even following rinsing with PBS, and proceeded to react with the Trypan blue stain causing the sample to stain a brown color. This process did not aid in characterizing a proper wound margin.

The results for the Trypan blue staining were found to be inconclusive. The foreskin stained in a similar manner as the cell viability test foreskins shown in Figure 49. It was not possible to differentiate between the burned and unburned regions (i.e. wound margins) of the foreskin following the Trypan blue staining. Unfortunately, the greatest difficulty came with analyzing the staining of the control sample (sample 5). Ideally, this sample should have remained pink following the stain, however there was speckles of blue on the surface of the skin. On the other hand, sample 4, should have stained completely blue, as it was submerged in liquid nitrogen for 20 minutes, however, this also was not true as sample 4 stained mostly pink. The reasons for such inconsistencies in the stained foreskins were not clear, but they did imply that new staining and burning methods were required.

Following testing, the team selected three samples (the positive and negative controls and sample 3, the 60 second cryoburn) on which to perform histological analysis, in order to confirm whether or not cells are viable in the foreskin post burning. Immediately following the burning of the samples, the histological process was started. Amanda Clement and Sharon Shaw, the histology core manager for WPI completed the majority of the histological analysis work.

The histology process involves chemical fixation of the samples and then a dehydration process completed with chemicals such as xylene (entire protocol for histology can be viewed in Appendix G). Amanda completed these steps for the group due to the lack of training. Following the dehydration, the samples were embedded in paraffin wax. Prior to embedding, the samples are cut in half and stood up on their side. Therefore the two halves are pointing upward with the center sections of each at the top of the embedded wax block. The samples were then sectioned by Sharon Shaw. For each slide, three sections were taken to generate samples obtained from different depths of the foreskin. Once the samples are sections, they are stained. Hematoxylin and Eosin (H&E) was used to stain the tissue. The hematoxylin stain penetrates the cell, staining nuclei blue while the eosin stains the elastin and collagen fibers in the cell pink, creating a pink skin image with small blue dots representing the nucleus. Following staining, the slides can be observed under a Nikon Eclipse E600 microscope to determine relative cell viability. Photographs of the skin were taken using an RT Color Spot camera and the SPOT version 4.6 imaging software. H & E staining also clearly shows the epidermal layer. In healthy tissue, the epidermal layer is fixed securely on the dermal layer. In burned or wounded tissue, the epidermal layer flakes off as a result of cellular disruption and death. Further characterization of cell death in histological slides can be analyzed due to the number of nuclei present in the tissue. Healthy tissue will have nuclei present throughout the epidermal and dermal layers. Unhealthy (dead) tissue will not have such nuclei present. If there is death of the nuclei, small white holes will be seen within the stained tissue where healthy nuclei were when the tissue was viable. (Garlick J, 2006).

Seen below in Figure 50 is an image of the live control. There are numerous nuclei visible (white arrow) and the epidermal layer (black arrows) is solid and intact on top of the dermis. These is little visible flaking of the epidermis off of the skin, indicating relatively alive tissue.

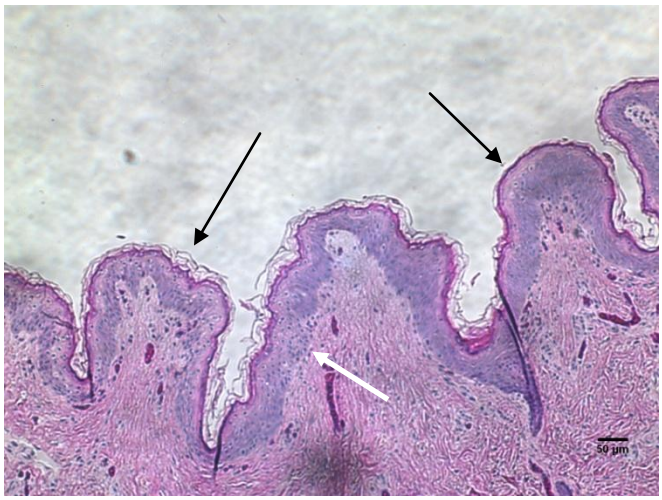

Figure 50: Live Foreskin Control (10X)

Seen below in Figure 51 is an image of the 60 second liquid nitrogen burned skin. Similar to the control picture, minimal flaking of the epidermal layer is shown (arrow points to epidermal layer), indicating that the 60 second burn did not successfully create a full-thickness burn on the skin.

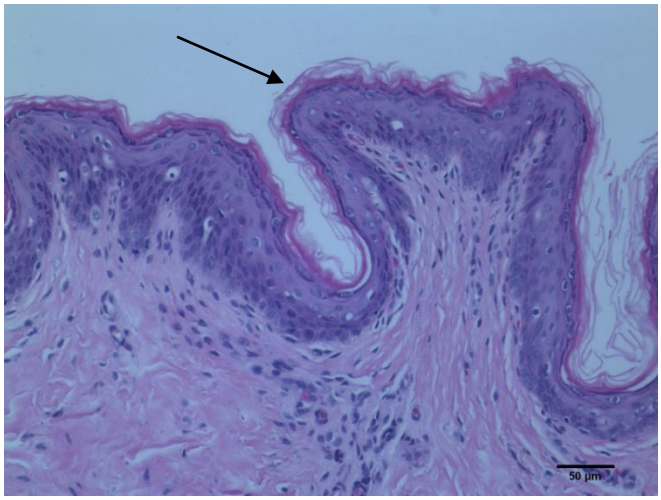

Figure 51: 60 Second Liquid Nitrogen burn (20X)

Finally, seen below in Figure 52 is an image of the foreskin that was submerged in the liquid nitrogen for 10 minutes. There has been some damage inflicted on this sample as the epidermal layer is visibly flaking off of the skin (black arrows), indicating a wound, however, the presence of nuclei (white arrows) within the skin indicate viable tissue.

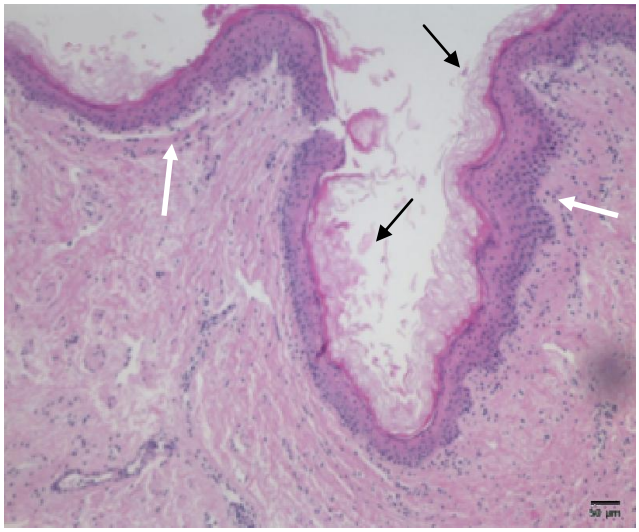

Figure 52: Liquid Nitrogen-submerged sample (10X)

The results of this histology analysis were beneficial. There was clearly a difference in viability between the submerged sample and the control sample; however, the 60 second liquid nitrogen contact burn did not appear to be strong enough to create a full-thickness wound on the foreskin samples There is minimal damage observed to the basal lamina analog on the liquid-nitrogen-submerged sample and little difference in the number of nuclei present in the two skin samples (Garlick J, 2006). Further testing is therefore required to generate a better wounding model.

After discussing the results with the advisors, the team concluded that the reason for the staining at random areas of the control sample could be due to some cells that were starting to die with foreskins that were more than two days old. To better quantify the viability of the foreskins, the team decided to us an MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, in order to deduce whether the Trypan blue staining was the reason for the discrepancies in the results. Unlike Trypan blue, the MTT assay stains for metabolic activity within the cells. This means that only the live tissue is stained purple, whereas the dead tissue remains a pink, white or brown color depending on the presence of the burn and the native skin color of the samples.

6.4 Cryorod Burns with MTT Cell Viability Assay

The next batch of foreskin testing was carried out with an MTT assay. To prepare the MTT solution, the team followed a procedure outlined by Katie Bush, PhD, a former student in

the Pins lab, in which 1 mg of MTT powder per 1 ml of dPBS was used. Once the foreskins were burned, they were immersed in the MTT solution for 2 hours and then washed three times with PBS before examination and imaging.

A new method of cryoburning (freeze-thaw) was also investigated, wherein the rod was immersed in liquid nitrogen in the usual manner and then immediately placed on the foreskin sample for various times depending on the sample. Following the initial burn, the sample was allowed to thaw completely and the cryorod was applied to the same spot again. These steps were repeated a varied number of times to find the optimum cycle to create a characterizeable burn on the foreskin samples.

A total of eight foreskins were used for this series of tests. One of the eight was cut in half to create a positive control (unburned) and a negative control (completely burned in liquid nitrogen). Sample 7 (positive control) was placed on the Petri dish unburned. Sample 8 (negative control) was placed in liquid nitrogen for 20 minutes, removed and set aside to thaw. It was then placed into the liquid nitrogen for another 20 minutes (to mimic the freeze-thaw method completed during this experiment). Samples 9-12 were cryoburned using the freeze-thaw cycle. The cryorod was applied to sample 9 for 30 seconds, removed from the sample and then allowed to thaw. After approximately 2 minutes of thawing, the cryorod was reapplied to the foreskin sample and then allowed to thaw again. During the 2 minutes of thawing, the cryorod was placed in liquid nitrogen until boiling stopped for one minute, removed and immediately placed on the sample. This cycle was repeated 6 times for sample 9. Samples 10, 11 and 12 underwent three 30 second, three 60 second and 3 90 second freeze-thaw applications, respectively.

Single application cryoburns were produced on samples 13-15. The cryorod was placed in the liquid nitrogen until boiling stopped for one minute and then applied to sample 13 for 60 seconds, sample 14 for 90 seconds and sample 15 for 120 seconds. Following the burning, all eight the samples were placed in a 12-well plate and the MTT solution was added to each well until the foreskin samples were completely submerged in the solution. The plate was then left in the laminar flow hood for 2 hours to allow for the MTT to stain the samples. After 2 hours, the MTT solution was removed from each well. Each sample was washed with distilled phosphate buffered saline (dPBS) three times before burn evaluations were made.

Figure 53, shows the nine samples after the MTT assay. Each sample (although previously described) is labeled in Figure 51with “F/T” abbreviating the “freeze-thaw” samples,

"fried" indicating the sample that was submerged in the liquid nitrogen and "burn" simply indicating that the particular sample was burned with the liquid nitrogen-cooled rod for the period of seconds listed about the sample. An unpredicted result involved the positive control sample which stained purple (alive) only at the edges, a disappointing finding, as the live control sample was expected to contain more viable cells in the center of the sample than the burned samples. The negative control sample stained completely purple (indicating live tissue), again a deviation from the expected results, since the sample was expected to have fewer viable cells than the live tissue as a result of being submerged in liquid nitrogen for 40 minutes. The group was very careful to leave the samples separated and properly labeled throughout the testing process, resulting in little chance of an accidental switch.

The freeze-thaw samples in Figure 53 created a burned area in the center of the foreskin; however, Sample 11 (Figure 53E) was stained completely purple when the cryoburned region should have not stained. It was later noticed that sample 11 was actually photographed with the epidermal layer of the sample facing down, not allowing for the burned region to be seen.

