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Design of a Co-culture System Using Collagen Microthreads to Facilitate Neovascularization

A Major Qualifying Project Report

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Abstract

Several co-culture systems incorporating fibroblasts and endothelial cells have been used to characterize cell-cell and cell-matrix interactions critical for angiogenesis in wound healing. However, these models do not provide a means for translation into therapies for chronic non-healing wounds. We developed an approach that may facilitate *in vivo* neovascularization utilizing the advantages of both proangiogenic co-culture and collagen microthreads. This system could also serve as an *in vitro* tool to study angiogenic mechanisms. After design validation pilot studies, self-assembled collagen microthreads populated with human dermal fibroblasts (HDFs) were extruded using a novel method. Human umbilical vein endothelial cells (HUVECs) were then seeded on the surface of the HDF-populated collagen microthreads. Fluorescence microscopy confirmed the presence of both cell types after 48 hours of co-culture, and phalloidin staining was used to visualize cell morphology. These results demonstrate that co-culture can be established on a collagen microthread platform with these seeding methods, and that this system is viable after 48 hours of co-culture. Future studies of these co-cultured constructs should investigate their cellular interactions, angiogenic potential, mechanical properties, and implantation feasibility.

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1. Introduction

Ischemia-related diseases, such as cardiovascular disease and chronic wounds, are prevalent medical issues, affecting over 80 billion patients and costing over $480 billion annually [1, 2]. These diseases are characterized by tissue death, insufficient blood flow, and diminished functionality. Many groups have investigated potential treatments for these diseases. Among these treatments are options for cell delivery to the injured tissue. For example, cardiac ischemia or infarction might be treated with cardiomyocytes or skeletal myoblasts [3, 4]. It has been found that these therapies demonstrate distinct benefits over some traditional treatments. In particular, the formation of new blood vessels through angiogenic pathways has emerged as a potentially effective solution for these diseases [5].

The enhancement of angiogenesis toward more efficient and complete wound healing has been studied extensively both *in vivo* and *in vitro*. Physiological angiogenesis occurs through a highly-regulated cascade of endothelial cell activation, migration, proliferation, and tubule formation whereby new vasculature sprouts from pre-existing vessels [6]. Though endothelial cells play a critical role in neovascular formation, the delivery of endothelial cells (ECs) alone is not sufficient because the cells are not presented with the full complement of environmental cues required for proper angiogenesis [7]. A number of angiogenic and angiostatic growth factors are required for angiogenesis to occur [8, 9]. *In vivo*, periendothelial cells including fibroblasts serve as an abundant source of such angiogenic factors, activating ECs, promoting EC cell-cell contact, and directing angiogenesis [10]. Further, these cells produce extracellular matrix proteins that provide structural and biochemical support for the neovasculature [11].

Several groups have developed co-culture systems consisting of these two cell types to assess the role of each in angiogenesis. Using a co-culture model system with endothelial cells and

fibroblasts in a collagen gel, such studies have demonstrated induced angiogenesis *in vitro* as evidenced by EC activation, migration, and the formation of tube-like structures [6]. These models used collagen hydrogels and sponges to provide essential three-dimensional support and signaling templates for cellular interactions. However, the effectiveness of such scaffolds may be restricted by generally poor mechanical properties, limited mass transport, and an inability to translate directly to clinical application [12]. As such, there remains a need for an adequate delivery platform that will facilitate guided angiogenesis in a medical context.

Previously, small diameter fiber scaffolds derived from natural polymers have demonstrated improved mechanical strength as well as the ability to facilitate contact guidance, alignment, and orientation of cells [13]. These scaffolds can be composed of various biomaterials, such as collagen, and have been shown to endure *in vivo* implantation.

This basic principle was adapted into a novel extrusion method to enable and facilitate the embedding of cells within the microthread. Human dermal fibroblasts (HDFs) were co-extruded with a collagen suspension, creating HDF-populated microthreads. Cell seeding on a cylindrical surface provides a number of challenges, so a custom-built PDMS seeding device was created to provide constrained volume and geometry. This device promotes cell contact and adhesion to the curved microthread surface. Human umbilical vein endothelial cells (HUVECs) were seeded onto the surface of the collagen microthread using this device.

HDF-HUVEC co-cultured threads were characterized using fluorescent microscopy. Phalloidin was used to visualize cell morphology, and Hoechst was used to identify cell nuclei. To differentiate between the two cell types, the HUVECs were pre-loaded with DiI-ac-LDL, an endothelial cell-specific dye. Visualization using fluorescence microscopy was used to verify the spatial location of the cells as well as to determine viability based on cell morphology over

extended culture periods. All fluorescent images of the co-culture samples confirmed the presence of HDFs within, and HUVECs on the threads at all time points. Changes in HDF and HUVEC morphology were observed in both mono- and co-cultured threads as a function of time.

The developed technology, particularly the methods to independently seed two cell types, could also be utilized to construct threads for housing and studying other co-culture systems, not just pro-angiogenic ones. This opens up countless possibilities for creating easily deliverable cellular therapies and studying other *in vivo* physiologic mechanisms.

In the subsequent chapters, this project will elaborate on the importance of angiogenesis in wound healing, our project strategy, and alternative designs and verification. It will provide a discussion of results and the final design validation. It will also contain conclusions based on the analysis of the system, and recommendations for future work.

2. Literature Review

2.1 Clinical Motivation

2.1.1 The Problem

Ischemia occurs with insufficient blood supply or blood flow to a part of the body. This lack of oxygen and nutrients can lead to tissue damage or dysfunction [14, 15]. Ischemia is caused by the narrowing or restriction of blood vessels, which can be triggered by various sources including atherosclerosis, thrombosis, blood clots, diseases such as sickle-cell disease and peripheral vascular disease, congenital heart defects, and tachycardia. This lack of perfusion also occurs in chronic wounds, especially venous ulcers, diabetic ulcers, and pressure ulcers [16]. Major types of ischemia include cardiac, cerebral, mesenteric and cutaneous ischemia.

Infarction involves the death of tissue caused by a lack of perfusion to the tissue (ischemia). In a myocardial infarction, this is usually due to blockage in a coronary artery. After 20 to 40 minutes of severe ischemia, irreversible necrosis, or tissue death, will occur. The dead tissue is eventually replaced by scar tissue, which in some tissues, especially the heart, restricts operation and functionality [17]. An illustration of this is shown in Figure 1.

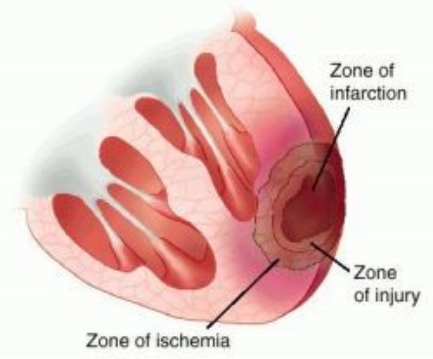

Figure 1: Myocardial Infarction Cross-Section. An example of a myocardial infarction shown in cross-section of the heart (ventricles only) with zone of infarction, zone of injury, and zone of ischemia. *Image from http://medical-dictionary.hefreedictionary.com/venous+infarction.*

In the case of a myocardial infarction, the dead myocardium and the resulting scar tissue cause ventricular fibrillation, which greatly impacts the functionality of the heart. Large amounts

of dead or scar tissue can lead to heart failure, as this tissue does not contract with the healthy heart tissue and leads to reduced blood flow. Ventricular fibrillation is a condition in which the heart cannot send normal electrical impulses to stimulate contraction, as the scar tissue is too prevalent, and instead the heartbeat becomes erratic and eventually stops, leading to death. The heart cannot sufficiently supply blood to the rest of the body promoting systemic ischemia. Diminished blood flow can contribute to additional cardiac ischemia and myocardial infarction by restricting the availability of oxygen and nutrients to the tissue, and can lead to failure in other organs, as well as brain damage and death [18].

2.1.2 Current Solutions

As lack of perfusion and the resulting tissue necrosis are widespread concerns that affect numerous areas of medicine, many treatments have been developed to address these conditions, specifically the causes and effects of dead tissue in the heart and the lack of blood flow to cells. Medications, surgical methods, and cellular therapies are currently in use as treatments.

Pharmaceuticals

Many pharmaceuticals have been developed to address the effects of ischemia and infarct, specifically the lack of blood to the injured tissue. For example, Angiotensin-Converting Enzyme (ACE) inhibitors work by blocking an enzyme responsible for the narrowing of blood vessels. With this enzyme blocked, vessels stay open and blood pressure is decreased; however respiratory complications are common, making this drug unsuitable for anyone with a pre-existing respiratory condition. A classification of medication known as vasodilators, such as nitroglycerin and other nitrates, widen and relax blood vessels to improve blood flow and allow more oxygen to reach ischemic tissues. This type of drug has few serious side effects, but

requires fastidious attention to other conditions and medications. Anti-platelet and anti-coagulant medications, commonly called “blood thinners”, are also prescribed to treat some conditions that can cause ischemia and infarction. Unfortunately, only a fraction of patients will have conditions that are treatable by this method, and there is a significant risk for the development of bleeding problems [19].

Surgical Methods

The percutaneous transluminal angioplasty (PTA) is a procedure used specifically in situations where plaque is the main cause of decreased blood flow. A balloon catheter is surgically inserted into an artery and is expanded. This action compacts the plaque into the wall of the artery and allows blood to flow freely. Often, this procedure is accompanied by the implantation of a stent, a small mesh-like tube, which holds the plaque against the artery wall, as shown in Figure 2. This process is potentially dangerous as the site of insertion of the PTA catheter can become infected, and the stent can also damage blood vessels around it [20]. In addition, this process has a limited success rate, as narrowed vessels have a 25% chance of recurring in the three to six months after surgery, especially in the presence of excessive scar tissue [21].

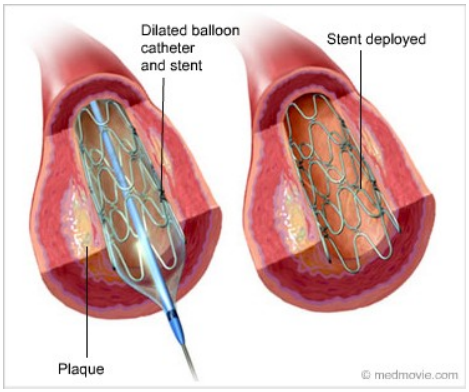

Figure 2: Balloon Catheter and Stent Deployed. The figure demonstrates the insertion and use of an arterial stent.

The artery bypass graft is another surgical procedure similar to PTA. Instead of an external, biocompatible synthetic, a small section of artery from another point in the body is grafted into the tissue. This surgery is, like the PTA, risky as it involves a major operation, and is therefore restricted to patients healthy enough to undergo invasive surgery [22].

Cellular Therapy

Cellular therapies are utilized to treat ischemic tissue and infarctions. The introduction of cells to the ischemic or infarcted tissue has been found to improve function by restoring the dead or injured tissue with new cells or by restoring perfusion to the injured tissue with the creation of neovascular structures. For example, a study, performed by Perin *et al*., was conducted with patients suffering from end-stage ischemic heart failure and followed the long-term effects of injection of bone marrow cells to the ischemic tissue. This study concluded that the introduction of these cells to the tissue delivers a long-term therapeutic effect through improved myocardial perfusion and regional contractility [23].

Currently, the most prevalent method for cell delivery is injection, either into the circulatory system through intravenous (IV) transport or directly into the injured tissue [24]. During intravenous transport, a solution of cells is prepared and introduced into the body through an IV. The cells travel through the circulatory system and make their way to the injury, where they will engraft to ischemic or infarcted tissue. This method has many flaws, including cell clumping and a slow rate of infusion [25]. In addition, there is no way to guarantee that the cells will localize to the targeted tissue. For example, introduced cardiac cells have been found in the spleen, kidney, and liver, and the lungs [25]. Direct injection of cells into the affected tissues can be accomplished in several ways. Some direct injection procedures, such as intracardiac injection, can be minimally invasive, as cells are injected through skin and muscle tissue into the injury

without surgery. A second procedure utilizes endoscopy, in which a tube that can be manipulated to access the injury and allows entry of medical instruments is inserted into the body, as a minimally invasive injection mechanism. A third method of direct injection is performed during surgery, in which a surgeon determines where the cells should be injected [24].

Despite successes in previous studies, direct injection of any kind has significant risks. Direct injection through the intervening skin and muscle layers has a long list of complications, such as the risk of additional damage within the tissue itself from the introduction of the needle as well as the risk to surrounding organs and tissues. Endoscopic procedures are not able to access all affected tissues, and endoscopy has additional risk of complications such as pain, bleeding, or infection, or perforations in the intermediate tissues [26]. While a more direct and specific approach to injection, this method does include all the risks of major surgery, including complications and death. Also, injected cells usually do not have sufficient ability to migrate, reducing the range of possible tissue repair and decreasing the effectiveness of these methods in large ischemic or infarcted areas [24].

With both intravenous transport and direct injection, it has been demonstrated that less than 3% of introduced cells will engraft to the site of the injury due to the delivery procedure [24]. From these results, we can conclude that delivery of therapeutic cells to injured tissue is has positive effects on cardiac tissue. However, the cell delivery method still reminds to be a problem needed to be addressed.

2.2.1 Biomaterial Scaffolds

Biomaterial scaffolds have been investigated to address the need, left by the previously discussed injection methods, for more effective and economical delivery of cells to injured tissue. Throughout the body, the biochemical and mechanical interplay between cells and their

underlying matrix are of paramount importance to the development, functioning, and repair of tissue. Therefore, in building engineered tissue constructs, which are fabricated by coupling isolated tissue-specific cells with biomaterial scaffolds, we seek to recreate the natural conditions of the cellular microenvironment as closely as possible. To achieve this, the biochemistry and structural organization of biomaterial scaffolds have been precisely controlled, which directs cellular attachment, proliferation, and ordered development [27]. Several crucial requirements of implantable scaffolds for tissue engineering have been identified [28, 29]:

i. Biodegradability with tunable degradation rate matching that of new tissue deposition.
ii. Biocompatibility through all stages of degradation.
iii. Mechanical properties analogous to native tissue throughout the regeneration process.
iv. Biofunctionality – the ability to support cellular proliferation and differentiation of both implanted and native cells, ECM secretion, and the formation of functional tissue.

Naturally derived polymer hydrogels have been characterized for use in several tissue engineering applications because they have macromolecular properties similar to – or in some cases, identical to – the native ECM [30]. Materials such as collagen, fibrin, alginate, chitosan, HA, and silk demonstrate highly desirable intrinsic biocompatibility and biofunctionality and have been studied extensively as scaffolds for engineering connective tissues, skin, muscle, and several other types of tissue. In particular, the collagens, which are the most abundant proteins in the extracellular matrix of mammalian tissues [31], have shown exceptional potential as biomaterial scaffolds. Originally used as delivery vehicles for cultured skin cells and therapeutic drugs in skin replacement and burn wound applications [32], collagen scaffolds have more

recently been used in bioengineered tissues such as blood vessels, heart valves, and ligaments [33]. While collagen films, hydrogels, and sponges provide essential three-dimensional support and signaling templates for regenerating tissue, the effectiveness of such collagen based scaffolds for tissue engineering is restricted by generally poor mechanical properties [12].

Fibrous scaffolds with controllable, well-characterized mechanical properties have been produced from several naturally derived biomaterials including fibrin, silk, and collagen. The original motivation for this type of biomaterial scaffold was to promote regeneration of tendons and ligaments; Kato *et al.* found that fibers extruded from type I collagen demonstrated mechanical, structural, and biochemical homology to native tendon and ligament [34]. Studies have shown that cylindrical substrata of less than 100 μm (i.e. fiber based scaffolds) facilitate contact guidance, alignment, and orientation of cells as well as regeneration-focused cell functions such as ECM deposition [35, 36]. In addition to mimicking native tissue and enhancing tissue regeneration, fiber-based scaffolds, such as collagen microthreads, have demonstrated an ability to be bundled, woven, or braided into larger, more complex structures [13, 37]. It has been shown that bundled structures of silk fiber scaffolds increase surface area for cell attachment and ECM deposition while minimizing mass transfer limitations [38]. Several groups have used cell-seeded fibrous scaffolds as delivery vehicles for cells as well as bench-top model systems to characterize cell-matrix interactions [13, 37].

2.2.2 Cellular Co-culture

Though these previously studied scaffolds have yielded generally positive results, most cell delivery and tissue engineering approaches have focused on systems incorporating a single cell

type. However, *in vivo* tissues consist of multiple cell types influenced by each other through cell-cell interactions.

In vivo, cells reside in a highly interactive environment. The phenotype, function, and behavior of one cell type are affected by other cell types in the body. These cellular interactions have been largely studied in recent years. The general types of cell-cell interactions are described in Figure 3 [39].

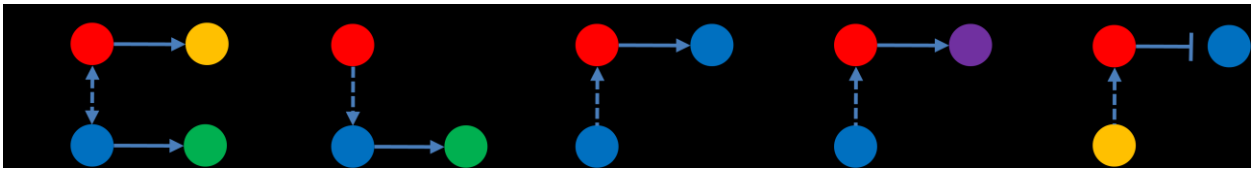

Figure 3: Classification of Cell Interactions in Co-Culture. Cell interactions in co-culture can be classified into several distinct categories [39]. *A:* The two cell types interact to promote their respective physiological function or differentiation. *B:* One cell type promotes the physiological function or differentiation of a second cell type. *C:* One cell type influences a second cell type to transdifferentiate into lineage similar to that of the first. *D:* The first cell type influences the second cell type to differentiate to a lineage different from either of the two original cell types. *E:* One cell type inhibits the differentiation of a second cell type. *Figure and caption adapted from [39].*

Engineered tissue constructs, fabricated by coupling cells with biomaterial scaffolds, seek to mimic the natural conditions of the cellular microenvironment as much as possible. Specific tissues are designed as *in vitro* models for controlled analysis of cell function and tissue development under normal and compromised conditions. A common method of better mimicking the *in vivo* environment is cellular co-culture. This involves the culturing of two cell types in the same microenvironment. According to Hendriks et al. [39], co-culture is utilized in tissue engineering to create a multi-cellular tissue or organ replacement to maintain and support each cell type's specific lineage

While co-culture systems were initially used to study cell-cell interactions, they are becoming increasingly common tools in creating tissue models as they provide several advantages over single-cell type models. Since natural tissues are mostly multi-cellular, co-

culture approaches to engineering tissues can be used as an effective culture environment to generate grafts that will more precisely mimic the natural development of the tissue.

2.3 Angiogenesis

2.3.1 Angiogenesis in Wound Healing

The formation of new blood vessels, either through angiogenesis or vasculogenesis is an essential part of wound healing. It is required so that the new tissue receives proper perfusion of oxygen and nutrients. Wound healing can be divided into four distinct phases: coagulation, inflammation, proliferation, and remodeling. Coagulation involves clot formation to seal off the wound area, fibrin synthesis, and the release of cytokines that activate the next phase. Inflammation, typically starting shortly after injury and lasting for a few days involves vascular dilation, leukocyte arrival at the wound site, and formation of granulation tissue. Granulation tissue is highly vascular due to the occurrence of vasculogenesis and angiogenesis during its formation. Following this is the proliferation phase, characterized by fibroblast proliferation and scar tissue formation. This replaces much of the granulation tissue with scar tissue. Remodeling ensues in which a balance of collagen synthesis and degradation is established to provide scar tissue with its characteristic mechanical properties [40].

The large role that vasculogenesis and angiogenesis play in wound healing has made it a primary target of research in the field of both chronic and ischemic wounds. Essential to developing a greater understanding of angiogenesis is the establishment of relevant accessible *in vitro* models [41, 42] based on the *in vivo* mechanisms of the process. From a tissue engineering perspective, translation of these models into therapeutic treatments is a critical goal [43].

2.3.2 *In vivo* Mechanisms

The formation of new blood vessels is known as neovascularization and can be divided into two categories: vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* formation of new blood vessels, while angiogenesis is the formation of new capillaries from preexisting blood vessels [40, 44]. The process of angiogenesis involves the cooperation of a stromal cell layer, such as fibroblasts, in conjunction with endothelial cells as driven by a complex signaling cascade. This co-culture system involves two cell types (Figure 4). This process as it occurs *in vivo* can be summarized and divided into several distinct steps (adapted from [42]):

1. Stimulation of endothelial cells via growth factor and cytokine binding to cell receptors
2. Expression of a specific profile of matrix degrading enzymes (proteases, MMPs, TIMPs, etc.) and matrix remodeling
3. Proliferation and migration of endothelial cells
4. Differentiation of endothelial cells and tube-like structure formation
5. Pericyte and smooth muscle cell (SMC) stabilization of new vessel structures

Activation of angiogenesis involves the stimulation of endothelial cells via growth factors and cytokines released from platelets, SMCs, monocytes/macrophages, and fibroblasts [40]. This induces the endothelial cells to produce specific proteases including MMPs, TIMPs, serine proteases, and urokinase plasminogen activator. The profile of proteases produced depends on the composition of the ECM that the endothelial cells are exposed to upon initial digestion of the basement membrane. The breakdown of the ECM allows the endothelial cells to migrate and proliferate. Migration of the endothelial cells is driven by a cytokine gradient and is mediated primarily by cell adhesion [40]. Following migration, the endothelial cells eventually differentiate and form tube-like structures through mechanisms that are poorly understood.

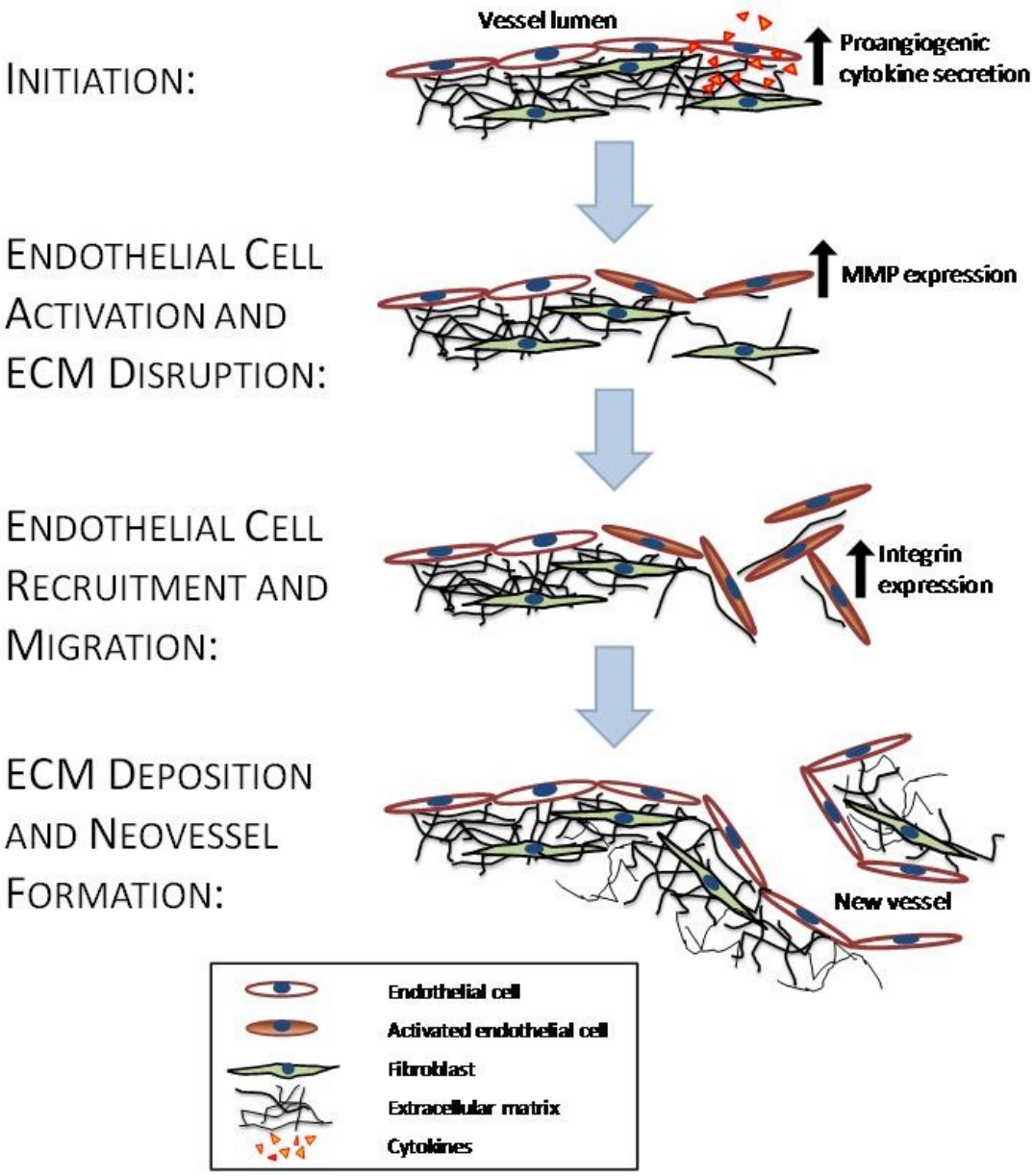

Figure 4: The Process of Angiogenesis.

2.3.3 Emulating Angiogenesis Strategies *in vitro*

Models of angiogenesis are used for several purposes. Uses of these models include but are not limited to elucidation of how the angiogenesis process works, developing pro-angiogenic therapeutics, and developing methods of angiogenesis inhibition to treat tumors [44]. *In vitro* models of angiogenesis seek to closely mimic the steps of the process as it occurs *in*

vivo. In 1997, Jain et al. described the ideal angiogenesis model which included the following (adapted from [44]):

1. Known release rates, spatial and temporal concentrations, and distributions of angiogenic factors and inhibitors for forming dose-dependent curves.
2. Ability to quantify the structure of the new vasculature.
3. Ability to quantify endothelial cell migration and proliferation, etc.
4. Confirm *in vitro* responses *in vivo*.

In vitro angiogenesis models are usually based around the culturing of endothelial cells, as they are the primary cell type required for the process to occur [45]. There are two main types of *in vitro* angiogenesis models: organ culture models and cell culture models. Organ culture models most often involve the isolation and culture of rat aortic ring or chick aortic arch followed by quantification of endothelial cell outgrowth. The advantage of organ culture models is that the endothelial cells are cultured with native stromal cells, which better emulates the *in vivo* environment [44]. Cell culture models range from 2-D culturing of endothelial cells on ECM-coated surfaces to co-culture systems of endothelial cells and fibroblasts seeded in 3-D matrices.

From a tissue engineering perspective, one effective method of modeling angiogenesis in an *in vitro* cell culture model is through co-culture in an attempt to better mimic the environment that endothelial cells are exposed to during angiogenesis *in vivo*. Most angiogenesis co-culture models include endothelial cells and a supporting stromal cell type (fibroblasts, smooth muscle cells, etc.) [44]. Fibroblasts appear to be the most widely used stromal cell type for this particular co-culture variation. In addition, an ECM-based scaffold is often included to help further emulate the *in vivo* environment. This particular co-culture model of endothelial cells and fibroblasts has

shown many advantages over single culture of endothelial cells. Some widely observed advantages include:

- Increased tubule / tube-like structure formation [42, 43, 46]
- Decreased endothelial cell apoptosis rates with lowered proliferation activity [41, 43, 47]
- Increased endothelial cell migration [6]
- Expression of specific MMPs, TIMPs, proteases, and growth factors involved in angiogenesis [46, 48]

2.4 Scientific and Engineering Need

Increased perfusion assists in the restoration of functionality in ischemic tissues. For example, Hasche *et al.* conducted a study that compared the time for which a human subject suffered from cardiac ischemia to the resulting infarction size and subsequently to the heart's ability to function at a sufficient level. By inducing perfusion of ischemic tissues through therapeutic angiogenesis, it may be possible to enhance wound healing and tissue function [17]. In ischemic wounds and infarcted tissue, perfusion is inadequate and the process of angiogenesis is disrupted [49], eliminating the ability of such wounds to repair themselves since revascularization must occur before proper wound healing can take place. Therefore, there is a need for the development of novel clinical therapies for patients who suffer from tissue ischemia.

Because most of the comorbidities associated with delayed healing and chronic wounds result in tissue-level impairment of microcirculation, traditional large-scale revascularization strategies including surgical bypass, angioplasty, and stent procedures are not effective [40]. Alternative clinical approaches to enhance wound healing by means of neovascularization are needed. Treatment options that aim to stimulate neovessel formation and wound healing at the cellular level have shown some promise. However, despite the critical role of endothelial cells in

neovascular formation, stromal cell layer such as fibroblast is required to be presented with the full complement of environmental cues that are required for proper angiogenesis [7].

Temporal, spatial, and dose-controlled exposure to a number of angiogenic and angiostatic growth factors is required for proper angiogenesis, and thus wound healing, to occur [8, 9]. As previously discussed, several groups have developed *in vitro* models consisting of fibroblast-endothelial cell co-culture on biomaterial scaffolds that emulate angiogenesis. However, these pro-angiogenic co-culture systems have not yet been translated to clinical therapies, and effective translation will require a delivery vehicle to direct angiogenesis.