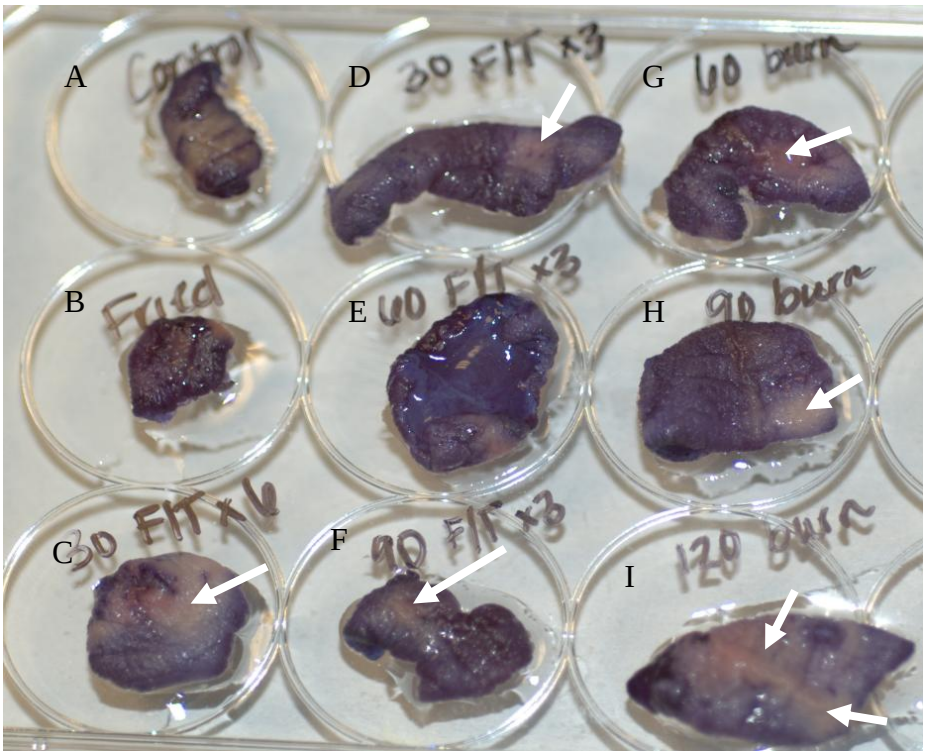

Figure 53: Samples 1-12 after an MTT assay (black arrows indicate wound margins).

The MTT assays, appeared to return more consistent results as a method of staining for cell viability when compared to Trypan blue. Consequently, all future burns were characterized

using MTT as the cell viability staining method. To identify a more successful burning method, the group decided to complete other designs that create burns on the foreskin samples.

6.5 Cryo and Thermal Burns with Histology

A second series of samples were put through the aforementioned histological analysis process to determine if different testing techniques results in a more useful (full-thickness) burn. This test involved five different samples. One sample was a positive control that was unburned. A second sample served as a negative control and was submerged in liquid nitrogen for 20 minutes, allowed to thaw and then re-submerged and thawed for the same time an additional two times. The third sample was burned for 30 seconds with the liquid nitrogen-cooled rod 6 separate times, with thawing between burn applications permitted. The fourth sample was burned with a single 120 second liquid nitrogen-cooled stainless steel rod. The final sample was burned with a heated (350°C) bolt that was applied to the skin for 5 seconds.

Seen below in Figure 54 is an image of the control (unburned) skin. There is minimal flaking of the epidermal layer (black arrows) as well as a relatively high number of cell nuclei (white arrows) when compared to images of burned tissue.

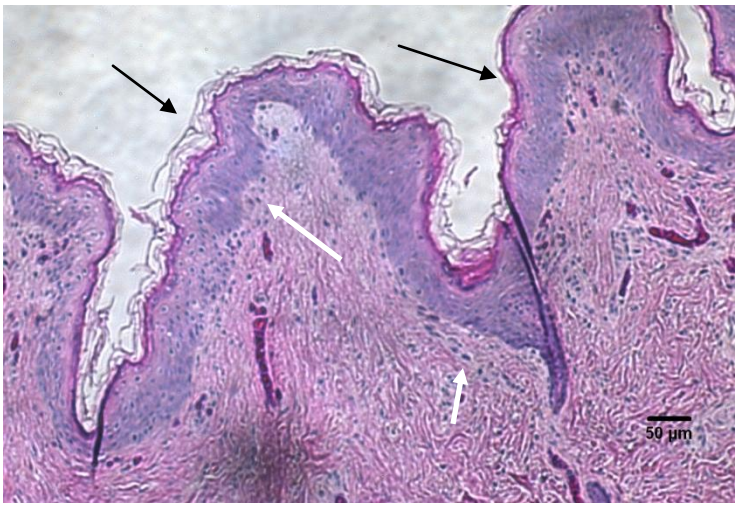

Figure 54: Live Control (10X)

Seen below in Figure 55 is an image of the liquid nitrogen submerged sample. It is difficult to distinguish a definitive epidermal layer (black arrows) as it appeared to be destroyed by the liquid nitrogen bath. There are also fewer nuclei (white arrow) visible in the dermal layer of the skin, indicating a possible wound.

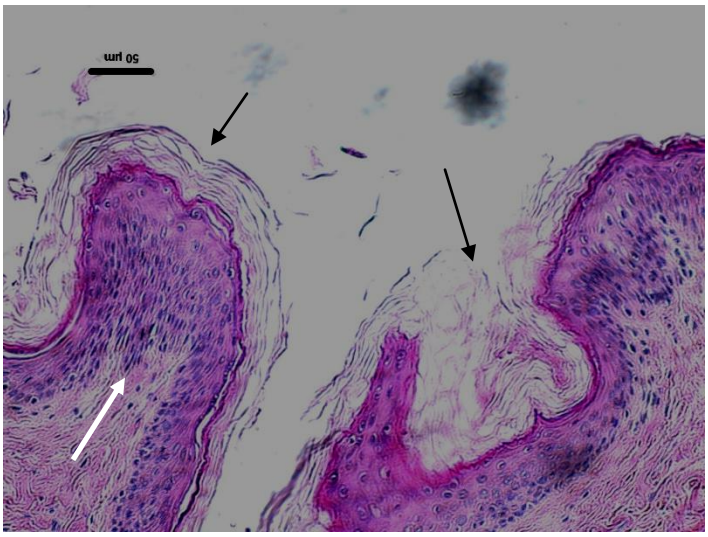

Figure 55: Liquid Nitrogen-submerged foreskin (20X)

Seen below in Figure 56 is an image of the 30 X 6 liquid nitrogen applications freeze thaw sample. There is visible damage to the epidermal layer indicated by the flaking off of this layer (black arrows), which may also be a sign of a thermal wound.

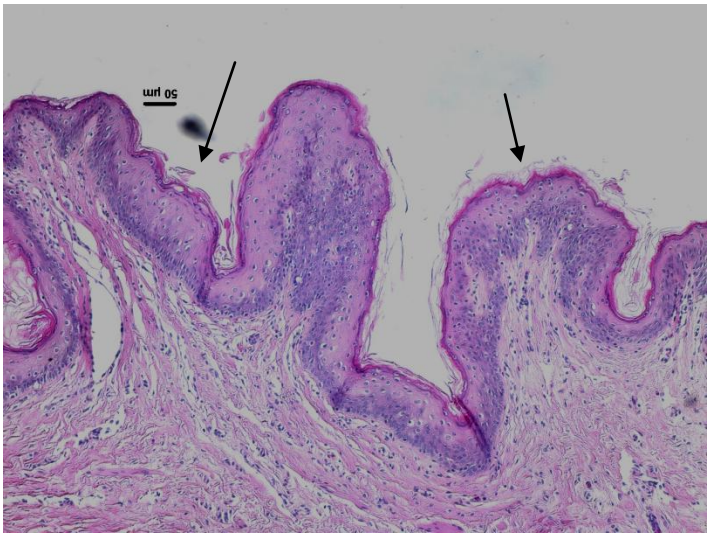

Figure 56: 30 seconds X 6 Applications Freeze Thaw Burn

Seen below in Figure 57 is an image of a foreskin sample that was burned with a single 120 second application of a liquid nitrogen cooled stainless steel rod. There is some disturbance of the epidermal layer (black arrows) indicating that this long duration (2 minute) burn may damage the skin more than shorter (60 second) burn applications.

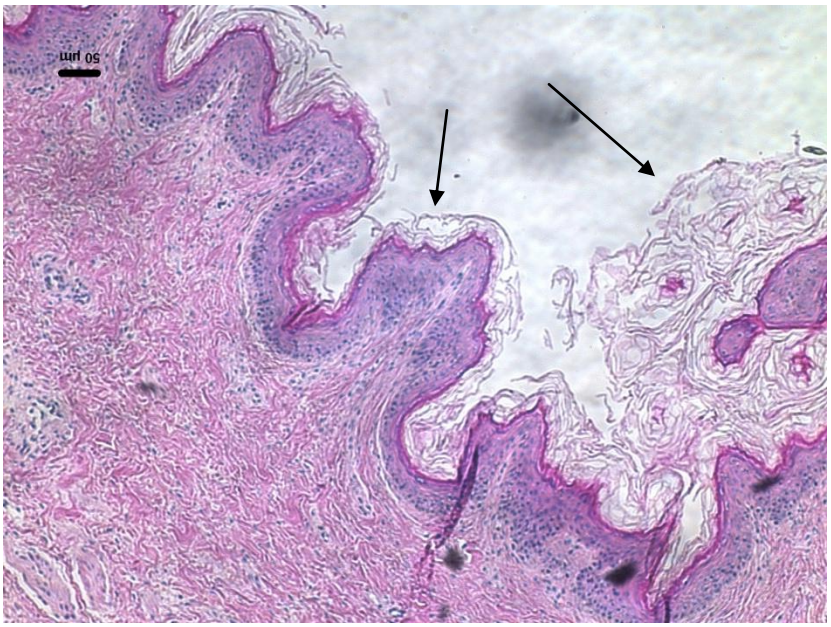

Figure 57: 120 second Liquid Nitrogen Burn

Finally, seen below in Figure 58 is the foreskin sample that was burned with the application of a bolt that was heated to 350°C on a hot plate. The heated bolt produced a severe epidermal damage on the surface of the foreskin as shown by the epidermal layer flaking off (black arrows) of the dermal layer.

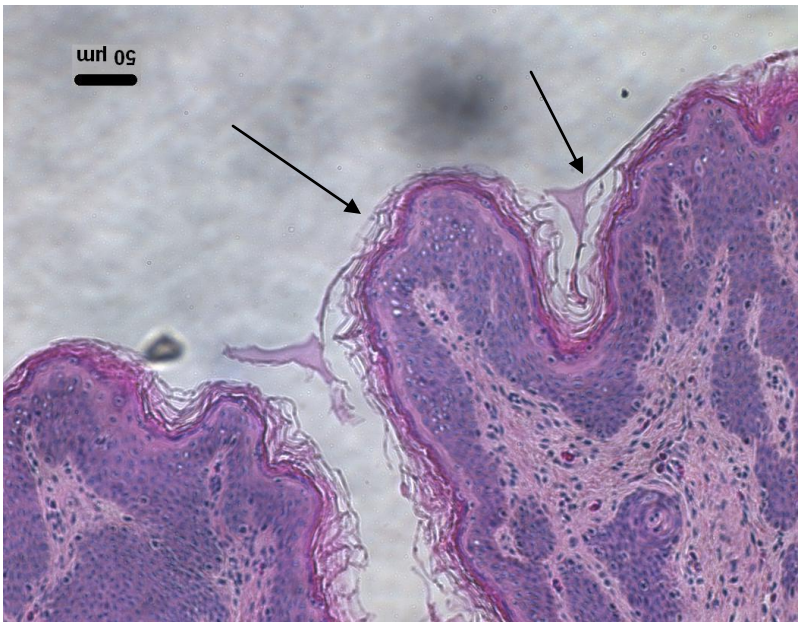

Figure 58: Hot Bolt Burn

6.6 Various Burning Methods with MTT Cell Viability Assay

The next step in the testing process was to carry out some more thermal burn tests along with some new cryoburning methods to determine if other wound model designs produce better burn results. An over-the-counter wart removal kit was used to model a thermal injury on the foreskin as the wart removal process involves chemically freezing the wart. This process of freezing skin was considered as a method of creating a full-thickness wound on the skin.

6.6.1 Thermal Burn Wounds

Initially, thermal burns were produced on two foreskins, while a third foreskin was kept aside as a positive control. Sample 16 was the positive control with no burns on the foreskin. In order to create the thermal burns, a hex bolt was heated on a hotplate at a temperature of 350°C and immediately placed on the foreskin sample. The hex bolt was applied to sample 17 for approximately 30 seconds, creating a noticeable burn on the epidermal layer (Figure 59A). Next, the hex bolt was heated again and applied to sample 18 for 3 seconds, which caused a noticeable but less severe burn and can be seen in Figure 59B.