3. Project Strategy

The goal of this project was to use biopolymer microthreads as a platform for delivering two cell types to promote angiogenesis. To best accomplish this goal, it would be necessary to combine the advantages of two technologies: *pro-angiogenic co-culture* and *biomaterials-based delivery systems*. This task presented a number of engineering challenges that would ultimately need to be overcome. This chapter will provide an overview of the strategic design process that was performed to first determine and then prioritize the design objectives. The final section of this chapter will discuss the broad approach that the design team developed to satisfy the client statement and achieve the design objectives.

3.1 Design

This section will describe in detail the process of designing a co-culture system on biopolymer microthreads. We will discuss the use of the engineering design process and its utility in helping designers make strategic, unbiased decisions. Broadly, the design process includes comprehensively defining the problem and subsequently utilizing specific quantification and comparison tools to direct decision-making.

Before the actual design stage can begin, it is essential to clearly identify the project's stakeholders – the clients, the users, and the designers. Typically, the clients are the motivating force behind the project since they deliver an initial statement describing the ultimate goal. For this project, the clients, Professors George Pins and Marsha Rolle, provided a project description that included an initial client statement, the context and need for the project, and the expected deliverables. Users include research groups at WPI who are investigating the use of microthreads as scaffolds for the delivery of therapeutic cells to compromised tissue. The role of the designers

– the MQP team: Shawn Carey, Jonathan Charest, Elizabeth Ellis, and Jason Hu – is ultimately to translate the wants, needs, and desires of all stakeholders into a single design.

3.2 Clarification of Design Goals

Following the identification of the stakeholders, it becomes necessary for the designers to fully understand the goals of the project. Since the design is established by the clients, we first reflected on the client statement presented to our design team, which stated:

> ***Design and develop a co-culture system on biopolymer microthreads to identify cellular interactions that direct cell proliferation and differentiation for regenerative therapies.***

To better understand the context of the problem, we used a series of informal interviews to ask our clients and users questions such as: (1) how could *you* use such a co-culture system? (2) what features or attributes would you like this system to demonstrate? and (3) is there an ideal tissue-specific application for this system? In addition to these discussions, our design team conducted extensive brainstorming sessions to identify the characteristics and attributes of a co-culture system on microthreads. The list below includes attributes which fall into three groups: objectives, constraints, and functions. Objectives are the goals for the system, as determined through coordination among all stakeholders, constraints are the conditions of design that must be met for the design to succeed, and functions are the requirements that the co-culture system will need to allow for or perform.

<u>Objectives:</u>
Co-culture two cell types
Identify or develop compatible conditions for more than one cell type
Utilize biopolymer microthreads as scaffold
Ensure microthread integrity
Generate consistent threads

Generate uniform threads
Develop consistent cell seeding methods
Promote uniform cell seeding
Develop means for fiber anchoring
Develop methods for system characterization
Promote ease of use
Time efficient
Modular
Cost effective

Constraints
Materials must be commercially available
System must be easy to maintain
Materials must not be cytotoxic
Materials must be sterilizable by known processes and with on-site resources
Culture conditions must be compatible for two cell types
Scaffold diameter must be less than 1mm

Functions of the Co-Culture System
Able to culture two cell types
Able to seed two cell types independently in a dosage and spatially controlled manner
Able to monitor select cell characteristics
Able to perform immunohistochemical assays
Able to perform viability assays
Able to confirm effectiveness of co-culture
Able to secure threads
Able to control thread properties

3.2.1 Objectives

As we began to more fully understand the motivation and significance of the project, we were able to draft a list of *pruned* project objectives. From this, we identified top-level objectives as well as sub-objectives and created an indented objectives list. After a series of revisions to this list, we developed a better organized, more focused set of project objectives as shown below.

I. **Create Effective Co-culture**
    A. Maintain cell viability
    B. Uniform cell seeding
    C. Consistent cell seeding
    D. Compatible cell types
II. **Utilize Biopolymer Microthreads as Scaffolds**
    A. Thread integrity
    B. Thread anchoring
    C. Uniform threads
    D. Thread stability (over time)
    E. Consistent threads (batch to batch)
III. **Develop Methods for System Characterization**
    A. Non-terminal system characterization
    B. Accurate
    C. Consistent
    D. Reliable
IV. **Cost Effective**
V. **User Friendly**
    A. Upgradeable
    B. Ease of use
    C. Modular
VI. **Time Efficient**

As shown in the indented objectives list, there are six top-tier project objectives: create effective co-culture, utilize biopolymer microthreads, develop methods for system characterization, cost effective, user friendly, and time efficient. The designers established that, to create effective co-culture in our system, it is important to maintain cell viability, demonstrate consistent (batch-to-batch) and uniform cell seeding, and utilize compatible cell types.

One of the primary requirements of the co-culture system is to utilize biopolymer microthreads as a scaffold for cells. To do so effectively, the design team determined that methods would have to be developed to ensure initial integrity and sustained stability of the threads, anchoring of the threads, and consistent and uniform threads.

Methods for system characterization serve as validation for a specific application (i.e. cardiac, orthopedic, etc.) and more specifically, allow the user to assess whether the system functions as desired in terms of cell survival, migration, and function. These methods should be accurate (the characterization observation corresponds with what is actually happening in the co-culture system), consistent (several characterization analyses on the same sample generate the same results), reliable, and non-terminal. Finally, the system must be user friendly; that is, it will be upgradeable (an individual component of the system could be improved), easy to use, and modular (can accommodate several different cell types).

3.2.2 Quantitative Analysis of Objectives

Since the design process relies on a strategic approach to decision making, a weighted objectives tree must be formulated from the indented objectives list to prioritize the goals of the project. A tool that is commonly used to rank items relative to one another is a pairwise comparison chart. Objectives at the same level, such as the major, top-tier objectives or all of the sub-objectives branching off of a major objective, are methodically compared. When using a pairwise comparison chart to compare two items, the more important of the two receives a score of 1 while the less important receives a 0. If the compared items are equally important to the design, each is given a score of 0.5. After the scores for each objective were summed, they were normalized by adding one, which is a standard method to generate only nonzero values [50]. These values can then be compared as weights, which would be impossible with a score of zero. For this project, the following weights were assigned to the stakeholders for these comparisons: client George Pins (1/3), client Marsha Rolle (1/3), design team (1/3). The results of the top-tier

pairwise comparison charts are shown in Table 1 with the design team's score in the left columns and the sum of the clients' scores in the right columns.

Table 1: Pairwise Comparison Chart of First-Tier Objectives.

Project Objectives	I		II		III		IV		V		VI		Score	Normalized Score	Weight
I. Create Effective Co-Culture			0.5	1.0	1.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0	13.5	14.5	0.28
II. Utilize Biopolymer Microthreads	0.5	1.0			1.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0	13.5	14.5	0.28
III. Methods for System Characterization	0.0	0.0	0.0	0.0			1.0	1.5	1.0	2.0	1.0	2.0	8.5	9.5	0.19
IV. Cost Effective	0.0	0.0	0.0	0.0	0.0	0.5			1.0	1.0	0.0	1.5	4.0	5.0	0.10
V. User Friendly	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0			0.0	0.5	1.5	2.5	0.05
VI. Time Efficient	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.5	1.0	1.5			4.0	5.0	0.10

Comparison of the six primary objectives demonstrates that although all of the project objectives are essential, some are clearly more important to consider in the design process. The stakeholders identified the two major objectives Create Effective Co-Culture and Utilize Biopolymer Microthreads as Scaffolds as the most important, followed by Include Methods for System Characterization, Cost Effective, Time Efficient, and User Friendly. The following tables compare the sub-objectives within each of the major objectives.

Table 2: Pairwise Comparison Chart of Sub-Objectives I.

I. Create Effective Co-Culture	A		B		C		D		Score	Normalized Score	Weight
A. Maintain Cell Viability			1.0	2.0	1.0	2.0	1.0	1.0	8.0	9.0	0.41
B. Uniform Cell Seeding	0.0	0.0			0.0	1.5	0.0	0.5	2.0	3.0	0.14
C. Consistent Cell Seeding	0.0	0.0	1.0	0.5			0.0	0.5	2.0	3.0	0.14
D. Compatible Cell Types	0.0	1.0	1.0	1.5	1.0	1.5			6.0	7.0	0.32

Within the major objective of Create Effective Co-Culture, the designers and clients established that Maintaining Cell Viability was the highest priority in this tier by awarding it the highest weighting. This sub-objective was followed by the use of Compatible Cell Types, and demonstrating Uniform and Consistent Cell Seeding.

Table 3: Pairwise Comparison Chart of Sub-Objectives II.

II. Utilize Biopolymer Microthreads	A		B		C		D		E		Score	Normalized Score	Weight
A. Thread Integrity			1.0	2.0	1.0	1.5	0.5	2.0	1.0	0.5	9.5	10.5	0.32
B. Thread Anchoring	0.0	0.0			1.0	1.0	1.0	0.5	1.0	0.0	4.5	5.5	0.17
C. Uniform Threads	0.0	0.5	0.0	1.0			0.0	1.0	0.0	0.0	2.5	3.5	0.11
D. Thread Stability (over time)	0.5	0.0	0.0	1.5	1.0	1.0			0.5	1.0	5.5	6.5	0.20
E. Consistent Threads (batch to batch)	0.0	1.5	0.0	0.0	1.0	2.0	0.5	1.0			6.0	7.0	0.21

The stakeholders determined that, with respect to the biopolymer microthreads, in order from most to least important, the system must maintain Thread Integrity, produce Consistent and Stable Threads, enable Thread Anchoring, and generate Uniform Threads.

Table 4: Pairwise Comparison Chart of Sub-Objectives III.

III. Include Methods for System Characterization	A		B		C		D		Score	Normalized Score	Weight
A. Non-terminal System Characterization			0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.05
B. Accurate	1.0	2.0			1.0	0.5	1.0	1.0	6.5	7.5	0.34
C. Consistent	1.0	2.0	0.0	1.5			0.0	2.0	6.5	7.5	0.34
D. Reliable	1.0	2.0	0.0	1.0	1.0	0.0			5.0	6.0	0.27

The most important sub-objectives under the Include Methods for System Characterization tier were that the characterization system be Accurate and Consistent. Furthermore, the system should be Reliable in that it delivers definitive results. Non-terminal System Characterization would be ideal, but this sub-objective is a relatively low priority because the goal of this project is to develop a proof-of-concept system.

Table 5: Pairwise Comparison Chart of Sub-Objectives V.

V. User Friendly	A		B		C		Score	Normalized Score	Weight
A. Upgradeable			0.0	0.0	0.0	0.5	0.5	1.5	0.13
B. Ease of Use	1.0	2.0			1.0	2.0	6.0	7.0	0.58
C. Modular	1.0	1.5	0.0	0.0			2.5	3.5	0.29

Finally, under the top-tier objective of User Friendly, Ease of Use was the highest weighted characteristic. The second-ranked sub-objective in this category was Modular (i.e. using the microthread scaffold as a platform for any number of different cell types and applications); development of an Upgradeable (scalable) system ranked least important of the three sub-objectives.

After using the pairwise comparison charts to quantify and prioritize the stakeholders' interests in developing this novel co-culture system, the design team created a weighted objectives tree to summarize our results (see Figure 5). The first number in each box represents that objective's relative weighting within its branch; the boldface value defines where each sub-objective ranks with respect to all other objectives at the same level (i.e. major objectives, sub-objectives).

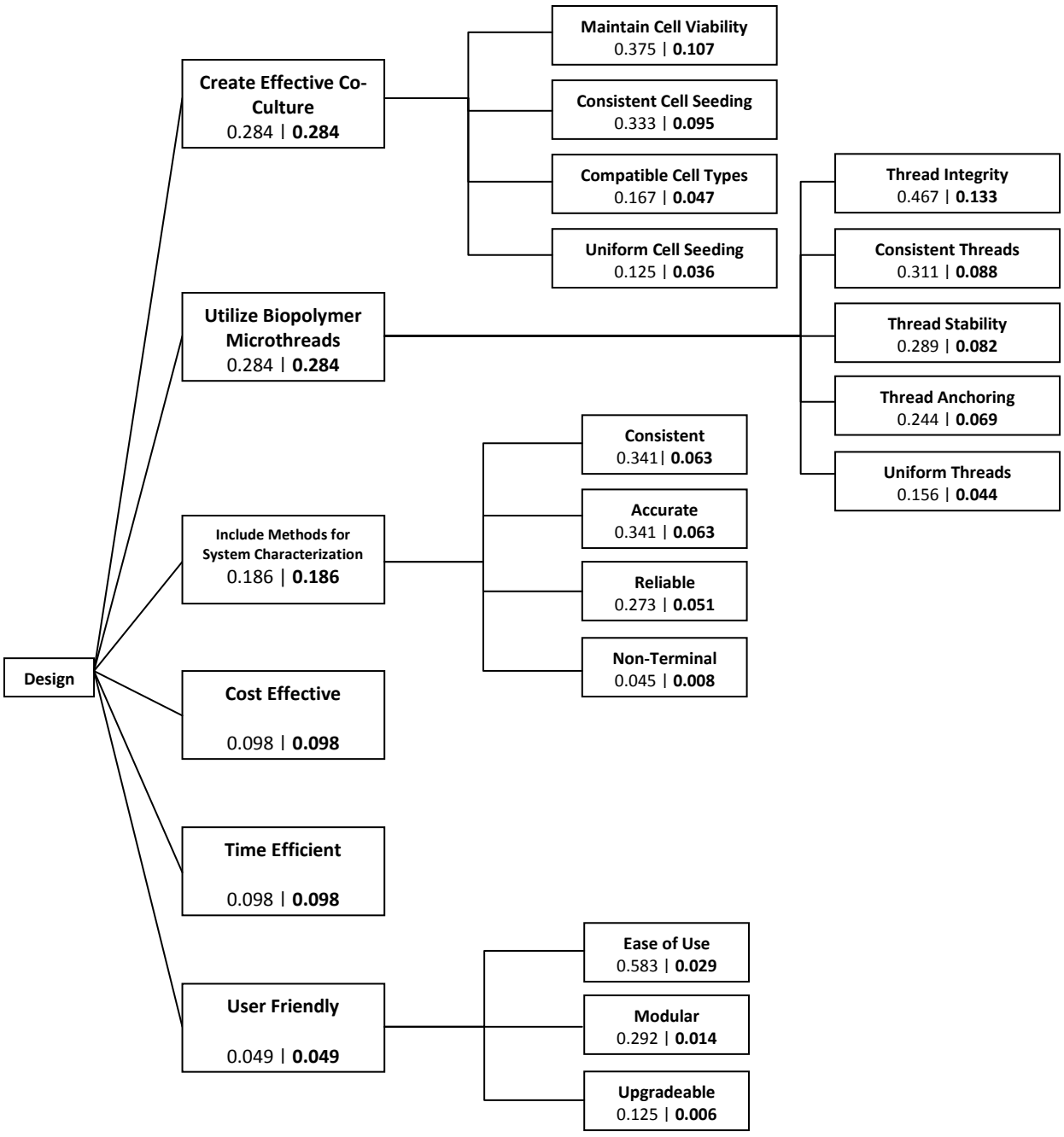

Figure 5: Weighted Objectives Tree.