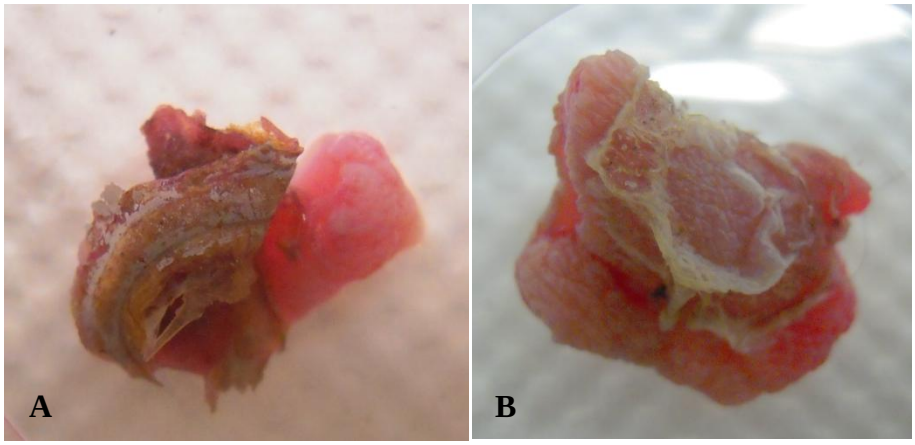

Figure 59: Sample 17 (A) and sample 18 (B) immediately after a thermal burn.

Figure 60 below shows some foreskin epidermal layer residue left behind on the hex bolt after it was applied to one of the samples. The partial removal of skin from the thermal burned samples could be one of the reasons for the poorly defined wound margins noticed following staining.

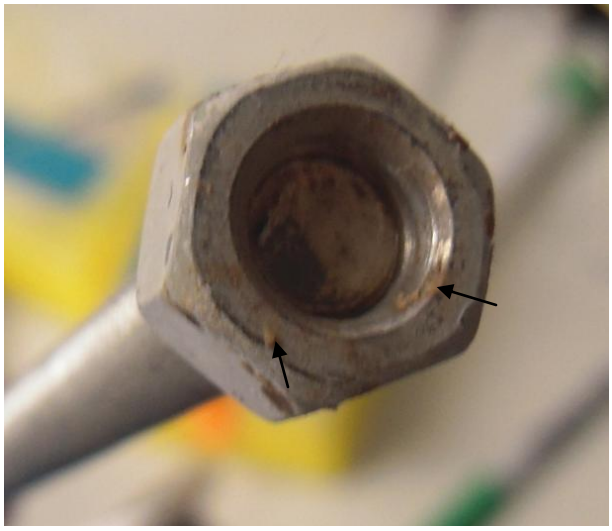

Figure 60: Epidermal layer residue (black arrows) on hex bolt.

6.6.2 Wart Removal Kit

After the thermal burns, the wart removal kit was used to produce cryoburns on samples 19 and 20. The wart removal kit was used in order to test different means of producing a definitive and characterizeable burn margin on the samples. The wart removal kit, Compound-W, manufactured by Medtech Products (Irvington, NY) and consisted of a can of wart removal solution that creates a chemical reaction with dimethyl ether and propane resulting in super-cooled liquid. The applicator tip was dipped into the wart removal solution container for approximately 5 seconds (as recommended on the box label and immediately applied to surface of sample 19 for 30 seconds. This application left a white (frozen) spot on the sample, as can be seen in Figure 61 below. The white spot disappeared once the sample was allowed to thaw.

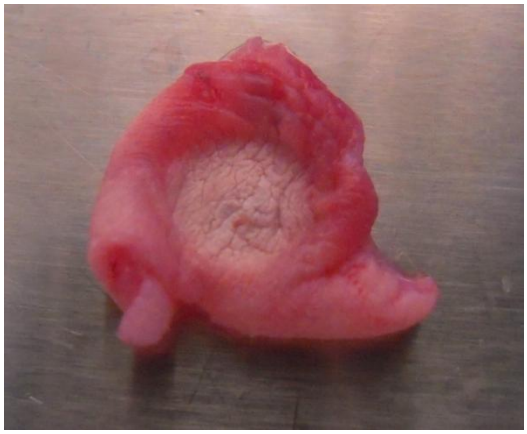

Figure 61: A wart-removal kit cryoburned region on sample 19.

The cryo-applicator from the wart removal kit was then applied to sample 20 in a freeze-thaw cycle of two 30 second applications. After being held in the cryo-solution for 5 seconds, the applicator was applied to sample 20 for 30 seconds. The skin was allowed to thaw for 2 minutes and the applicator was placed on the skin again for 30 seconds.

6.6.3 MTT Cell Viability Assay

Once the heat and cryoburning was complete, the samples were stained and examined using the MTT assay (Figure 62). Sample 21 (Figure 62A), the positive control, contained a large amount of dead tissue, as noticed in earlier assays as well. Figure 62B shows that the burned area of sample 22 had turned a brown color s a result of being burned when the heated metal was applied and that area ended up staining a dark blue color. It was also noted, however, that the cells of the region around the burn had died off. Similar results were observed on sample 23 (Figure 62C). The burned area stained a dark blue color however, the surrounding area remained pink, signifying dead tissue. Figure 62D shows sample 24 with a clear pink spot where the wart removal applicator was applied for 30 seconds. This showed that all the tissue around the wound margin had viable cells, but the cells completely died where the applicator was placed on the sample. Finally, Figure 62E shows sample 25 with a similar bright pink spot where the applicator was placed in a freeze-thaw cycle and the tissue around the wound still had some viable cells.

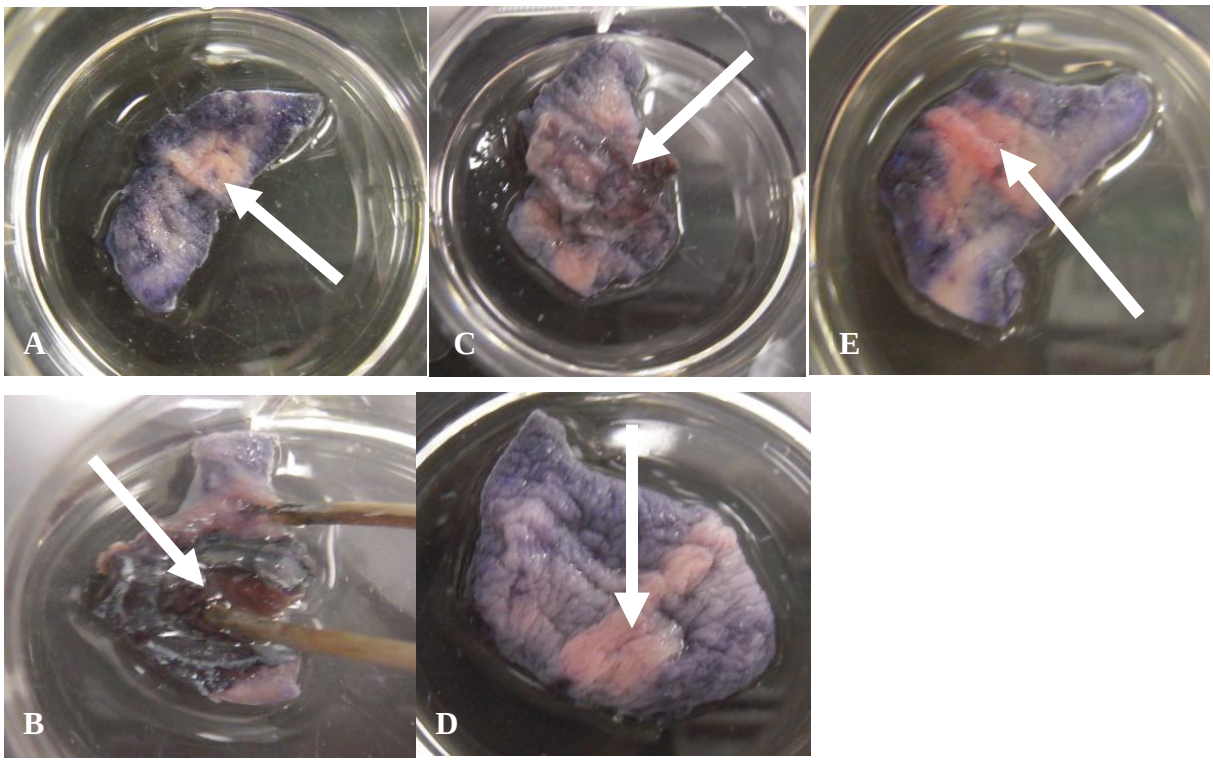

Figure 62: Heat (B and C) and cryoburn (D and E) samples after the MTT assay.

As more tests were being carried out, more promising results were being observed than the initial testing however, the consistency and reproducibility were yet to be achieved with the burns. Again, it was speculated that the reason for the small region of dead tissue on the positive control sample, could be because some of the tissue died as the foreskin became older.

6.7 Rapid Cooling Spray with MTT Cell Viability Assay

A final set of tests were carried out using the rapid cooling spray Cytocool™ that was obtained from Professor Pins' laboratory. Cytocool is used to rapidly cool and freeze tissue for histological evaluation purposes in the Pins' laboratory thus was expected to freeze and harm the foreskins. The team also wanted to test as many burning methods as possible before choosing one particular method of burning the foreskin samples. Also, in order to ensure that the foreskin media was not interfering with the reactions between the foreskin epidermis and the MTT solution, each foreskin sample was blotted dry with a Kim wipe before producing a burn.

Three foreskin samples were used for these tests and an MTT assay was performed on all the samples at the end. The Cytocool container stored a rapid cooling solution called tetraflouroethane. The container also consisted of a trigger handle and a thin tube attached to the

nozzle that sprayed the solution onto the sample directly. The most significant issue with the Cytocool testing was the inability to control the spraying to a single area on the samples.

The first sample was obtained from the foreskin media and placed on a Petri dish. The nozzle was then held approximately 2 inches away from the epidermal layer of the sample and the trigger handle was pressed. The force and pressure, at which the Cytocool solution came out of the nozzle, caused the foreskin sample to move around within the Petri dish. In order to control the movement of the foreskin during the spray application, a pair of forceps was used to hold the foreskin sample in place.

Seen in Figure 63A, the Cytocool sprayed all over the Petri dish, creating a layer of frost on the Petri dish while the foreskin sat submerged in the Cytocool solution. Figure 63B shows a foreskin after Cytocool solution evaporated from the Petri dish and the foreskin sample began to thaw as seen by the pinkish skin color indicated by an arrow.

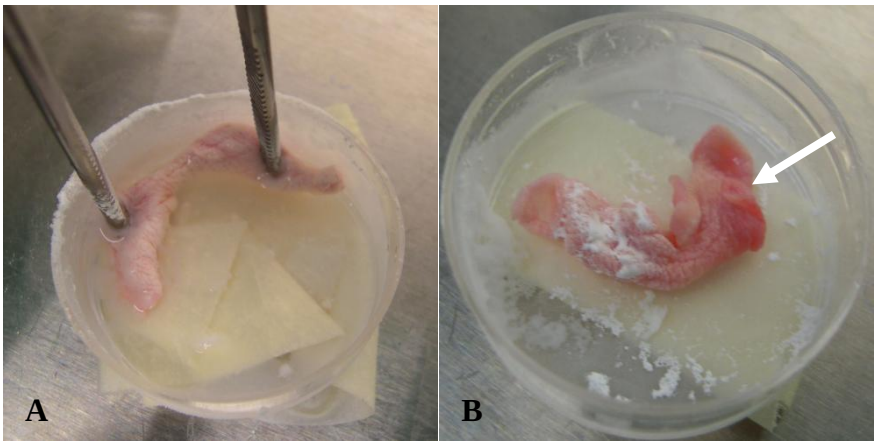

Figure 63: Foreskin sample immediately after Cytocool spray application (A)

A foreskin sample beginning to thaw (B).

This method was used to create a rapid freezing cryoburns on samples 27 and 28 using the freeze-thaw cycle applications. Sample 26 was set aside as a positive (live) control. The Cytocool was applied onto sample 27 using two freeze-thaw spray applications, each 10 seconds long. Sample 28 was sprayed twice for 20 seconds each, using the freeze-thaw cycle again. After the spray applications, each sample was completely covered in the MTT solution (Figure 64) and left in the tissue culture hood for 2 hours.

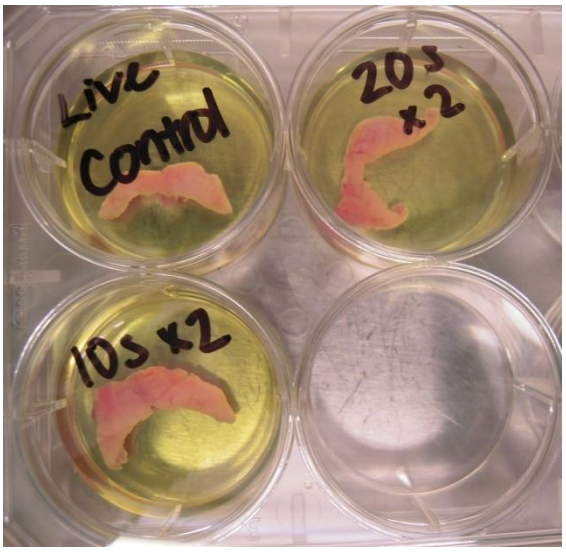

Figure 64: Cytocool foreskin samples submerged in MTT solution.

Figure 65, shows the results of the MTT assay for the Cytocool burn samples. The control tissue (Figure 65A) was observed to have some dead tissue, however it was mostly viable (see arrows). Sample 27 (Figure 65B) stained a dark purple only on one edge of the foreskin. The rest of the sample was stained a light pink, possibly signifying a large amount of cell death. Finally, sample 28 (Figure 65C) showed prominent cell death on either side of the dark purple stain. The tissue death on the edges of the sample could be because of its submergence in the tetraflouroethane solution for 10 minutes after the sample was sprayed. These observations indicate that the tetraflouroethane spray alone is ineffective in destroying the cells in the foreskin. The samples that were left in the bath of tetraflouroethane for a period of 10 minutes showed some cell death. Ultimately, there is a lack of controllability and reproducibility associated with this method of thermal injury.

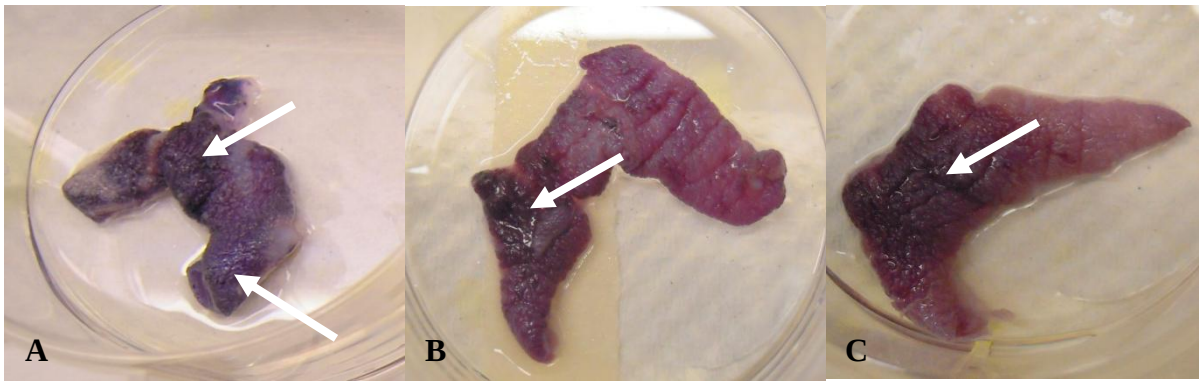

Figure 65: Cytocool samples 26-28, after the MTT assay.

6.8 Post-Cryoburn Culture Method with Histological Analysis and MTT

Following the promising, but still rather inconclusive results of the cryo and thermal burns investigated by the team using histology and described in Section 6.5 Cryo and Thermal Burns with Histology, the team returned to the literature. This research led to the discovery of a fundamental difference between the team's initial burning protocol and the protocols described by some of the leading researchers also inducing wounds on BES: the concept of post-injury culture. The team found that several other research groups were culturing their tissues for up to 72 hours after inducing injuries(Ghalbzouri AE, 2004). After discussing these findings with their advisors, the team developed a new burning protocol that included a post-injury culture period. The full details of this protocol can be found in Appendix F: Testing Procedures.

In order to prepare their samples for cryoburning and culture the team removed excess fat layers from the dermal side of six foreskin samples, then rinsed each tissue in an antibiotic rinse containing gentamicin, penicillin, and streptomysin. Each sample was then placed in one of the Pins lab culture devices and cultured for 24 hours at 37°C at the air liquid interface in order to dry the surface of the tissue. At 24 hours after preparation, four of the samples were assigned for burning for 30, 60, 90, or 120 seconds respectively, and two samples were designated as unburned, live controls. Immediately prior to burning approximately 500µL of 100% EtOH was applied to the surface of each foreskin sample in order prevent the formation of an ice layer and increase the penetration of the cold through the skin as described by a cryobranding procedure for livestock discovered by the team and their graduate advisor (L&H Branding Irons). Each foreskin tissue was then burned with the stainless steel cryorod, in culture devices for the designated length of time. In order to remember where the cryorod was applied, the approximate burn location was drawn on the cover of the six-well plate and was later used as a guide for marking with histology paint. After burning the six-well plate containing the cryoburned foreskins and controls was incubated at 37°C for 72 hours at the air-liquid interface with standard foreskin medium prepared by the Pins lab.

Following the 72 hour culture period the surface of each foreskin was thoroughly dried using sterilized paper towels. The wound margin marks on the cover of the six-well plate was then used as a guide to mark the wound margins of the foreskin tissues with a surgical swab handle dipped in blue tissue marking dye (Cancer Diagnostics Inc.). Samples were then cut in half with one half of each sample designated for histology and one for MTT analysis. The

samples designated for histology were placed in formalin and then later processed and sent out for sectioning. The team performed a hemotoxylin and eosin stain using the protocol described in Appendix F: Testing Procedures and imaged the stained slides using the Nikon Eclipse E600 microscope, RT Color Spot camera, and the SPOT version 4.6 software.

6.8.1 Post-Injury Culture - Histology

In Figure 66, below the results of the 30 second cyroburn and an unburned control can be seen. While the 30 second cryoburn tissue shows minor disorganization (arrows) of the epidermal layer and a slightly lower density of visible cells in the epidermal layer, both of which are indications of wounded tissue, the two tissues appear overall very similar. This comparison lead the team to determine that 30 seconds is not a long enough burn time to sufficiently injure the foreskin tissue.

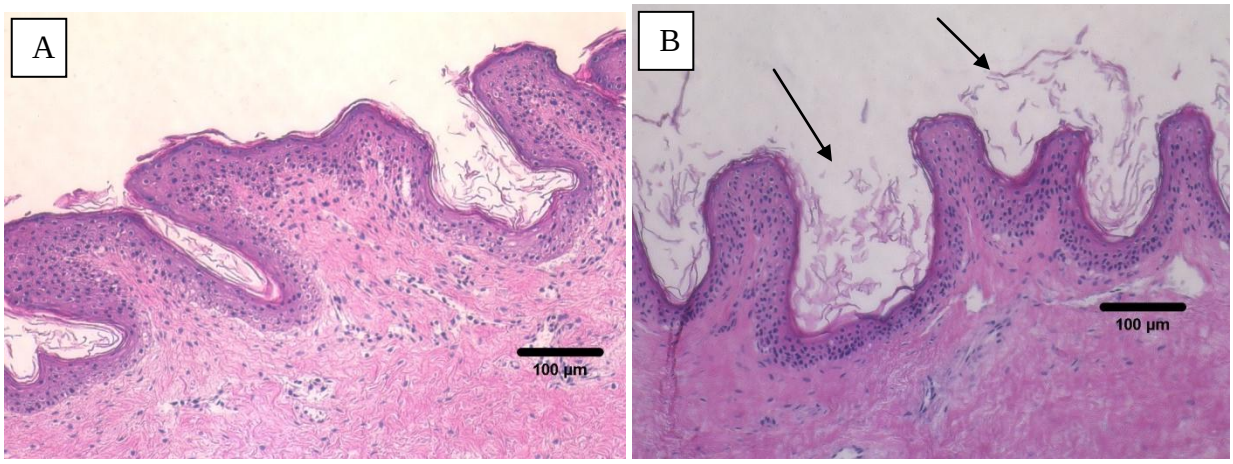

Figure 66: A unburned control tissue (A) and a 30 second cryoburn (B), both cultured 72 hours post injury period

Figure 67 below shows a 60 second cryoburn, with its wound margin marked by the blue tissue marking dye. This tissue has a distinct disorganization of the epidermal layer (arrow A), a separation of the epidermal and dermal layers of the tissue (arrow B) as well as a drastic decrease in epidermal and dermal cell density than that seen in the control tissue in Figure 66. All of these markers are evidence of a major injury to this tissue.

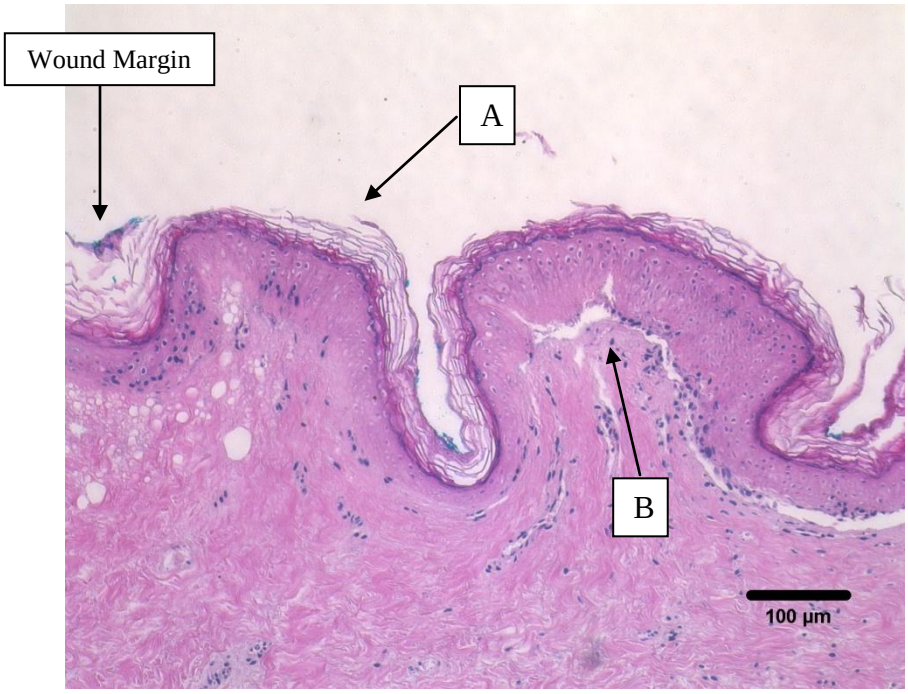

Figure 67: 60 second cryoburn, cultured 72 hours post-injury

The image of the 90 second cryoburn shown in Figure 68 highlights the wound margin, which was also marked with the blue tissue marking dye as well as the distinct differences between the burned and unburned tissue. Circles compare the disorganized epidermal layer of the burned side of the tissue to the more orderly epidermal layer of the unburned side. The arrow shows an area where the epidermal layer has been completely disrupted.