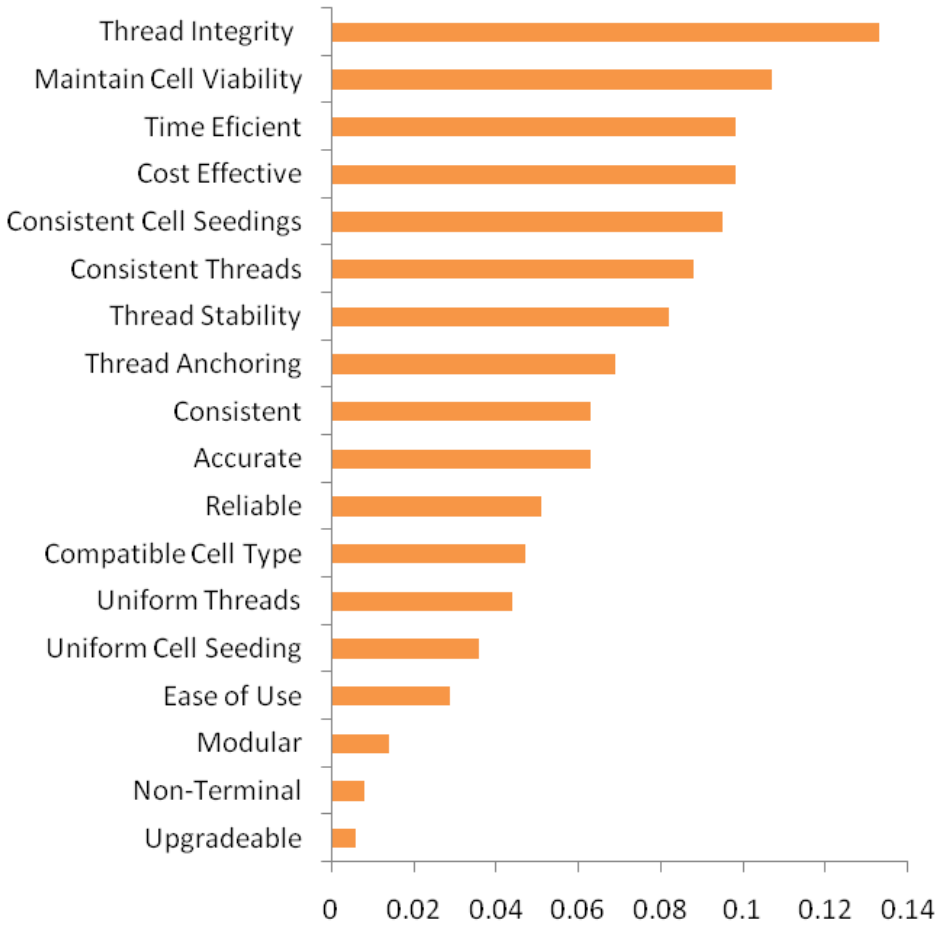

Figure 6: Summary of Weighted Objectives Tree.

3.2.2 Development of Revised Client Statement

Based on the objectives analysis and further research, the original client statement "Design and develop a co-culture system on biopolymer microthreads to identify cellular interactions that direct cell proliferation and differentiation for regenerative therapies." was revised.

The revised client statement was:

> **Design and develop a cellular co-culture system on biopolymer microthreads that will facilitate guided angiogenesis.**

The most important objective of this project was to develop a co-culture system for cell delivery using biopolymer microthreads as scaffolds. Co-culture of the chosen cell types must

demonstrate significant potential for promoting and directing angiogenesis relative to a single cell system. The design of this co-culture system should ensure thread integrity and cell viability for at least three days in culture. The system should be easy to use, time efficient, cost effective, and modular. The design should minimize batch-to-batch variability. A set of comprehensive characterization procedures should be developed to monitor the viability, morphology, migration, and cell-cell signaling for the co-culture system.

3.3 Project Approach

The design team developed a two-step approach to facilitate the independent seeding of two cell types. The first cell type would be embedded within the collagen microthread scaffold (I in Figure 7). After a period of incubation, the second cell type would be seeded on the surface of the cell-populated collagen microthread scaffold (II in Figure 7).

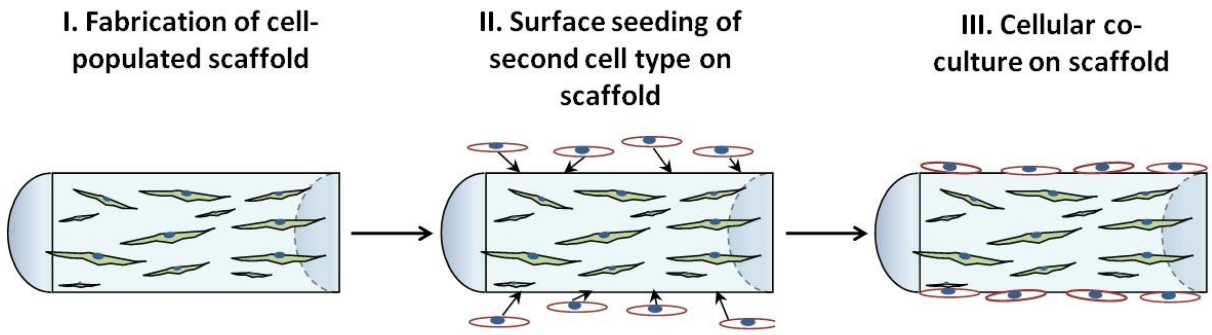

Figure 7: Novel Approach to Co-Culture on a Collagen Microthread Scaffold. This approach allows for independent seeding of two cell types.

Because of the complexity of the proposed system and the anticipated challenges in implementing this approach, we broke it into three *Specific Aims*. These broad goals represent benchmarks that we determined must be met in order to achieve the design objectives.

Specific Aim 1: **Provide a 3-D culture environment for therapeutic cell types by embedding one of the cell types in a collagen microthread.**

There are two motivations for utilizing microthread scaffolds in this way. Ongoing research has shown that cues from the extracellular matrix in 3-D microenvironments can significantly affect biochemical signaling pathways between and within cells. Providing a culture microenvironment that more closely resembles the native ECM will lead to more natural cellular activity and ultimately a more effective culture system. Embedding the first cell type within the thread will also allow for independent seeding of the second cell type as well as dose, spatial, and temporal control over cell seeding.

Specific Aim 2: **Seed second cell type on the surface of a collagen microthread. The objective of this aim is to develop a seeding technique that has the potential to more effectively seed a chosen cell type on the surface of the microthread.**

The effectiveness of traditional methods of seeding cells on microthreads such as droplet seeding is limited because of insufficient cell attachment (as seen in Figure 8). Further, these methods give the user little control over cell location. Therefore, new methods must be developed to improve the seeding efficiency and control.

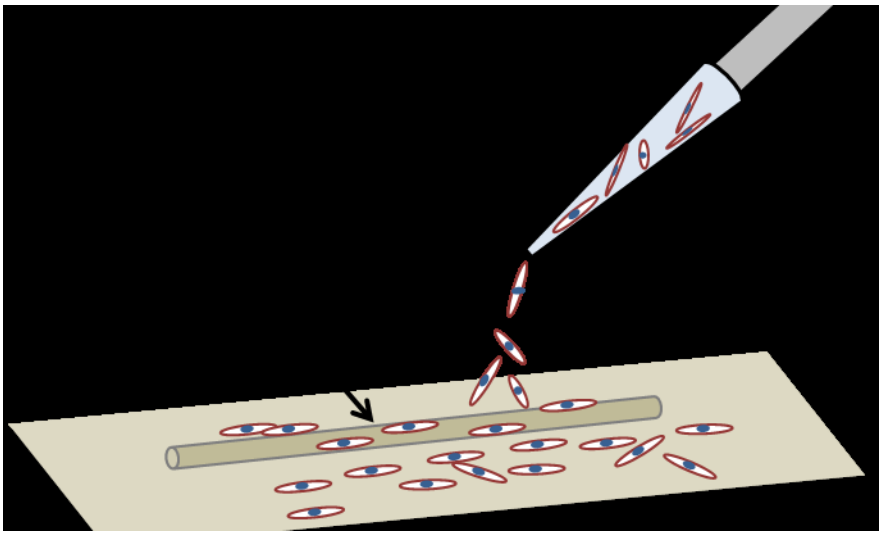

Figure 8: Inefficiency of Traditional Seeding Methods on a Microthread Scaffold.

Specific Aim 3: **Demonstrate the ability to maintain a long-term cellular co-culture system.**

After methods have been developed to I) effectively embed one cell type within a collagen microthread and II) effectively seed the second type on the surface of the thread, it will be critical to combine the two methods to evaluate the feasibility of establishing a co-culture system. As discussed in the Literature Review chapter, co-culture systems have shown many advantages over single culture systems. However, such systems have never been studied on biopolymer microthreads. Validating the feasibility of such therapeutic co-culture systems on a microthread would promote future development toward use as delivery vehicles for therapeutic cells.

4. Alternative Designs

4.1 Conceptual Designs

4.1.1 Development of Design Alternatives for Fabrication

To facilitate the development of alternative designs, the team held a brainstorming session for all stakeholders in this project. Nineteen fabrication techniques for the co-culture system were brainstormed and, because many of the proposed ideas shared common mechanisms for the fabrication of a co-culture system, eight design alternatives were identified. Each of these alternatives was investigated and an illustration, description, and pros and cons are discussed below.

Extrusion:

Designs under this category utilize a collagen thread extrusion method adapted from the Pins Lab [51]. Collagen and cell suspensions are extruded through small diameter tubing into a temperature and pH-controlled buffer solution to promote collagen polymerization (Figure 9).

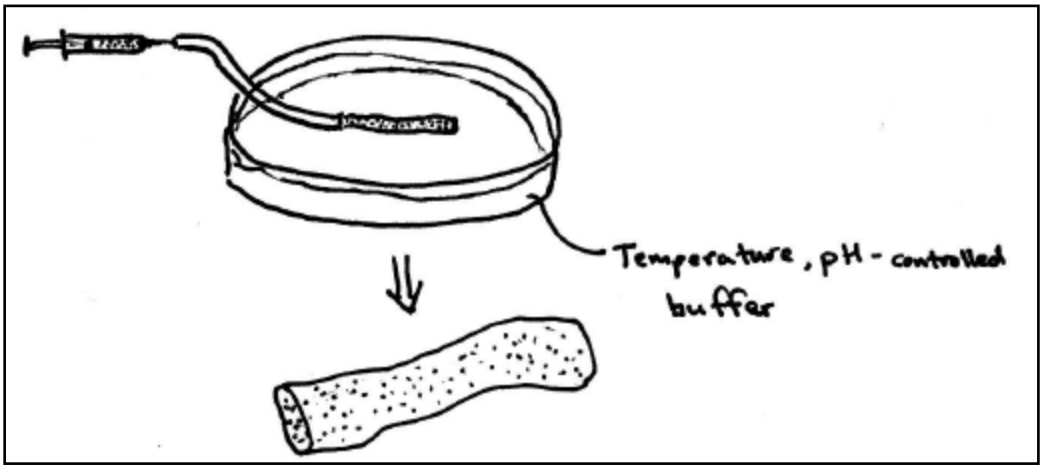

Figure 9: Cartoon Illustrating Basic Extrusion Procedure.

Pros:

- Allows for cell embedding
- Control over thread dimensions
- Allows for media diffusion
- Well-characterized technique

Cons:

- Potential for premature polymerization in extrusion tube
- Potential adverse cellular response to shear stresses during extrusion
- Incomplete mixing/uneven cell distribution

Molding:

Channels with defined dimensions are created on a non-adhesive and biocompatible material such as Polydimethylsiloxane (PDMS) or agarose. Collagen and cell suspensions are mixed and injected into these channels; the device is submerged in a temperature and pH-controlled buffer solution for collagen polymerization (Figure 10).

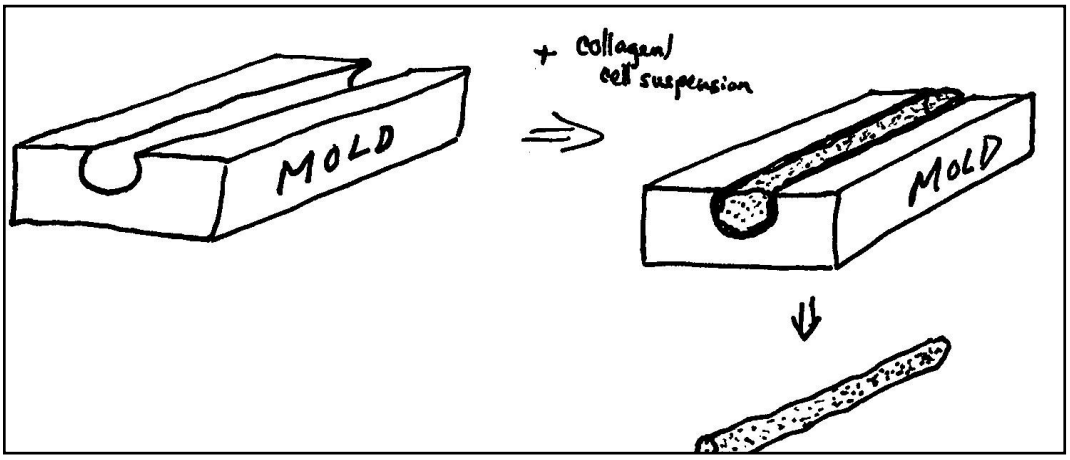

Figure 10: Cartoon Illustrating Basic Molding Procedure.

Pros:

- Allows for cell embedding
- Control over thread dimensions
- Individual channels prevent tangling during production
- Could allow for seeding of two cell types

Cons:

- Restricted media diffusion due to mold
- Does not allow for independent seeding of two cell types
- Not a true "thread" morphology
- Lack of thread integrity
- Difficult to harvest threads from mold
- Settling of cells in collagen resulting in uneven distribution
- Requires machining of mold

Parfait Molding:

Similar to molding described above; collagen is mixed independently with suspensions of cell types A and B. The resulting solutions are injected alternately into channels with defined dimensions to create a "parfait" consisting of both cell types embedded. This approach could be used with a horizontal mold as shown below or in a vertical cylindrical mold (Figure 11).