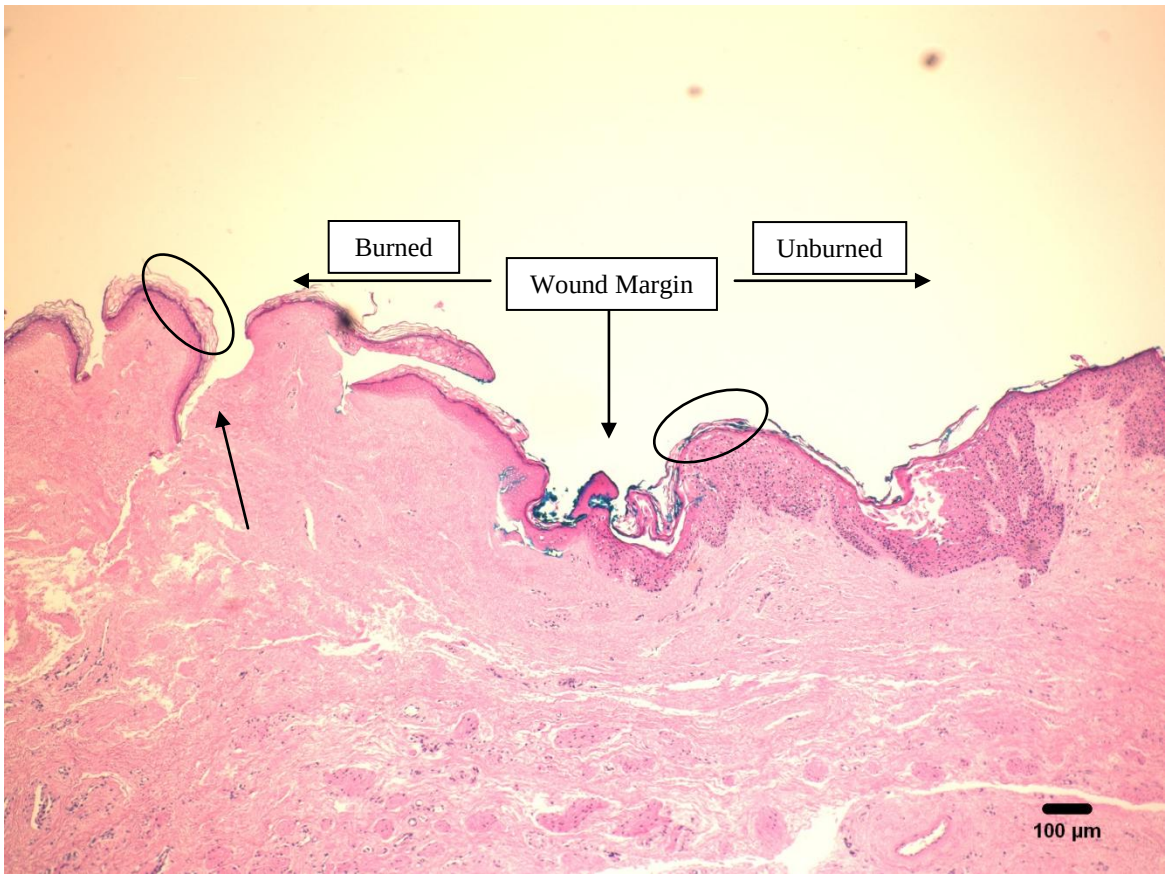

Figure 68: 90 second cryoburn, cultured 72 hours post injury (4x)

In the higher magnification image of the same 90 second cryoburn shown in Figure 69, a clear difference in the cell density between the two sides of the tissue can be observed (circles). Additionally, in the unburned side of the tissue there is an almost complete absence of cells in both the dermal and epidermal layers of the tissue, which may indicate apoptosis, or cell death. Finally, the arrow points out an area where the epidermal and dermal layers have separated. Each of the attributes identified in Figure 68 and Figure 69 are indications of a major injury.

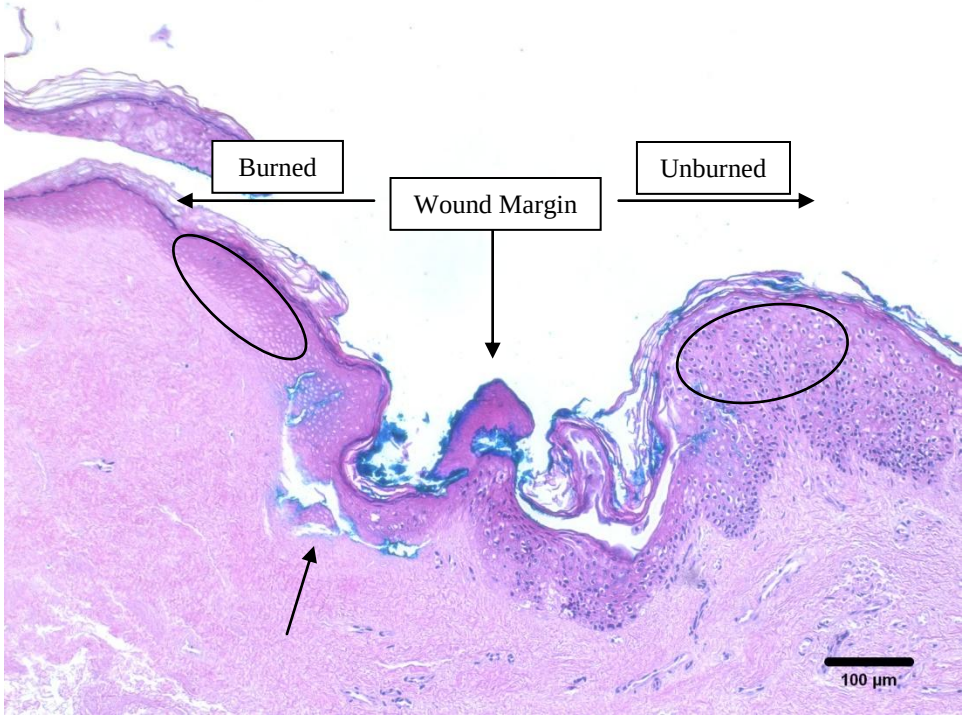

Figure 69: 90 second cryoburn, cultured 72 hours post injury (10x)

The final cryoburn from the 72 hour post-injury culture experiment was a 120 second cryoburn shown in Figure 70. This image shows many of the same burn indicators as the 90 second sample, but also shows some evidence of damage to the underlying dermal matrix (arrow). This damage could be an indication that the 120 seconds is a longer than ideal burn time for foreskin tissue.

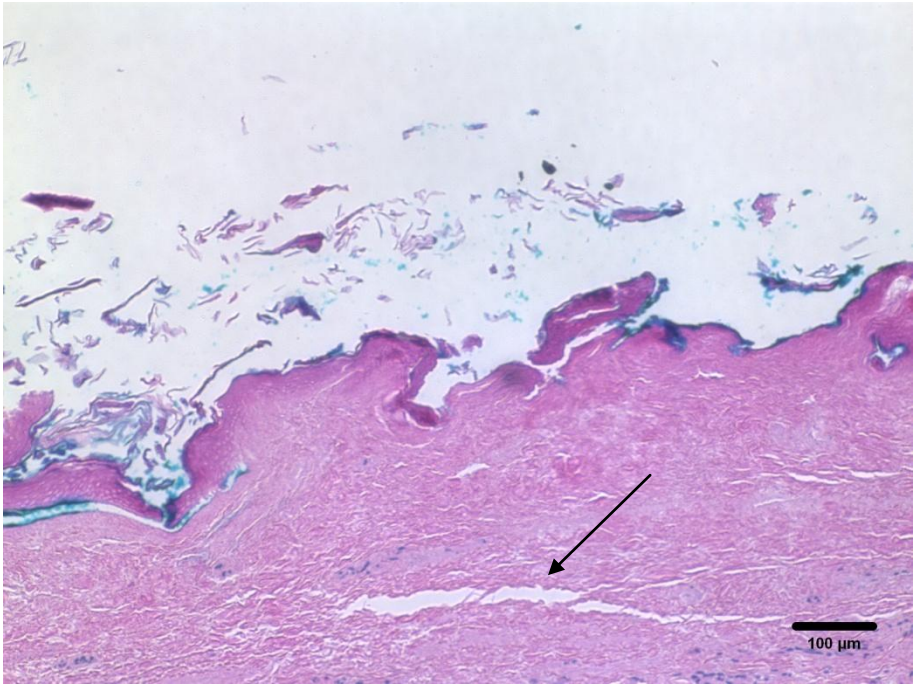

Figure 70: 120 second cryoburn, cultured 72 hours post injury

6.8.2 Post-Injury Culture - MTT

One half of each post-injury culture sample was reserved for MTT analysis which was carried out according to the procedure outlined in Appendix F: Testing Procedures. The results of this MTT were rather inconsistent with the MTT results experienced in the past and the promising results shown by the histology from this particular experiment. While the expectation was that the MTT would stain live tissue a dark purple and the dead or burned tissue would remain pink, the team found that after the two-hour incubation with MTT, the entire tissue remained a pink-white color. Additionally, the MTT solution liquid in each well remained a distinct yellow color where in other MTT experiments it had dulled to almost clear by the end of the two hour incubation. The team suspects that this may be due to old MTT solution, which had been in solution for several weeks. This is further evidence for the need to identify a better bench-top marker for cell viability for use in conjunction with the burning method. Unfortunately, due to time constraints, the team was unable to repeat the MTT portion of this experiment with fresh MTT solution, in order to confirm the hypothesis about the age of the solution's contribution to the poor results.

7. Final Design

At the conclusion of the project time line the team has identified two major accomplishments that are considered to encompass a final design:

1. The development of a functioning cryoburning device
2. The development of a method for inducing full thickness cryoburns with definitive wound margins on neonatal foreskin tissue samples

The team believes that both of these accomplishments are in line with the objectives and functions of the project and will be very useful for both the client and user.

7.2 Final Cryoburning Device Design

The final cryoburning device, which was designed and manufactured by the team is pictured in Figure 71. It is comprised of two Lexan trays fixed with stainless steel spacers and fixtures. The top tray has interchangeable masks with holes and serves to guide the stainless steel cryorod down to the tissue samples that will be contained in the six-well plate held by the lower Lexan tray. The finished device with cryorod stands approximately 14 inches tall and has a foot print of approximately 4.7 by 6.5 inches.

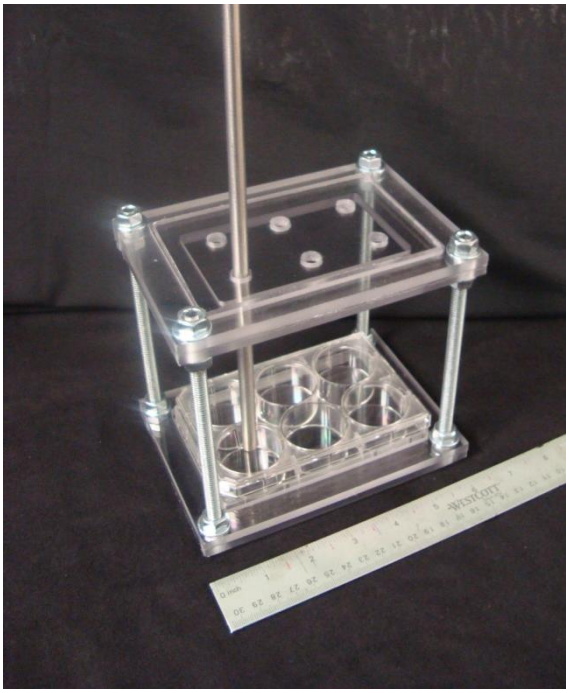

Figure 71: Photograph of Final Cryoburning Device

7.3 Final Wounding Model Design

In addition to the cryoburning device, the team has developed a protocol for inducing cryoburns on the primary test tissue: neonatal foreskin. This protocol was developed from the experiments performed by the team in which tissues were cultured for 72 hours post-injury and can be viewed in full in Appendix F: Testing Procedures. Additionally, based on the results of the histology from the initial experiments, the team has determined that the most appropriate cryorod-tissue exposure time for neonatal foreskin is 90 seconds, as this duration creates a visible, full thickness burn, with definitive wound margins, that can be characterized by histological analysis.

8. Discussion

Throughout the design and testing process several external factors impacted by the team's device were considered and evaluated. Some important factors that were considered by the team were the device's economic impact, environmental impact, societal impact, political ramifications, ethical concerns, health and safety issues, manufacturability and sustainability.

8.1 Device Design

After brainstorming several design alternatives, the team decided upon the cryoprobe hanger design discussed in chapter 5. The use of a probe hanger and weighted rod grant the user more control over the cryorod's movement and ensure that the burn is reproducible on neonatal foreskin samples suspended in the six-well plate on the lower-most tray. The entire device is composed of two materials: stainless steel and Lexan to allow for easy sterilization in the Pins laboratory.

8.2 Economic Impact

The probe-hanger cryoburn device was designed to be user-friendly and efficient in creating reproducible burns on BES; more specifically the Composite Bioengineered Skin Substitutes (CBSS) created in the Pins Lab. The team also considered financial factors during the design process, making the materials used and the production of the device cost-effective for consumers. Materials such as Lexan and stainless steel proved to be economical, since both these materials are not only readily available but can also be purchased and machined at relatively low costs.

There is a strong clinical need for the wounding model device in the field of regenerative medicine. For researchers studying *in vitro* thermal injuries to BES, this device can be used to create reproducible full-thickness wounds with definitive wound margins. The team believes that the device's unique simple design could serve as a wounding model for other research laboratories.

With the market introduction of this device, the study of burns and chronic ulcers will progress, resulting in more effective treatment options for rapid regeneration and wound healing. This research could greatly impact the pharmaceutical market for burn wound treatment and also improve the economical aspect of the ongoing research in this field.

8.3 Environmental Impact

Both stainless steel and Lexan (polycarbonate) are reusable and recyclable material respectively. Therefore, if the device needs to be discarded for any reason, it would be economical to recycle or reuse the components of the device. The device was also designed to be used repeatedly without the need for disposable components. All components of the device, including the cryorod, can be sterilized and reused multiple times. This sterilization will not have any negative impact on the environment.

8.4 Societal Influence/Impact

If used outside of the laboratory for which it was designed, this device has the potential to positively impact the society. Using this device, researchers would be able to easily create reproducible burns, which would allow them to characterize and study wounding healing and what is involved in regeneration so they could create an off-the-shelf BES capable of rapid regeneration. Any progress made in the treatment of burns and chronic ulcers due to use of the team's device would improve the quality of life for patients.

8.5 Political Ramifications

Several countries around the world believe that the use of neonatal foreskins for research is not socially or religiously acceptable. For this reason, there could be a significant issue with the procedures used in the development of this device or the device itself if neonatal foreskins continue to be the tissue model for this project. While realizing the oppositions to neonatal foreskins is an important factor, it is also important to understand the importance of their contribution towards implementing successful treatments for patients suffering from severe burn injuries and chronic ulcers. It is also important to note that future research does not necessarily include the use of foreskin tissue, and there are other tissue options that could be used as a sample tissue should issues arise.

8.6 Ethical Concerns

The team does not believe that the use of neonatal foreskin violates any ethical codes within the United States. The foreskin samples used for testing purposes was collected from the UMMS Hospital. These foreskins are considered waste tissue and would have been discarded post-surgery if not utilized by the team. The samples are from anonymous donors and all samples were screened for diseases and drug use prior to being given to the team. The use of the foreskin

samples provide means of producing *in vitro* burns to further research in this important field of medicine. Furthermore, the no animal or clinical testing was completed during this project.

8.7 Health and Safety Impacts/Concerns/Issues

As mentioned earlier, the wound model device contributes to the field of regenerative medicine including the treatment of severe burns and chronic ulcer wounds. Since this device is not used clinically, there is no potential for health or safety concerns. However, the device is carefully fabricated with a simple and safe design, in order to ensure the safety of the user. The team avoided using any toxic or harmful materials during manufacturing while working in the manufacturing labs in Goddard Laboratories where all safety protocols were followed. In order to ensure the sterility of the foreskin samples and the device, all tests were carried out under a laminar fume hood with sterile instruments.

8.8 Manufacturability

The device's simple design makes it easy to manufacture. The device only uses two materials: stainless steel and Lexan, both of which are readily available The simple design also makes the device durable and easy to repair, allowing the user and manufacturer to save money on repairs when compared to more sophisticated devices. A small, focused market is predicted for this device as it is meant to be used in laboratories that are studying the impact of burn wounds on BES. For this reason, this device would be manufactured in small volumes per order. The device would not only be economical to purchase, but also economical to manufacture and introduce into the market.

9. Future Plans

The design team has created a device that facilitates a reproducible injury model on neonatal foreskins. This device is suitable for use in the Pins' Lab with the culture devices that are used to seed and fabricate their Composite Bioengineered Skin Substitutes (CBSS). To optimize space, the device is capable of injuring a total of six CBSS at one time in the six well plate resting on the lower tray of the device assembly.

This project has shown that the device is effective in creating a full-thickness wound on neonatal foreskins after exposing the tissue to a liquid nitrogen cooled stainless steel rod and for 90 seconds followed by 72 hours of post-injury culture at 37°C. In the future, the appropriate exposure time will need to be established for the CBSS. Due to the mechanical integrity and structure of CBSS, it is presumed that this exposure time will be shorter than that of the foreskin. The teams' hypotheses will need to be confirmed with further testing on the CBSS samples until an optimal burn time is determined. The team understands that as the exposure time increases visible indicators of a full-thickness burn will appear (Garlick J, 2006) and the user should use these indicators to determine the optimal burn time for CBSS based on the extent of damage desired.

Initially the team sought a simple, reliable, bench-top assay for characterizing the cryoburns they created, but after a great deal of testing the minimally invasive MTT and Trypan blue stains created equivocal results which made it difficult to conclude whether or not a burn was created on the foreskins and the team relied histological analysis for burn characterization. Because histological analysis required several days before results are available, a reliable bench top assay that is capable of indicating more clearly if a burn is present still needs to be identified.

Following the adaptation of the team's device and wounding procedure to CBSS, the client and user will use this design to further analyze the burn and the resulting reepithelialization, migration and proliferation that occurs in the CBSS *in vitro*. They will also use this device to create burn models in which they will attempt to characterize paracrine signaling during regeneration and reepithelialization in the CBSS. This will be an *in vitro* attempt to model cellular signaling that naturally occurs at the interface of the implant with the native tissue in burn and ulcer treatments. Improving the ability of the CBSS to rapidly integrate with native tissue, could be a step toward creating a clinically available skin substitute that outperforms the current treatments like Apligraf® and Integra®.

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Appendix A: Pairwise Comparison Charts

Table 23: Pairwise Comparison Chart for Main Objectives

Top Level Objectives	Cost Effective	Device Should be Useful	Device Should be Accurate	Burn Model Should be Able to be Characterized	Ease of Use	Device Should be Precise	Total	Weight
Cost Effective								
Device Should be Useful								
Device Should be Accurate								
Burn Model Should be Able to be Characterized								
Ease of Use								
Device Should be Precise								

Table 24: Pairwise Comparison Chart for Sub-Objectives of Device Should Be Useful

Objective: Device Should be Useful	Maintain the BES matrix	Compatible with client BES substitutes	Create a reproducible wound	Total	Weight
Maintain the BES matrix					
Compatible with client BES substitutes					
Create a reproducible wound					

Table 25: Pairwise Comparison Chart of Sub-Objective Ease of Use

Objective: Ease of Use	User Friendly	Durable	Small Footprint	Sterilizable	Ease of Assembly	Total	Weight
User Friendly							
Durable							
Small Footprint							
Sterilizable							
Ease of Assembly							

Table 26: Pairwise Comparison Chart of Sub-Objective Device Should Be Precise

Objective: Device should be precise	Consistency	Reproducibility	Total	Weight
Consistency				
Reproducibility				

Table 27: Pairwise Comparison Chart of Sub-Objective Cost Effective

Objective: Cost Effective	High Throughput	Reusable	Total	Weight
High Throughput				
Reusable				

Table 28: Pairwise Comparison Chart of Sub-Objective Burn Model Should Be Able to Be Characterized

Objective: Burn Model should be able to be characterized	Analytical Chemistry	Histological Analysis	Non-Invasive Imaging	Total	Weight
Analytical Chemistry					
Histological Analysis					
Non-Invasive Imaging					

Appendix B: Client's Completed Pairwise Comparison Charts

Table 29: Client's Completed Pairwise Comparison Chart for Main Objectives

Top Level Objectives	Cost Effective	Device Should be Useful	Device should be accurate	Burn Model should be able to be characterized	Ease of Use	Device should be precise	Total	Weight
Cost Effective	**X**	0	1	0	1	0	2	0.13
Device Should be Useful	1	**X**	1	0.5	1	0.5	4	0.27
Device should be accurate	0	0	**X**	0	0	0	0	0
Burn Model should be able to be characterized	1	0.5	1	**X**	1	0.5	4	0.27
Ease of Use	0	0	1	0	**X**	0	1	0.07
Device should be precise	1	0.5	1	0.5	1	**X**	4	0.27

Table 30: Client's Completed Pairwise Comparison Chart for Sub-Objectives of Device Should Be Useful

Objective: Device Should be Useful	Maintain the BES matrix	Compatible with client BES substitutes	Create a reproducible wound	Total	Weight
Maintain the BES matrix	**X**	0.5	0	0.5	0.17
Compatible with client BES substitutes	0.5	**X**	0.5	1.0	0.33
Create a reproducible wound	1	0.5	**X**	1.5	0.50

Table 31: Client's Completed Pairwise Comparison Chart of Sub-Objective Ease of Use

Objective: Ease of Use	User Friendly	Durable	Small Footprint	Sterilizable	Ease of Assembly	Portable	Total	Weight
User Friendly	**X**	1	1	0	0.5	0.5	3	0.20
Durable	0	**X**	0	0	0	0	0	0
Small Footprint	0	1	**X**	0	0	0	1	0.07
Sterilizable	1	1	1	**X**	0.5	0.5	4	0.27
Ease of Assembly	0.5	1	1	0.5	**X**	0.5	3.5	0.23
Portable	0.5	1	1	0.5	0.5	**X**	3.5	0.23

Table 32: Client's Completed Pairwise Comparison Chart of Sub-Objective Device Should Be Precise

Objective: Device should be precise	Consistency	Reproducibility	Total	Weight
Consistency	**X**	0.5	0.5	0.50
Reproducibility	0.5	**X**	0.5	0.50

Table 33: Client's Completed Pairwise Comparison Chart of Sub-Objective Cost Effective

Objective: Cost Effective	High Throughput	Reusable	Total	Weight
High Throughput	**X**	1	1	1
Reusable	0	**X**	0	0

Table 34: Client's Completed Pairwise Comparison Chart of Sub-Objective Burn Model Should Be Able to Be Characterized

Objective: Burn Model should be able to be characterized	Analytical Chemistry	Histological Analysis	Non-Invasive Imaging	Total	Weight
Analytical Chemistry	**X**	0.5	1	1.5	0.50
Histological Analysis	0.5	**X**	1	1.5	0.50
Non-Invasive Imaging	0	0	**X**	0	0

Appendix C: Designer's Completed Pairwise Comparison Charts

Table 35: Designer's Completed Pairwise Comparison Chart for Main Objectives

Top Level Objectives	Cost Effective	Device Should be Useful	Device should be accurate	Burn Model should be able to be characterized	Ease of Use	Device should be precise	Total	Weight
Cost Effective	X	0	0	0	0	0	0	0
Device Should be Useful	1	X	1	1	1	1	5	0.33
Device should be accurate	1	0	X	1	.5	.5	3	0.20
Burn Model should be able to be characterized	1	0	0	X	1	.5	2.5	0.17
Ease of Use	1	0	.5	0	X	0	1.5	0.10
Device should be precise	1	0	.5	.5	1	X	3	0.20

Table 36: Designer's Completed Pairwise Comparison Chart for Sub-Objectives of Device Should Be Useful

Objective: Device Should be Useful	Maintain the BES matrix	Compatible with client BES substitutes	Create a reproducible wound	Total	Weight
Maintain the BES matrix	X	.5	0	.5	0.17
Compatible with client BES substitutes	.5	X	.5	1	0.33
Create a reproducible wound	1	.5	X	1.5	0.10