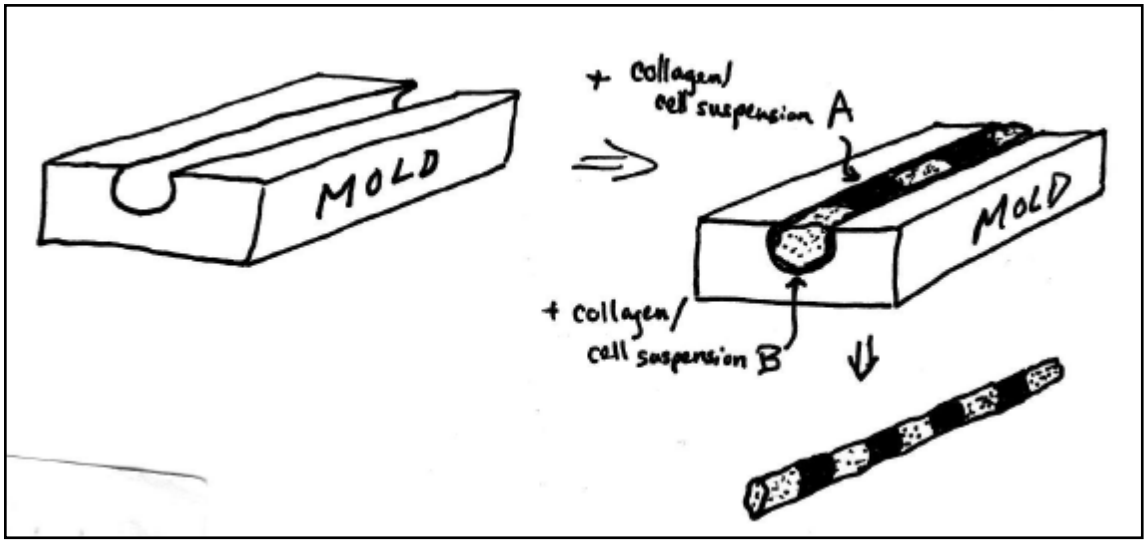

Figure 11: Cartoon Illustrating Parfait Molding Procedure.

Pros:

- Allows for cell embedding
- May allow for localization of both cell types and somewhat independent seeding
- Control over thread dimensions
- Individual channels prevent tangling during production

Cons:

- Restricted media perfusion due to mold
- Not a true "thread" structure
- Lack of thread integrity, especially due to interfacial boundaries
- Difficult to harvest threads from mold
- Settling of cells in collagen resulting in uneven distribution
- Requires machining of mold

Extrusion/Microfluidic Mixing:

Essentially, this procedure provides more effective mixing prior to either extrusion or molding. Small microfluidic channels are created on Teflon or another biomaterial. The collagen solution and cell suspensions are pumped into the channel, mixed thoroughly, and exit the

mixing channel homogenously mixed (Figure 12); the resulting solution can be molded or extruded.

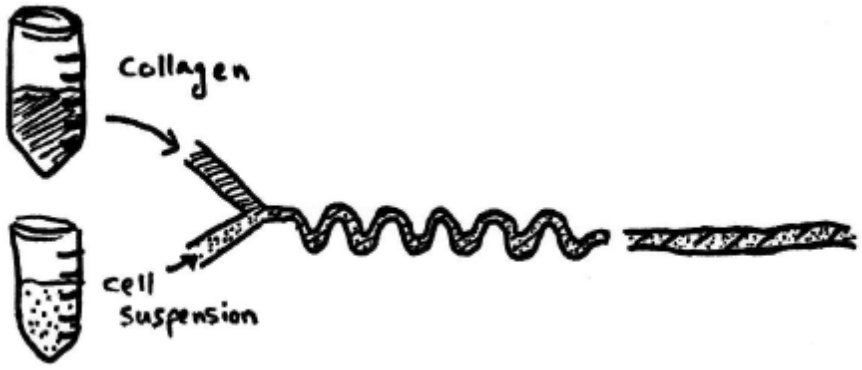

Figure 12: Cartoon Illustrating Microfluidic Mixing Procedure.

Pros:

- Allows for cell embedding
- Thorough mixing/even cell distribution
- Homogenous mixing minimizes variation in mechanical integrity of the thread
- Used to pre-mix solutions for extrusion or molding

Cons:

- Does not allow for independent seeding of two cell types
- Potential adverse cellular reaction to turbulent mixing
- Additional step and equipment
- Potential for premature polymerization
- Requires machining of device

Rolling:

A thin collagen gel or film is seeded with cells and incubated to allow for adequate cell attachment. The number, density, and location of one or more cell types can be controlled. The cell-seeded gel is removed from the dish and rolled upon itself to create a cylindrical construct (Figure 13).

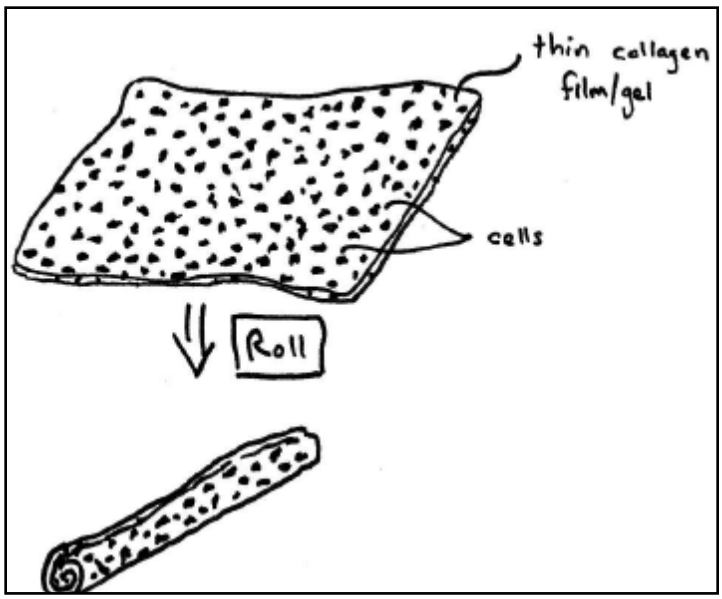

Figure 13: Cartoon Illustrating Rolling Procedure.

Pros:

- Allows for cell embedding
- Ability to deliver more than one cell type
- Ability to control number, density, and location of cells
- Maximize seeding efficiency

Cons:

- Not a true microthread
- Much larger dimensions than microthreads
- Requires handling and manipulation during production

Magnetic Seeding:

Iron atoms are incorporated within the microthread through co-extrusion as well as loaded into the cells that are to be seeded on the thread. The thread and cells are magnetized so that the cells are attracted to the thread when exposed to a magnetic field (Figure 14).

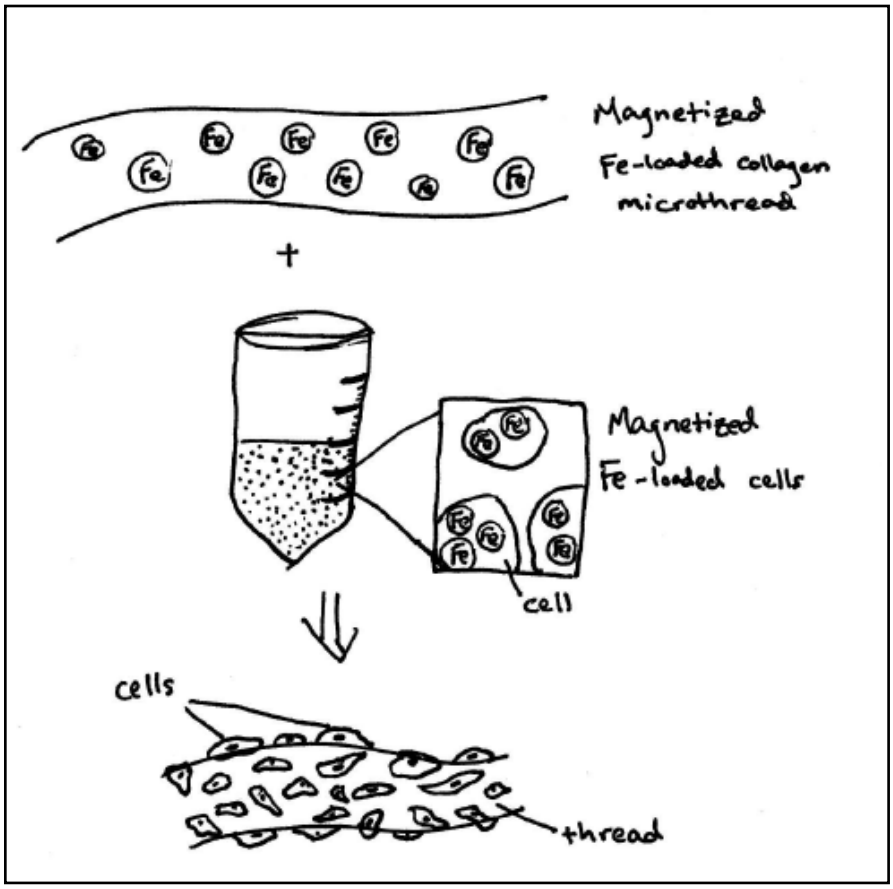

Figure 14: Cartoon Illustrating Magnetic Seeding Procedure.

Pros:

- Utilizes established extrusion techniques
- Control over thread dimensions
- Potential for cell tracking using iron-loading cells

Cons:

- Surface seeding only
- Does not involve second cell type, secondary seeding required
- No spatially-selective seeding
- Potential adverse cellular reaction to iron

Spatial Chemical Seeding:

Surface treatments such as ionic charge and/or polymeric coatings are applied in a controlled manner to the microthreads to selectively control cell adhesion of one or more cell types. Surface treatments used to block attachment of cell type A are subsequently removed to allow cell type B to attach, resulting in a controlled distribution and number of both cell types. The advantage of this method is that it is possible to independently seed two cell types (Figure 15).

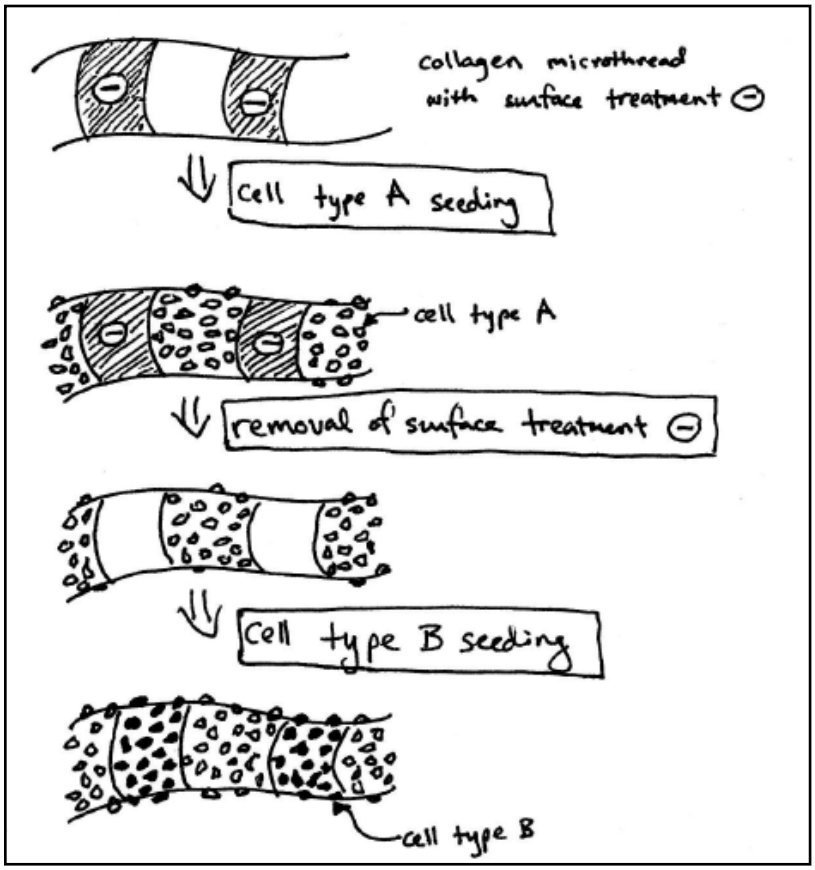

Figure 15: Cartoon Illustrating Spatial Chemical Seeding Procedure.

Pros:

- Utilizes established extrusion techniques
- Control over thread dimensions
- Control over seeding concentration, distribution
- Ability to seed two cell types independently

Cons:

- Surface seeding only
- Inefficient seeding as a result of selective seeding
- Potential adverse cellular reaction to chemical manipulation
- Potential adverse biomaterial reaction to chemical manipulation

Centrifugation:

Biopolymer microthreads are secured on the surface of a non-adhesive culture plate. Cell suspensions are added to the plate before centrifugation. The centripetal force will drive the cells to adhere to the microthreads. This strategy could be coupled with one of the selective seeding techniques described above (Figure 16).

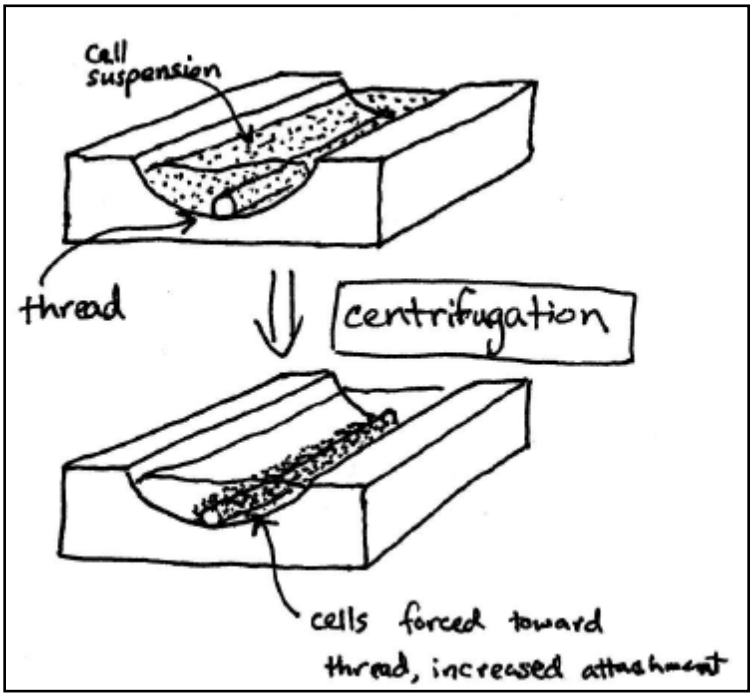

Figure 16: Cartoon Illustrating Centrifugation Seeding Procedure.

Pros:

- Utilizes traditional extrusion techniques
- Maximize seeding efficiency

Cons:

- Surface seeding only
- Potential thread deformation and damage due to high forces
- Potential damage to cells due to high forces

4.1.2 Strategic Analysis of Design Alternatives

After the generation of design alternatives, it was necessary to determine which design best conformed to the project objectives while satisfying the project constraints. Only those design alternatives that fulfilled all of the constraints were to be included in the strategic decision-making process. It was concluded that designs utilizing the *rolling* technique would not be able to produce true "microthreads" and there may be some issues with thread integrity and long-term stability if the construct were to unroll. Furthermore, production of cell-seeded scaffolds by this

method would require extensive handling and manipulation. Therefore, this technique could not be used as it failed to meet all of the constraints.

A Best of Class chart (Figure 17) was used to compare the relative ability of each of the remaining seven design alternatives to satisfy the weighted objectives. The design alternatives were ranked from 1-7 as to how well they address each project objective. The design that best fulfilled any given objective was awarded a score of 1. If two designs achieved a given objective equally well and were both determined to be the best alternative for that objective, the resulting score for both alternatives was the average of 1 and 2 (1.5). This technique is commonly used in Best of Class charts and guarantees proportional comparison. Weighted scores were calculated by multiplying the Best of Class chart scores by the objectives' weights that the design team had previously assigned. The alternatives with the lowest total weighted scores were deemed to be the best choices for fabrication techniques. Objectives that were not relevant to the choice of fabrication technique were omitted from this analysis.

	Designs ->	Extrusion		Molding		Parfait Molding		Spatial Magnetic Seeding		Spatial Chemical Seeding		Microfluidic Mixing		Spin-down Method	
Objectives	Weight	Score	WS	S	WS	S	WS	S	WS	S	WS	S	WS	S	WS
I: Maintain cell viability	0.107	3	0.321	1.5	0.1605	1.5	0.1605	6.5	0.6955	6.5	0.6955	5	0.535	4	0.428
I: Uniform cell seeding	0.036	1.5	0.054	6	0.216	7	0.252	3.5	0.126	3.5	0.126	1.5	0.054	5	0.18
I: Consistent cell seeding	0.095	3	0.285	6	0.57	7	0.665	3	0.285	3	0.285	1	0.095	5	0.475
I: Compatible cell types	0.047														
II: Thread integrity	0.133	3	0.399	3	0.399	6	0.798	3	0.399	7	0.931	3	0.399	3	0.399
II: Thread anchoring	0.069	4	0.276	4	0.276	4	0.276	4	0.276	4	0.276	4	0.276	4	0.276
II: Uniform threads	0.044	6	0.264	2	0.088	2	0.088	4.5	0.198	4.5	0.198	7	0.308	2	0.088
II: Thread stability	0.082	4	0.328	1.5	0.123	1.5	0.123	6	0.492	7	0.574	4	0.328	4	0.328
II: Consistent threads	0.088	4	0.352	1.5	0.132	1.5	0.132	6	0.528	7	0.616	4	0.352	4	0.352
III: Non-terminal system characterization	0.008														
III: Accurate	0.063														
III: Consistent	0.063														
III: Reliable	0.051														
IV: Time efficient	0.098	3	0.294	3	0.294	5	0.49	6.5	0.637	6.5	0.637	4	0.392	3	0.294
V: Ease of use	0.029	3	0.087	3	0.087	3	0.087	6	0.174	7	0.203	3	0.087	3	0.087
V: Modular	0.014	3	0.042	3	0.042	3	0.042	6.5	0.091	6.5	0.091	3	0.042	3	0.042
V: Upgradeable	0.006	3	0.018	3	0.018	3	0.018	6	0.036	6	0.036	4	0.024	6	0.036
VI: Cost effective	0.098	4	0.392	4	0.392	4	0.392	6.5	0.637	6.5	0.637	5	0.49	4	0.392
Weighted Score		3.112		2.7975		3.5235		4.5745		5.3055		3.382		3.377	
Rank		2		1		5		6		7		4		3	

Figure 17: Best of Class Chart. Chart compares various fabrication techniques to weighted objectives. Score-dependent coloring is used to visualize rankings. Green scores indicate good anticipated fulfillment of the objective, while red scores indicate poor anticipated fulfillment.

The Best of Class chart indicated that the best fabrication methods given our constraints, objectives, and desired functions would be molding and extrusion. These processes would both allow for cell embedding, which would facilitate independent cell seeding when used in conjunction with a surface seeding technique. In particular, we anticipated that these methods would create uniform and mechanically stable threads with a high degree of cell viability. Through the design process, it was determined that the ability to create an actual "microthread" was especially important. The benefits of microthreads as scaffolds in cell-based applications have previously been identified in the Background section, and it was determined that several of the key features of biopolymer microthreads, including fibrillar alignment, rapid polymerization, and thread integrity, are imparted through the extrusion process (as described by Pins, *et al.* [51]).

4.2 Selection Process for Co-culture Cell Types

In the brainstorming session, possible cell types to use in this project were discussed. The following is a list of possible cell types:

Human dermal fibroblasts (HDFs)
Human mesenchymal stem cells (hMSCs)
Smooth muscle cells (SMCs)
Keratinocytes
Endothelial cells
Skeletal muscle cells
3T3-J2 cells
Cardiac myocytes
Induced pluripotent cells (IPCs)

Because one of the goals of the project was to utilize a co-culture system, it was necessary to determine the feasibility of using each of the cell types that was proposed during the brainstorming session. By constructing a matrix that specified all of the possible combinations of

cells, we organized the proposed co-culture systems in terms of co-culture outcomes. A thorough literature review revealed that only eleven of the combinations had been previously studied. Table 6 shows the eleven combinations with their studied measurable outcome.

Table 6: Possible Co-Culture Combinations.

Co-Culture Combination	Measureable Outcome
SMCs & hMSCs	↑ (increased) Expression of smooth muscle calponin & smooth muscle α-actin [52]
Keratinocytes & Dermal fibroblasts	↑ Expression of IL, KGF, & TGF ↑ Contractility in myofibroblasts [53, 54]
Skeletal myoblasts & hMSCs	↑ Proliferation rates of both cell types [55]
Skeletal myoblasts & SMCs	↑ [VEGF] causing myoblasts to turn into SMCs [56]
Endothelial cells & Dermal fibroblasts	↑ Angiogenesis *in vitro* [46]
Endothelial cells & hMSCs	Mimics development of vascularization [57]
Endothelial cells & SMCs	Activation of SMC protein kinase Akt [58]
3T3-J2s & Keratinocytes	↑ Keratinocyte production of FN, LN, and Col-IV [59]
Cardiac myocytes & hMSCs	↑ Expression of cardiac connexins in hMSCs and gap junction formation and induced hMSC expression of α-actin [52, 60]
Cardiac myocytes & Skeletal myoblasts	Induced cell fusion and morphological changes [61]
Cardiac myocytes & Endothelial cells	Induced endothelial cell expression of sarcomeric MHC, β-galactosidase, cardiac troponin-I, and active gap junctions [62]

Of these co-culture systems, the team chose to utilize combination of endothelial cells and dermal fibroblasts. This particular combination was chosen based on its well-characterized ability to emulate the process of angiogenesis *in vitro*. Further, there remains a need for a tissue engineered deliverable construct capable of promoting angiogenesis *in vivo*. The recapitulation of vascularization through induced angiogenesis evolved into a significant goal of the project. It

was determined that the endothelial cells would be seeded on the surface of the microthread based on previous studies done by Velazquez et al. [6] that indicate the preference of endothelial cells to migrate into a fibroblast-populated collagen gel. Furthermore, the team chose to incorporate the fibroblasts within the thread because they were more likely to survive the potentially harsh microenvironments of the extrusion process. By embedding the easily cultured fibroblasts inside a collagen microthread and subsequently seeding endothelial cells onto the surface, we hope to create a pro-angiogenic co-culture system on the microthread. The use of fibroblasts and endothelial cells was considered to be particularly advantageous because of their broad and well-characterized use in angiogenesis models and tissue engineering applications.

4.3 Preliminary Co-culture System Design

Taking into account the revised client statement, the results of the strategic selection methods and the resources available to the design team, a preliminary design was chosen. The design utilized extrusion of human dermal fibroblasts (HDFs) within the collagen threads and seeding of human umbilical vascular endothelial cells (HUVECs) onto the surface of the extruded threads. This approach is shown in Figure 18 and allowed for independent seeding of the two cell types and user control over cell-specific incubation times. The cell types were chosen because their interactions best promote angiogenesis in surrounding tissue. Furthermore, previous research has shown that endothelial cells seeded on a collagen gel will migrate into the gel and form tubules in response to gradients of factors secreted by the fibroblasts as well as cell-cell contact. The proposed system will serve as a platform for the further characterization of the cell-cell and cell-matrix interactions that are critical for therapeutic angiogenesis and may ultimately serve as a cell delivery vehicle that promotes guided angiogenesis *in vivo*.

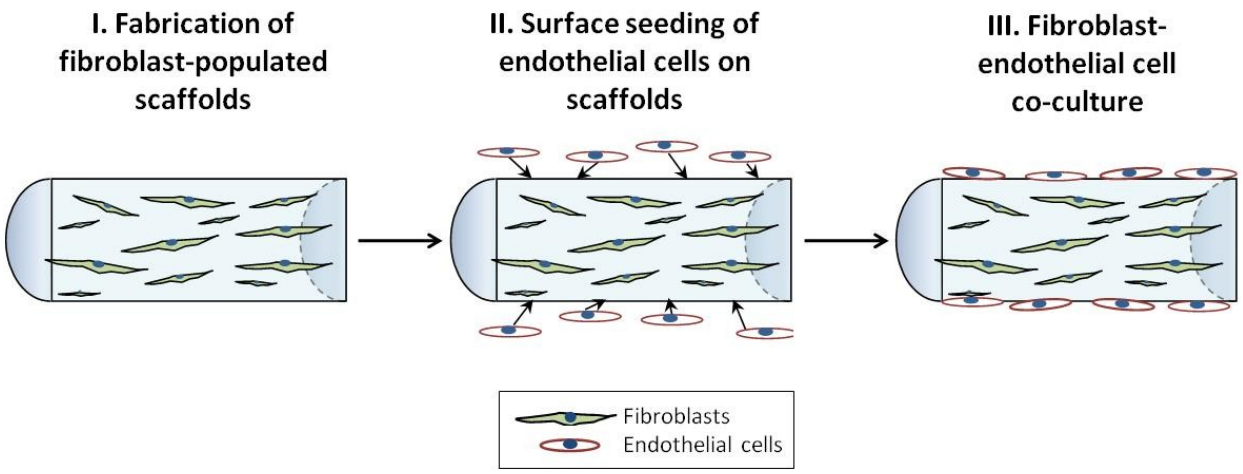

Figure 18: General Approach Schematic to Seed Fibroblasts and Endothelial Cells. This approach allows for independent seeding of the two cell types on a collagen microthread

5. Design Verification

The design process was used to determine that the novel co-culture system would consist of an extruded collagen microthread containing embedded human dermal fibroblasts and surface-seeded with human umbilical vein endothelial cells. Initial pilot studies were conducted to develop and validate effective methods for extruding the HDF-populated thread scaffolds under sterile conditions, surface seeding of HUVECs, visualizing cells both within and on the surface of the threads, and distinguishing between the two cell types during characterization. The complete protocols for all studies are in Appendix C. Construction of the co-culture system was then accomplished by combining the developed methods for independent seeding of the two cell types in and on the collagen threads (Figure 18).

5.1 HDF-Collagen Co-Extrusion Preliminary Studies

5.1.1 Pre-loading of HDFs with MitoTracker

Human dermal fibroblasts were pre-loaded with MitoTracker to facilitate non-terminal visualization. Following the manufacture protocol, HDFs were incubated in a 0.05% (V:V) MitoTracker/DMEM solution for 15 minutes. After rinsing twice with standard DMEM, cells were incubated in DMEM at 37 °C for at least 2 hours before use. A phase contrast/fluorescence overlaid image of HDFs with the green fluorescent MitoTracker can be seen in Figure 19. Minimal HDF autofluorescence was observed relative to the MitoTracker signal.

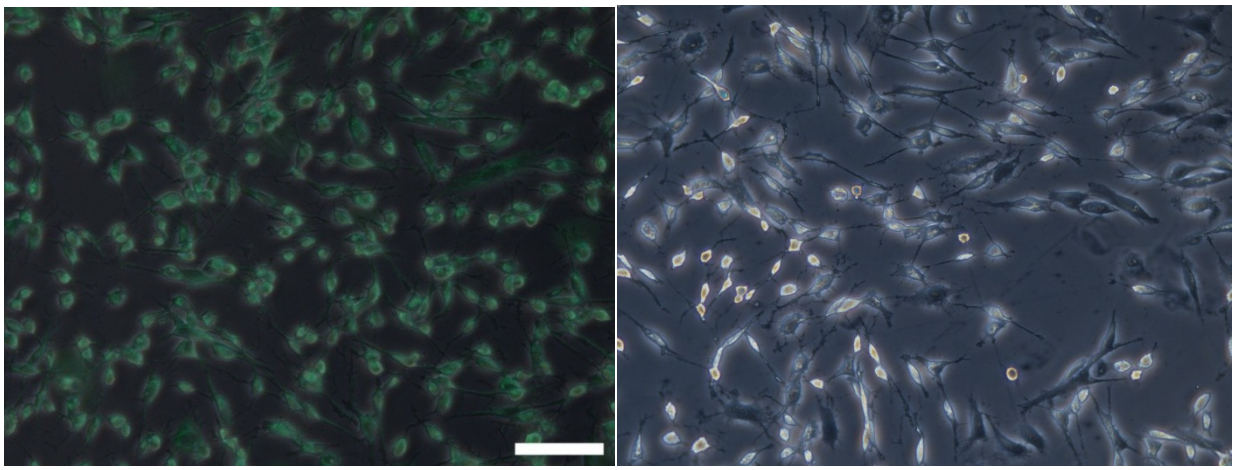

Figure 19: Pre-Loading of HDFs with MitoTracker. Phase contrast/fluorescence overlay image of MitoTracker Green-loaded human dermal fibroblasts (left). Unloaded fibroblasts showed no autofluorescence in green channel (right). Scale bar = 100 μm.