Table 37: Designer's Completed Pairwise Comparison Chart of Sub-Objective Ease of Use

Objective: Ease of Use	User Friendly	Durable	Small Footprint	Sterilizable	Ease of Assembly	Total	Weight
User Friendly	X	1	1	.5	1	3.5	0.35
Durable	0	X	.5	0	1	1.5	0.15
Small Footprint	0	.5	X	0	1	1.5	0.15
Sterilizable	.5	1	1	X	1	3.5	0.35
Ease of Assembly	0	0	0	0	X	0	0

Table 38: Designer's Completed Pairwise Comparison Chart of Sub-Objective Device Should Be Precise

Objective: Device should be precise	Consistency	Reproducibility	Total	Weight
Consistency	X	.5	.5	0.50
Reproducibility	.5	X	.5	0.50

Table 39: Designer's Completed Pairwise Comparison Chart of Sub-Objective Cost Effective

Objective: Cost Effective	High Throughput	Reusable	Total	Weight
High Throughput	X	1	1	1
Reusable	0	X	0	0

Table 40: Designer's Completed Pairwise Comparison Chart of Sub-Objective Burn Model Should Be Able to Be Characterized

Objective: Burn Model should be able to be characterized	Analytical Chemistry	Histological Analysis	Non-Invasive Imaging	Total	Weight
Analytical Chemistry	X	.5	1	1.5	0.50
Histological Analysis	.5	X	1	1.5	0.50
Non-Invasive Imaging	0	0	X	0	0

Appendix D: User's Completed Pairwise Comparison Charts

Table 41: User's Completed Pairwise Comparison Chart for Main Objectives

Top Level Objectives	Cost Effective	Device Should be Useful	Device should be accurate	Burn Model should be able to be characterized	Ease of Use	Device should be precise	Total	Weight
Cost Effective	--	0	0	0	0	0	0	2
Device Should be Useful	1	--	1	1	1	1	5	33
Device should be accurate	1	0	--	0	1	0	2	13
Burn Model should be able to be characterized	1	0	1	--	1	1	4	26
Ease of Use	1	0	0	0	--	0	1	6
Device should be precise	1	0	1	0	1	--	3	20

Table 42: User's Completed Pairwise Comparison Chart for Sub-Objectives of Device Should Be Useful

Objective: Device Should be Useful	Maintain the ES matrix	Compatible with client ES substitutes	Create a reproducible wound	Total	Weight
Maintain the ES matrix	--	0	1	1	33
Compatible with client ES substitutes	1	--	1	2	66
Create a reproducible wound	0	0	--	0	1

Table 43: User's Completed Pairwise Comparison Chart of Sub-Objective Ease of Use

Objective: Ease of Use	User Friendly	Durable	Small Footprint	Sterilizable	Ease of Assembly	Portable	Total	Weight
User Friendly	--	1	1	0	0	1	3	20
Durable	0	--	1	0	0	0	1	7
Small Footprint	0	0	--	0	0	1	1	7
Sterilizable	1	1	1	--	1	1	5	33
Ease of Assembly	1	1	1	0	--	1	4	26
Portable	0	1	0	0	0	--	1	7

Table 44: User's Completed Pairwise Comparison Chart of Sub-Objective Device Should Be Precise

Objective: Device should be precise	Consistency	Reproducibility	Total	Weight
Consistency	--	.5	.5	50
Reproducibility	.5	--	.5	50

Table 45: User's Completed Pairwise Comparison Chart of Sub-Objective Cost Effective

Objective: Cost Effective	High Throughput	Reusable	Total	Weight
High Throughput	--	1	1	99
Reusable	0	--	0	1

Table 46: User's Completed Pairwise Comparison Chart of Sub-Objective Burn Model Should Be Able to Be Characterized

Objective: Burn Model should be able to be characterized	Analytical Chemistry	Histological Analysis	Non-Invasive Imaging	Total	Weight
Analytical Chemistry	--	0	1	1	33
Histological Analysis	1	--	1	2	66
Non-Invasive Imaging	0	0	--	0	1

Appendix E: Weighted Objectives Ranking Matrix

		Cryoshower		Cryoprobe		Hot Plate Disk	
	Weight	Score	**Weighted Score**	Score	**Weighted Score**	Score	**Weighted Score**
Cost Effective	**13**	1	**13**	0.66	**8.58**	0.33	**4.29**
Device Should be Useful	**27**	1	**27**	0.33	**8.91**	0.66	**17.82**
Device should be accurate	**0**	0	**0**	0	**0**	0	**0**
Burn Model should be able to be characterized	**27**	1	**27**	0.33	**8.91**	0.66	**17.82**
Ease of Use	**7**	1	**7**	0.5	**3.5**	0.5	**3.5**
Device should be precise	**27**	1	**27**	0.33	**8.91**	0.66	**17.82**
Maintain the BES matrix	**17**	0.66	**11.22**	0.33	**5.61**	0.66	**11.22**
Compatible with client BES substitutes	**33**	0.33	**10.89**	0.33	**10.89**	0.33	**10.89**
Create a reproducible wound	**50**	1	**50**	0.33	**16.5**	0.33	**16.5**
User Friendly	**20**	1	**20**	0.33	**6.6**	0.66	**13.2**
Small Footprint	**7**	0.66	**4.62**	0.33	**2.31**	1	**7**
Sterilizable	**27**	0.66	**17.82**	0.66	**17.82**	1	**27**

Ease of Assembly	**23**	1	**23**	0.66	**15.18**	0.33	**7.59**
Portable	**23**	0.33	**7.59**	0.33	**7.59**	0.66	**15.18**
Total	**301**		**246.14**		**121.31**		**169.83**
Percentage			**0.818**		**0.403**		**0.564**

Appendix F: Testing Procedures

Initial Concept Testing (Chicken Skin Testing)

Cryoprobe

Materials:

- Test tissue: chicken skin
- Small metallic rod: standard drill bits
- Insulated bucket
- 0.5L liquid nitrogen
- Petri dish
- Long forceps
- Scalpel

Procedure:

1. Prepare test tissue by using scalpel to cut approx. 3cm x 3cm square pieces and place them in Petri dish
2. Obtain 0.5 L liquid nitrogen in insulated bucket
3. Using forceps, place small metallic rod in liquid nitrogen
4. When rod stops “boiling” (when rod and liquid nitrogen temperatures are in equilibrium) remove from liquid nitrogen using forceps
5. Apply tip of frozen rod to surface of tissue for approx. 10 seconds
6. Record observations
7. Repeat using different skin contact times

Hot Plate Disks (also used with Foreskin as test tissue)

Materials:

- Test tissue: chicken skin
- Small metallic disks: various size washers and small ceramic tile

- Hot plate
- Petri dish
- Long forceps
- Scalpel

Procedure:

1. Prepare test tissue by using scalpel to cut approx. 3cm x 3cm square pieces and place them in Petri dish
2. Heat hot plate to 150°C
3. Using forceps, place washers and ceramic tiles on hot plate
4. After approximately 5 minutes remove washers and ceramic

5. Apply washers and ceramic to surface of tissue for approx. 10 seconds
6. Observe
7. Repeat using different hot plate temperatures, heating times, and skin contact times

Initial Cryoprobe Concept Testing

Materials:

- Test tissue: human foreskin
- Small metallic rod: standard drill bits or 5 in long by 1/8 in diameter stainless steel rods
- Insulated bucket
- 0.5L liquid nitrogen
- Petri dish
- Long forceps
- Small forceps
- Scalpel
- Pipette with tips
- Biosafety cabinet

Procedure:

1. Place all materials inside biosafety cabinet
2. Remove test tissue from collection tube containing media
3. Prepare test tissue by using scalpel to cut tube shaped foreskin from the center outwards to create a rectangle shape
4. Remove excess fat layers from dermal side of foreskin tissue using scalpel and small forceps
5. Obtain 0.5 L liquid nitrogen in insulated bucket
6. Using forceps, place small metallic rod in liquid nitrogen
7. When rod stops “boiling” (when rod and liquid nitrogen temperatures are in equilibrium) remove from liquid nitrogen using forceps
8. Apply tip of frozen rod to surface of tissue for desired contact time
9. Observe

10. If desired repeat using different skin contact times

Prototype Cryoprobe Testing

Materials:

- Test tissue: human foreskin
- Cryorod of desired material: stainless steel, ceramic, or other materials
- Insulated bucket
- 0.5L liquid nitrogen
- Petri dish
- Long forceps
- Small forceps
- Scalpel blade and handle
- Pipette with tips
- Biosafety cabinet

Procedure:

1. Place all materials inside biosafety cabinet
2. Remove test tissue from collection tube containing media and place in petri dish
3. Prepare test tissue by using scalpel to cut foreskin tissue such that it lies flat
4. Remove excess fat layers from dermal side of foreskin tissue using scalpel and small forceps
5. Obtain 0.5 L liquid nitrogen in insulated bucket
6. Using forceps, place cryorod of desired material in liquid nitrogen
7. When rod stops “boiling” (when rod and liquid nitrogen temperatures are in equilibrium) remove from liquid nitrogen using forceps
8. Apply tip of frozen rod to surface of tissue for desired contact time
9. Observe
10. If desired repeat using different skin contact times

Trypan Blue Tissue Viability Assay

Materials:

- Cryoburned foreskin samples
- 0.4% w/v Trypan blue dye
- PBS
- Pipette
- Pipette tips

Procedure:

1. Dilute Trypan blue to 0.1% w/v with PBS
2. Apply 500µL 0.1% w/v Trypan blue to the epidermal surface of foreskin sample
3. Allow to sit for approximately 30 seconds
4. Thoroughly rinse stained sample in 10 mL PBS
5. Observe
6. Repeat for each additional sample

MTT Tissue Viability Assay

Materials:

- MTT powder
- dPBS?
- PBS
- Six well plate
- Pipette
- Pipette tips
- Appropriate hazardous waste container with label

Procedure:

1. Prepare liquid MTT by diluting 1 mg powder in 1 mL PBS

2. Place each foreskin sample in a separate well of a six well plate and label
3. Add enough MTT liquid to completely cover each sample (approximately 3 mL)
4. Allow to incubate at room temperature for 2 hours
5. Rinse each sample 3x with PBS, disposing of waste liquid in hazardous waste container
6. Observe and image as desired

Histological Analysis

Sample Fixing, Processing, and Embedding

Following burning, samples were fixed and processed using overnight processing cycle on the Shandon Citadel 2000 Tissue Processor at WPI's Gateway Park Center for Life Sciences and Bioengineering. This device processes tissues for histological analysis by taking them through a series of different chemicals including:

1. Formalin
2. 70% alcohol
3. 80% alcohol
4. 95% alcohol
5. 95% alcohol
6. 100% alcohol
7. 100% alcohol
8. 100% alcohol
9. Xylene
10. Xylene
11. Paraffin
12. Paraffin

After processing, each sample was then embedded in paraffin wax using a Leica EG1160 Embedding Station.

Sample Sectioning

Following processing and embedding, samples were sent away to the WPI Histology Core at Gateway Park for sectioning and mounting onto slides.