5.1.2 Preliminary HDF-Collagen Co-Extrusion

To develop an extrusion protocol for embedding HDFs, a preliminary experiment was conducted under non-sterile, 4°C (cold room) conditions. The method used was adapted from a cell-seeded collagen gel protocol. Briefly, acid-soluble type I collagen was placed in a 1 mL syringe and a combination of 5X DMEM and human dermal fibroblast cell suspension (80,000 cells/mL) in standard culture media was placed in another 1 mL syringe. The contents of the syringes were extruded through a mixing connector tip and polyethylene tube with inner diameter of 0.86 mm into a temperature-controlled 37°C bath of DMEM culture media. After 15 minutes, the short lengths of extruded collagen microthread (2-3 cm) were fixed and stained with Hoechst for visual analysis. A representative bright field/fluorescence overlaid image is shown in Figure 20. This experiment demonstrated the ability to co-extrude collagen and HDFs as well as the utility of MitoTracker in short-term non-terminal visual characterization. However, there were limitations to this pilot study and several questions derived from the results. First, only short lengths of threads were produced and they were difficult to handle with forceps. Also, while MitoTracker-positive cells (indicated by co-localization of Hoechst and MitoTracker) were

identified within the boundaries of the threads as shown by white arrow heads, it was not clear whether these cells were actually within the thread. Furthermore, it was impossible to tell whether the cells were alive after exposure to the co-extrudants at this time point and using these methods. Finally, this pilot study was conducted under non-sterile conditions in a 4°C cold room to better control polymerization of the collagen. Longer-term culture (3-5 days) requires stable threads, effective embedding of HDFs, high cell viability, and sterile fabrication conditions. Additionally, it is also important to note that very little auto-fluorescence was exhibited by the collagen threads under the green filter.

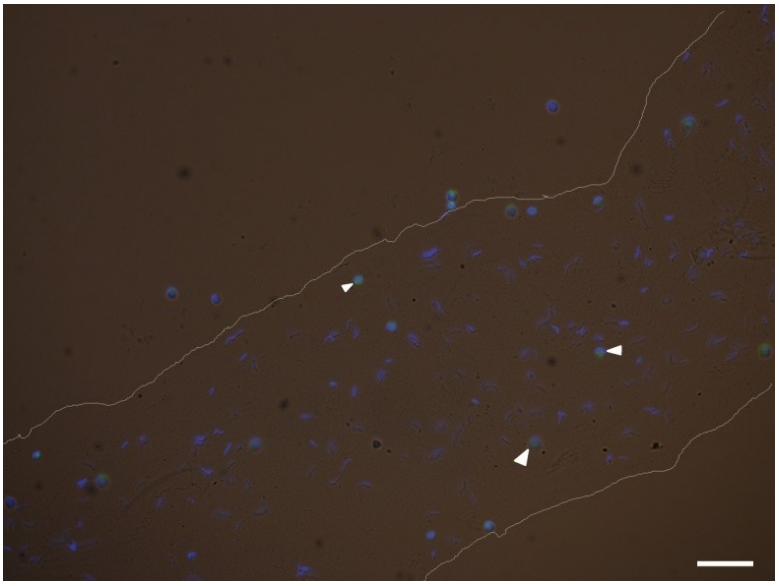

Figure 20: Preliminary HDF-Collagen Extrusion. Bright field/fluorescence overlay image of a thread containing MitoTracker-loaded HDFs (green) and stained with Hoechst (nuclei; blue); HDF presence was determined by the co-localization of MitoTracker-loaded cells with a nucleus. Smaller blue particles not associated with a MitoTracker-positive HDF were identified as the result of non-specific binding of Hoechst stain to impurities in the collagen. HDFs are indicated by white arrow heads and thread boundaries are highlighted by white lines. Scale bar = 100 μm.

5.1.3 Co-Extrusion Components Cytotoxicity Study

To evaluate the potential cytotoxicity of the components of the co-extrusion system, human dermal fibroblasts were exposed to the components individually for 10 minutes. MitoTracker-

positive HDFs in standard culture media exhibited no change in morphology when exposed to 5X DMEM and 10 mg/mL collagen (Figure 21). It was determined that short-term exposure to these components was not harmful to HDFs and therefore, the extrusion components were considered satisfactory.

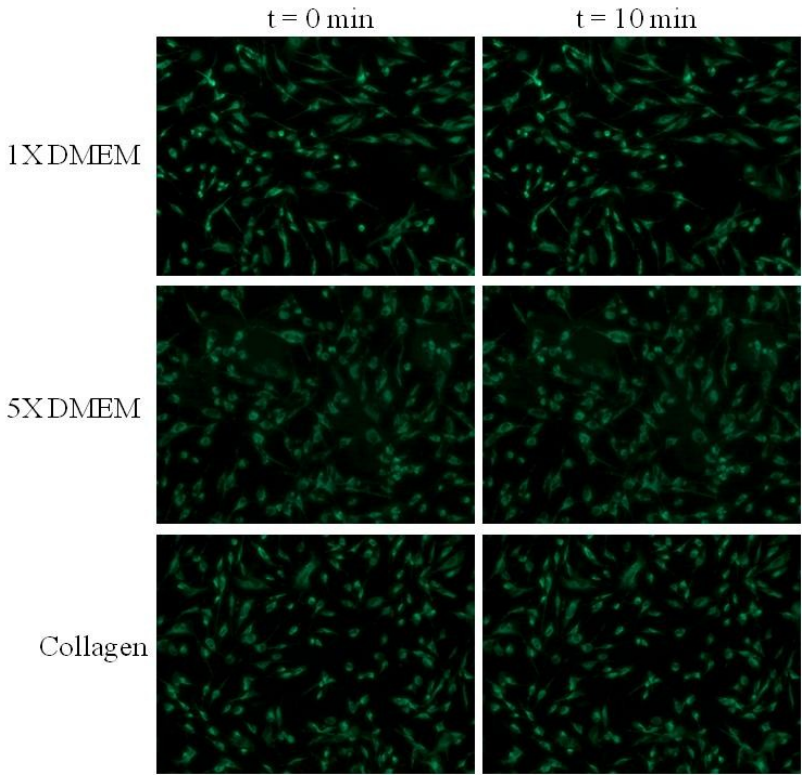

Figure 21: Co-Extrusion Components Cytotoxicity Study. Fluorescence images of MitoTracker-positive human dermal fibroblasts before (left column) and after (right column) 10 minutes of exposure to the co-extrusion components. No changes in morphology were observed.

5.1.4 Validation of Co-Extrusion Preliminary Study

The co-extrusion pilot study was repeated as previously described at room temperature rather than 4°C to better simulate the environment of the sterile tissue culture hood. Working at approximately 25°C with room temperature extrudants failed to produce intact threads, and other methods of fabrication were considered.

5.1.5 Acellular Co-Extrusion into FFB

Standard collagen microthread extrusion utilizes an optimized buffer to facilitate self-assembly. Therefore, a modification was made to the co-extrusion pilot study protocol where threads were extruded into 37°C fiber formation buffer (FFB; pH 7.42, 135 mM NaCl, 30 mM TrizmaBase, and 5 mM NaPO4 dibasic; Sigma, St. Louis, MO) to enhance gelling of the collagen and promote thread integrity (Figure 22A). Threads were incubated in FFB for either 30 (Figure 22C) or 60 (Figure 22D) minutes. After just 30 minutes in FFB, threads demonstrated suitable thread integrity by supporting their own weight (Figure 22B). Further, threads from the two groups were visually indistinguishable after 6 hr, suggesting 30 minutes in FFB is sufficient for promoting collagen gelling. The results of this study are summarized in Table 7.

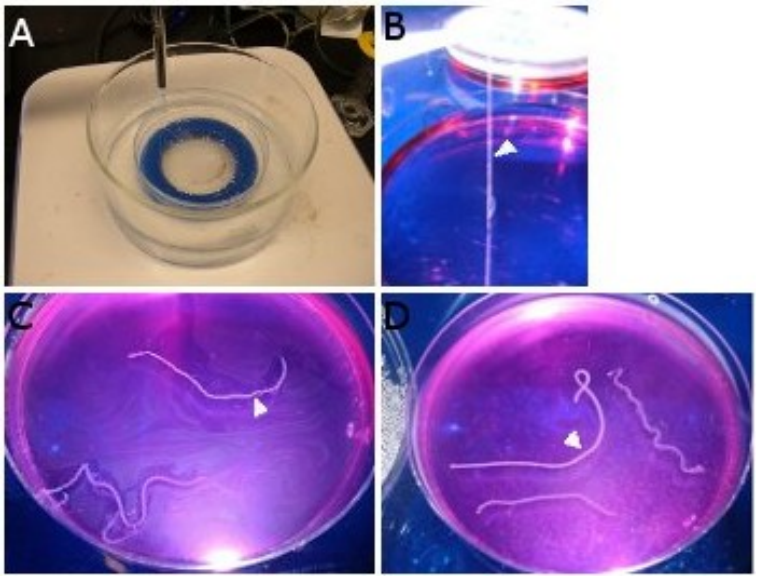

Figure 22: Acellular Co-Extrusion into 37°C FFB. A: Extrusion set-up; threads were extruded into 37 °C fiber formation buffer (FFB) and incubated for 30 or 60 minutes. **B:** Threads incubated in FFB for 30 minutes possess mechanical stability and ability to support their own weight. ***C* &*D*:** Threads extruded into FFB and incubated for 30 minutes (***C***) or 60 minutes (***D***) demonstrate no discernible differences. Threads are indicated by white arrow heads.

Time →	0 min	30 min	60 min	6 hr
FFB30 (30 min in FFB)	extruded	transfer to media - stable threads	- stable threads - supports own weight	- some loss of integrity - "frills" around thread - film developing on surface of media - able to be handled - supports own weight
FFB60 (60 min in FFB)	extruded	---	transfer to media - stable threads	

Table 7: Methods/Results of Acellular Co-Extrusion into 37°C FFB Study. Threads incubated for 30 (FFB30) and 60 (FFB60) minutes were intact, yet visually indistinguishable after 6 hours in culture.

Threads produced by this method had an accordion- or corkscrew-like structure. It was hypothesized that this was the result of non-homogeneous polymerization occurring during co-extrusion. With a constant flow rate, changes in the diameter of the container through which a fluid is flowing (syringe → needle → PE tubing) cause changes in the velocity of that fluid, which may have caused a "pile-up" effect within the tubing. Polymerization of the collagen also affected the viscosity, which further changes the flow properties of the gelling collagen solution.

Repeating this experiment failed to produce threads; it was determined that this was due to the use of room temperature collagen (instead of 4°C) and incomplete mixing of the extrudants. While incorporating FFB into the protocol was considered an improvement over previous methods, new techniques were to be evaluated for extrusion and fabrication of the threads. New methods will be discussed further in the following sections and will involve thorough mixing of the extrudants prior to being extruded to allow for more uniform flow and the fabrication of more uniform and cylindrical threads.

5.1.6 FFB Cytotoxicity Study

As was previously mentioned, the use of FFB as a bath to extrude threads into was thought to be an improvement over the use of culture media. To evaluate to potentially cytotoxic effects of

exposing human dermal fibroblasts to fiber formation buffer, MitoTracker-positive HDF cultures were incubated with PBS, FFB, and EtOH for 60 minutes. Figure 23 shows phase contrast/fluorescence images taken at 10×.

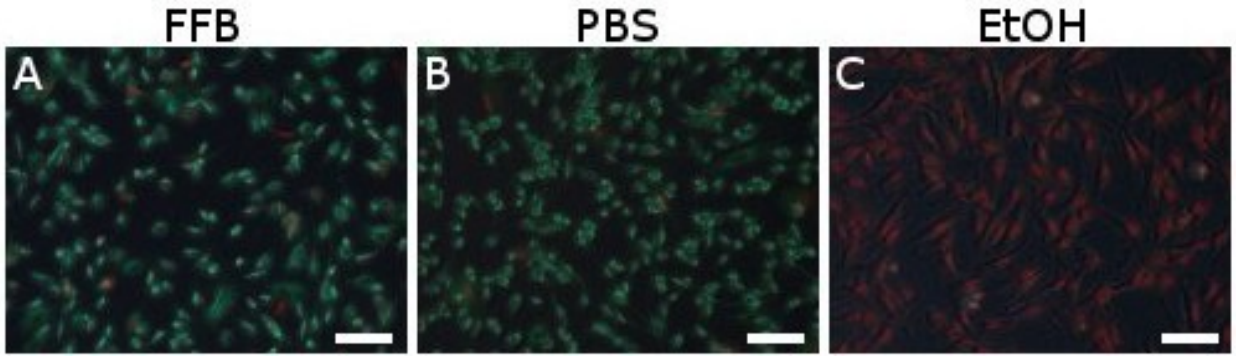

Figure 23: Fiber Formation Buffer (FFB) Cytotoxicity. Phase contrast/fluorescence overlay of MitoTracker Green-positive HDFs on tissue culture plastic exposed to FFB, PBS, or EtOH for 60 minutes (***A***, ***B***, ***C***, respectively). Samples were stained with 'Dead' component of Live/Dead stain (ethidium homodimer, EthD-1, red) following incubation. Cells incubated in FFB and PBS exhibited no discernable difference in viability, whereas all cells exposed to EtOH were dead (with no MitoTracker signal present). Scale bar = 100 μm.

5.1.7 Acellular and HDF-Collagen Cold-Mix Extrusion

One of the most significant objectives of the pilot studies was to develop a protocol to fabricate stable lengths of HDF-containing collagen microthreads in a reproducible manner. Previous attempts to repeat co-extrusion experiments into both DMEM and FFB failed to yield threads, likely due to insufficient and/or uncontrolled mixing of the extrudants and improper temperature control. To overcome this difficulty, the extrudants (collagen, 5X DMEM, and cell suspension) were mixed thoroughly by pipette before being drawn into a syringe for extrusion. Additionally, the collagen and 5X DMEM were kept on ice until just before use, optimizing the user's control over gelling conditions. HDFs were pre-loaded with MitoTracker as previously described. Briefly, the three solutions were mixed carefully as to not create bubbles at a 4:3:1 (collagen : DMEM + 180,000 HDFs/mL : 5X DMEM) ratio. The mixture was drawn into a syringe and extruded at a rate of 0.25 mL/min through 0.86 mm (ID) polyethylene tubing into

sterile 37°C FFB. After 30 minutes of incubation, the FFB was removed and replaced with standard culture media (DMEM). This study was conducted under sterile conditions in a tissue culture hood. This procedure is shown schematically in Figure 24 and a representative microthread in culture media is shown in Figure 25: HDF-Seeded Collagen Microthread Created by Cold-Mix Extrusion..