Hematoxylin and Eosin Staining

After receiving the sectioned and mounted samples, the team stained each sample slide using a standard Hematoxylin and Eosin Staining Procedure for Paraffin Sections already used by the Histology Core at Gateway Park. The procedure is as follows:

1. Xylene – 3 minutes
2. Xylene – 3 minutes
3. Xylene – 3 minutes
4. 100% EtOH – 3 minutes
5. 100% EtOH – 3 minutes
6. 95% EtOH – 1 minute
7. 70% EtOH – 1 minute
8. Rinse in running H_2O – until clear
9. Harris Hematoxylin – 5 minutes
10. Rinse in running H_2O – until clear
11. Differentiate sections in 1% HCL mixed in 70% EtOH (.25mL HCL to 100mL of 70% EtOH) – 2-3 quick dips
12. Rinse in running H_2O – 30 seconds
13. Blue sections in ammonia solution (3mL of 28% ammonia hydroxide in 1L tap water) – 30 seconds
14. Rinse in running warm H_2O – 5 minutes
15. 95% EtOH – 30 seconds
16. Conterstain in Eosin-Y – 1 minute
17. 70% EtOH – 30 seconds
18. 95% EtOH – 30 seconds
19. 95% EtOH – 30 seconds
20. 100% EtOH – 1 minute
21. 100% EtOH – 1 minute
22. 100% EtOH – 1 minute
23. Xylene – 1 minute
24. Xylene – 1 minute
25. Xylene – 1 minute

26. Coverslip slides using synthetic mounting medium

Cryoprobe Testing Followed by 72 hr Post-injury Culture

Materials:

- Test tissue: human foreskin
- Pins lab CBSS culture devices
- Small Allen wrench
- 6-well plate
- Foreskin medium
- Stainless steel cryorod
- Insulated bucket
- 0.5L liquid nitrogen
- Petri dish
- Long forceps
- Small forceps
- Scalpel blade and handle
- Pipette with tips
- Biosafety cabinet
- 100% EtOH
- Incubator at 37°C
- Sterile paper towels
- Blue tissue marking dye (Cancer Diagnostics Inc.)
- Surgical swab

Procedure:

Sample preparation:

- Place foreskins, culture devices, Allen wrench, forceps and scalpel in biosafety cabinet
- Remove test tissue from collection tube containing media and place in petri dish
- Prepare tissue for testing by using scalpel to cut foreskin tissue such that it lies flat
- Remove excess fat layers from dermal side of foreskin tissue using scalpel and small forceps
- Place foreskin tissues in culture devices and tighten screws using Allen wrench

- Place each culture device in well of a 6-well plate and add media underneath device
- Culture foreskins at air-liquid interface for approximately 24 hours at 37°C (in incubator)

Cryoburning:

- Obtain 0.5 L liquid nitrogen in insulated bucket
- Using forceps, place cryorod in liquid nitrogen
- Place foreskins in culture devices, 100% EtOH, histology paint, and surgical swab in biosafety cabinet
- When rod stops "boiling" (when rod and liquid nitrogen temperatures are in equilibrium) apply approximately 500µL 100% EtOH to tissue surface
- Immediately remove cryorod from liquid nitrogen using forceps and apply tip of frozen rod to surface of tissue for desired contact time
- Allow tissue to thaw to approximately room temperature
- Mark approximate wound margins on cover of six-well plate
- If desired repeat using different burning skin contact times
- Add more foreskin medium to 6-well plate and culture cryoburned foreskins at 37°C for 72 hours

Fixing of Samples:

- Following 72 hour post injury culture, dry tissue surface thoroughly using sterile paper towels
- Mark wound margins (as approximated on cover of six-well plate using handle of surgical swab dipped in histology paint
- Remove foreskin samples from culture devices
- Place each sample in formalin and proceed with standard histology procedure

Appendix G: Components of Tray Assemblies

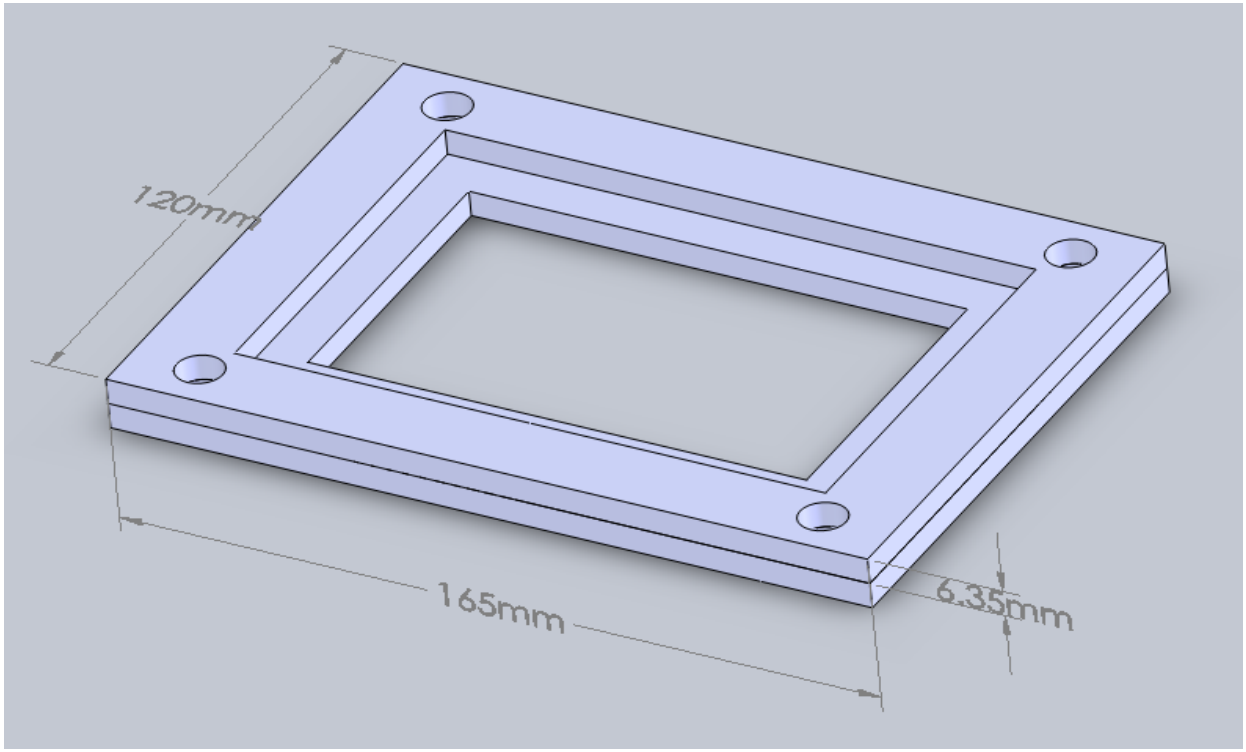

Figure 72: Top Tray Assembly without Mask

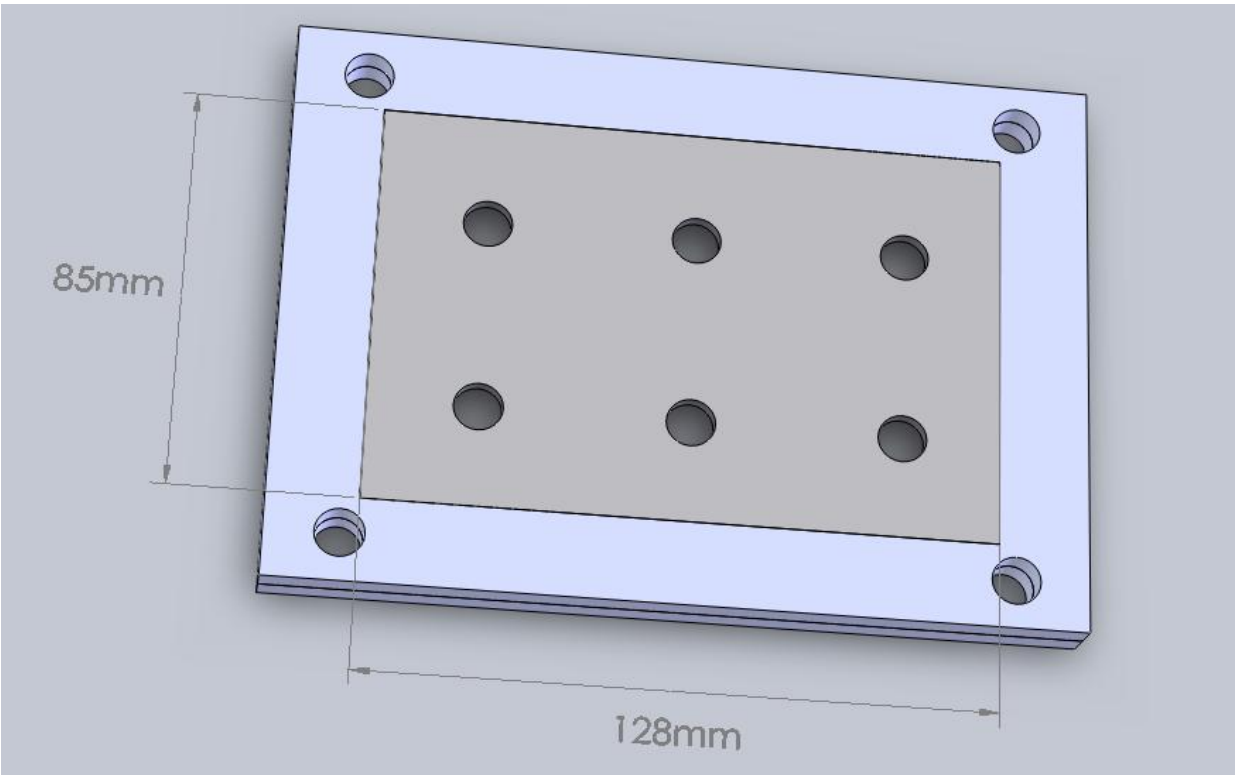

Figure 73: Top Tray Assembly with Mask

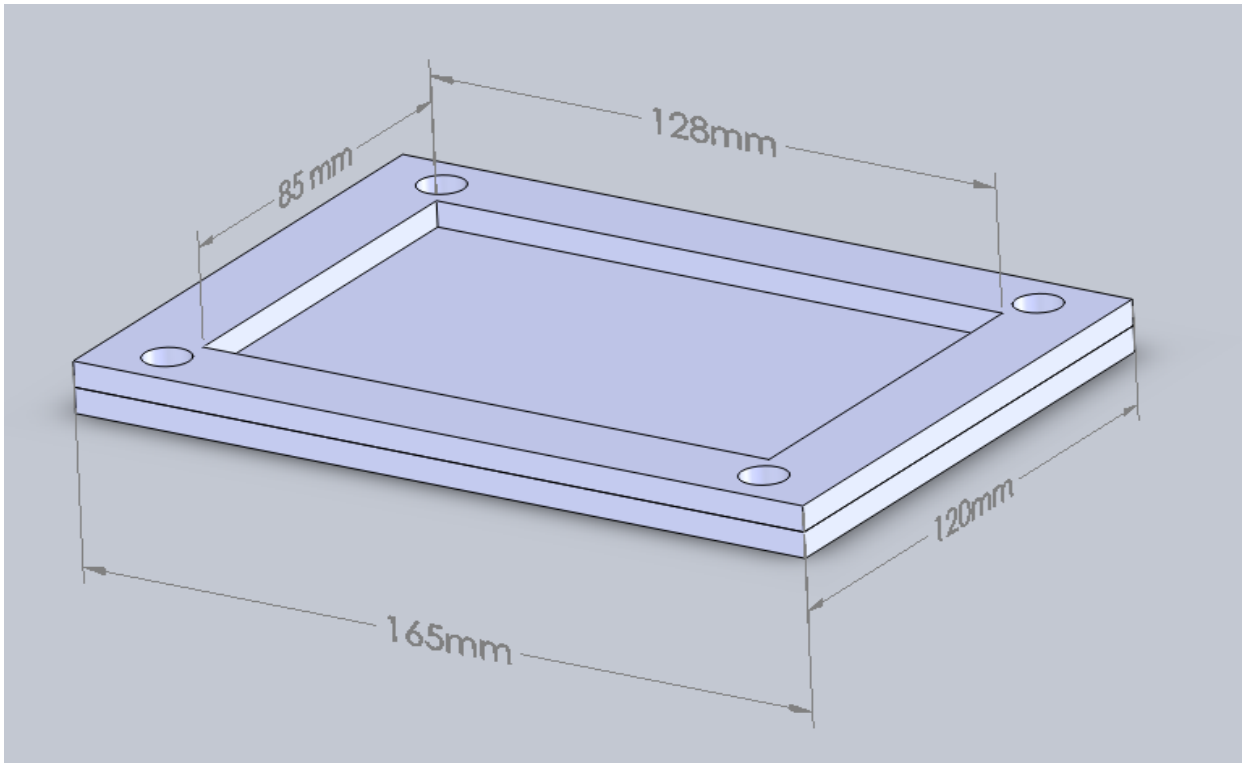

Figure 74: Bottom Tray Assembly