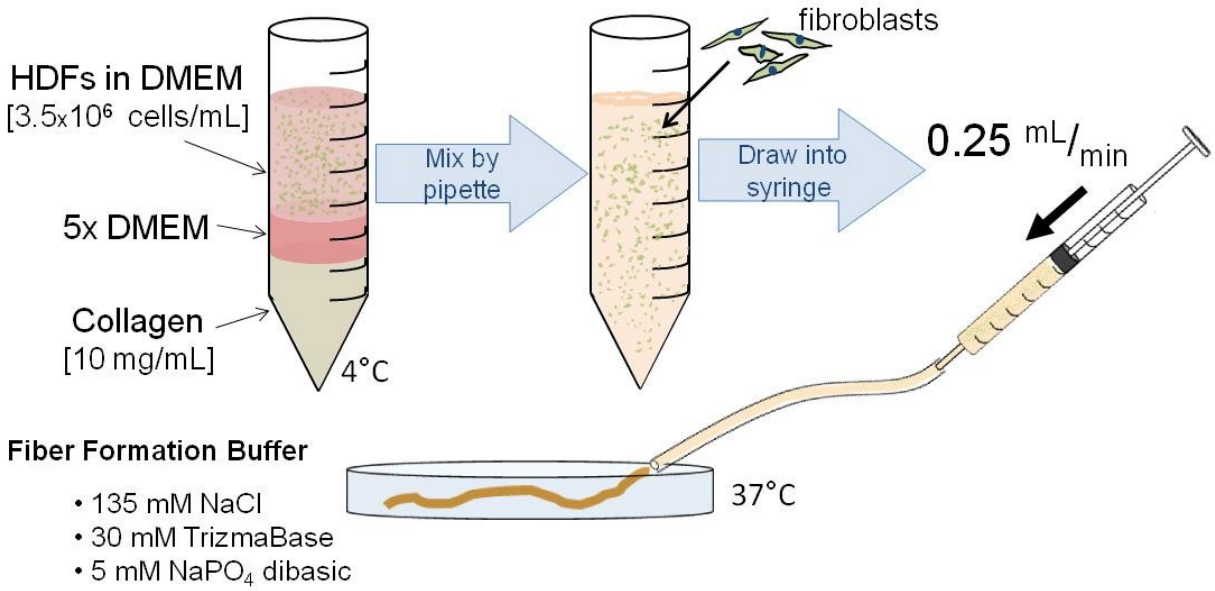

Figure 24: Cartoon Schematic of HDF-Collagen Cold-Mix Extrusion Method.

Figure 25: HDF-Seeded Collagen Microthread Created by Cold-Mix Extrusion.

Four hours after extrusion, HDF-populated collagen microthreads were removed from culture media and visually inspected using phase contrast and fluorescence microscopy. MitoTracker-loaded HDFs were distributed throughout the thickness of the thread and were within the boundaries of the thread (Figure 26).

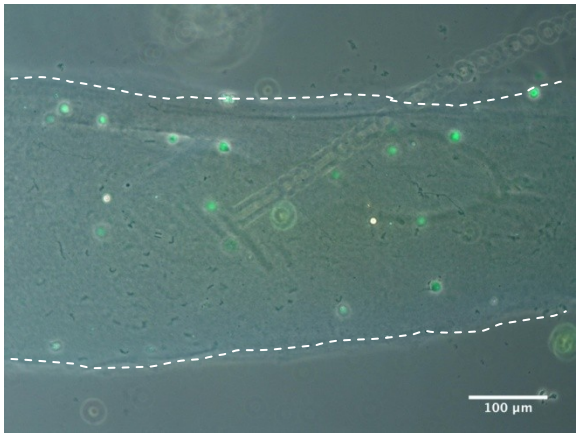

Figure 26: HDF-Seeded Collagen Microthreads 4h after Extrusion. Phase contrast/fluorescence overlay. Prior to cell spreading, HDFs appear as green balls. Scale bar = 100 µm.

While analysis 4h after extrusion demonstrated effective embedding of HDFs within collagen microthreads, this method did not confirm cell viability. To show that cell viability was maintained through the extrusion process, HDF-seeded collagen microthreads were fixed 4h and 24h after extrusion and stained with phalloidin (actin; green) and Hoechst (nuclei; blue) to visualize changes in cell morphology. As Figure 27 shows, 24h after extrusion, HDFs exhibited an increasingly spread morphology.

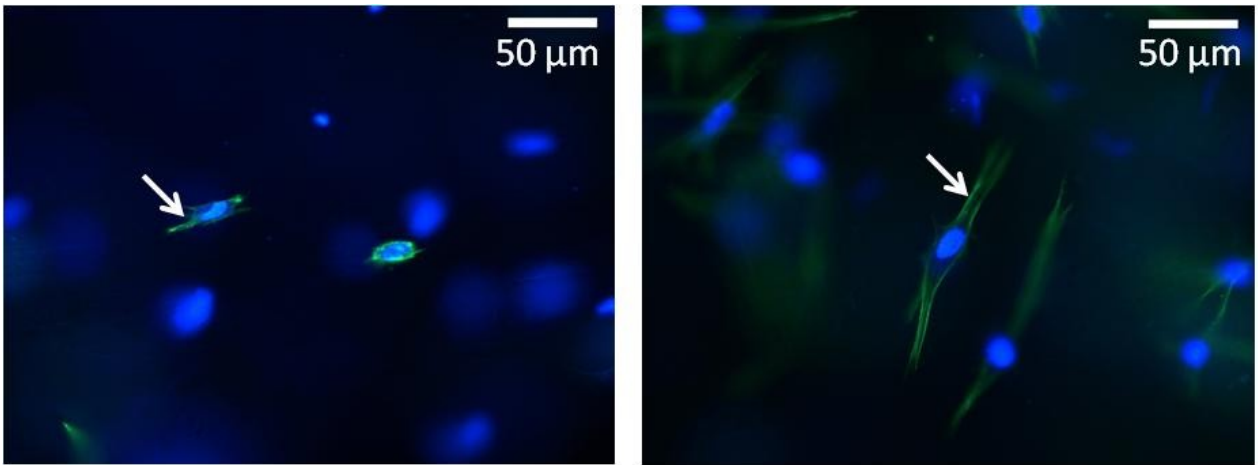

Figure 27: HDF Morphology 4h and 24h after Extrusion. Fluorescent images of HDFs in collagen threads 4h (left) and 24h (right) after the cold-mix extrusion process. White arrows indicate changes in HDF morphology. Samples were stained with phalloidin (actin; green) and Hoechst (nuclei; blue).

5.2 HUVEC Surface Seeding Preliminary Studies

Once an effective method of embedding HDFs within the thread was established, it was necessary to pursue a method for seeding the HUVECs on the thread surface. Two specific challenges needed to be addressed to accomplish this. First, the HUVECs needed to be visually distinguishable by microscopy from the HDFs both in culture and after fixation. Second, an efficient and effective method of seeding needed to be developed.

5.2.1 Pre-Loading of HUVECs with DiI-ac-LDL

One technique that would allow for both terminal and non-terminal imaging of the HUVECs was to pre-load the cells with an endothelial cell-specific fluorescent marker. DiI (a fluorescent molecule) conjugated to acetylated LDL (low-density lipoprotein) is one such marker. When incubated with the marker, endothelial cells will endocytose the molecule, localizing it within the cytoplasm and making the cells easily identifiable. HUVECs were incubated with DiI-Ac-LDL (BT-902, Biomedical Technologies Inc., Stoughton, MA) at 10 μg/mL in EGM for 4 hours, rinsed with PBS, and left to rest in EC culture media for at least 4 hours before use. Pre-loaded HUVECs were imaged at 10× and are shown in Figure 28.

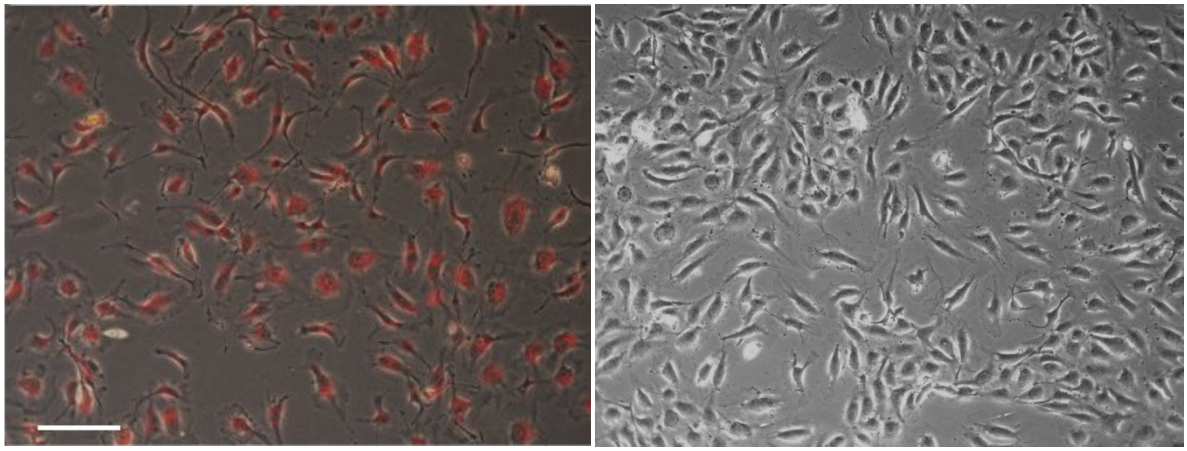

Figure 28: DiI-ac-LDL Labeled HUVECs. Phase contrast/fluorescence overlaid image of human umbilical vein endothelial cells following DiI-ac-LD uptake (left panel). Unlabeled human umbilical vein endothelial cells exhibit no fluorescence in the orange channel (right panel). Scale bar = 100μm.

5.2.2 Fabrication of HUVEC Surface Seeding Device

To effectively seed HUVECs on the surface of the threads, a concept similar to the molding design alternative was utilized. The initial idea was to place the thread on a culture plate and incubate it in a suspension of cells for a short period of time. While this would accomplish the task of getting cells onto the surface of the thread, it has been shown to be very inefficient

and yields a small number of cells attached. An improved method of seeding cells on the thread surface was developed using a modified version of a device created by Darshan Parekh, a graduate student in the Rolle Lab at WPI. This device would contain channels that would prevent movement of the thread during the surface seeding process and would facilitate more effective surface seeding by providing a constrained geometry and volume. A custom-built mold was made from polycarbonate using CNC machining. This mold acted as a negative template for the molding of PDMS seeding device (Figure 29; Figure 30). Polydimethylsiloxane (PDMS; Sylgard 184 silicone elastomer kit, Dow Corning, Midland, MI) was mixed with a curing agent at a 10:1 ratio (w/w). The mixture was then poured onto the polycarbonate mold and degassed under vacuum for 30 minutes before being cured at 60°C for 3 hours. The final PDMS seeding device is shown in the bottom-right panel of Figure 30.

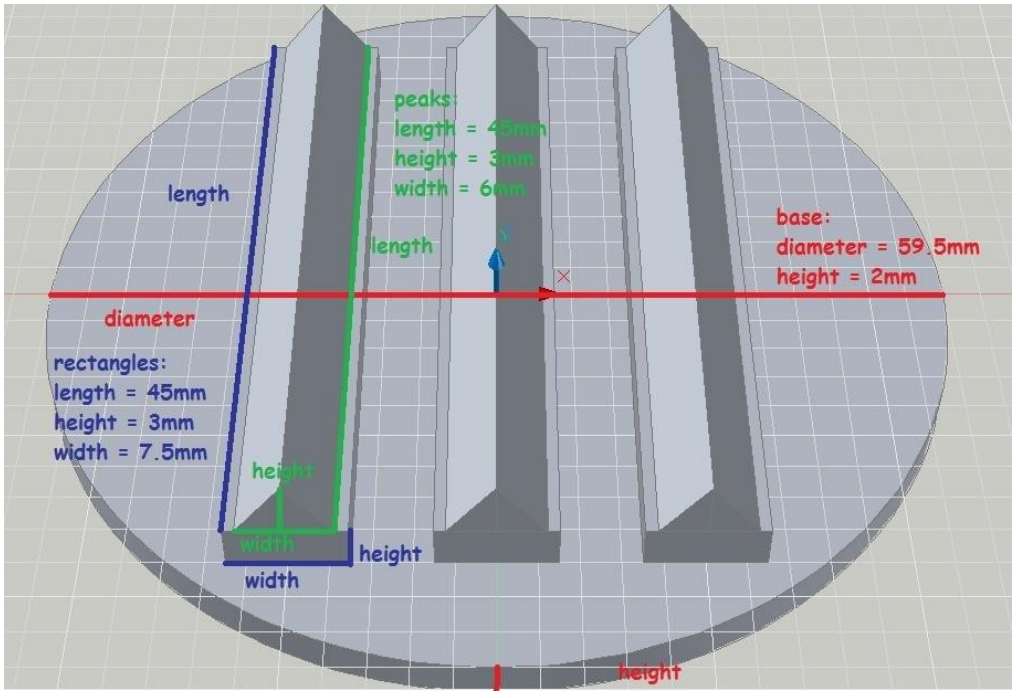

Figure 29: CAD Drawing of Custom PDMS Mold. Drawing includes dimensions.

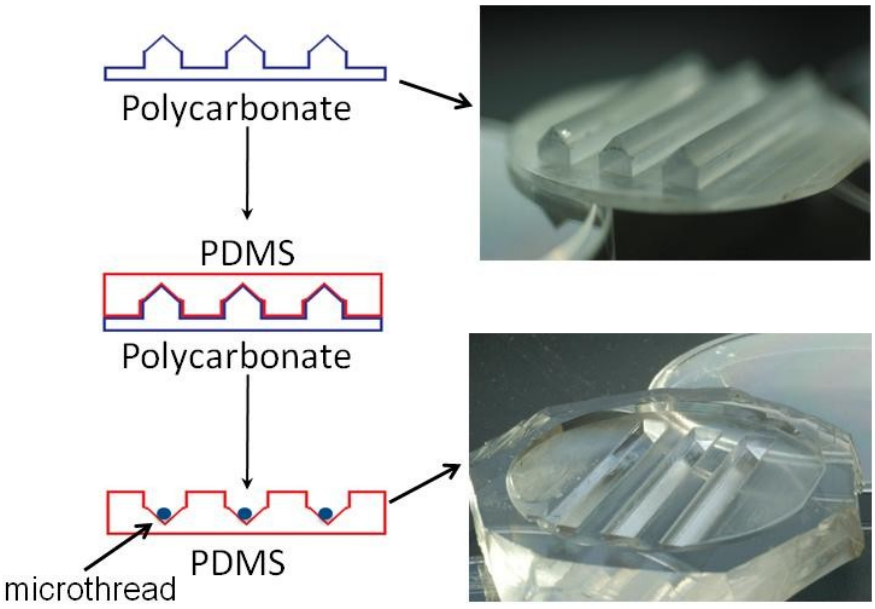

Figure 30: Schematic and Images of a HUVEC Surface Seeding Device.

5.2.2 HUVEC Surface Seeding

Briefly, acellular threads (produced by cold-mix extrusion) were removed from culture media, rinsed with PBS, and placed in the custom-built surface seeding device. As was previously discussed, this device provides a constrained volume and geometry that facilitates cell contact and adhesion to the curved surface of the collagen microthreads. One hundred-fifty microliters of DiI-Ac-LDL pre-loaded HUVEC cell suspension (66,000 cells/mL) were pipetted onto the surface of each microthread as shown in Figure 31. The cells were allowed to adhere for 4 hours. After the incubation period, the threads were rinsed with PBS and inspected using fluorescence microscopy (Figure 32).

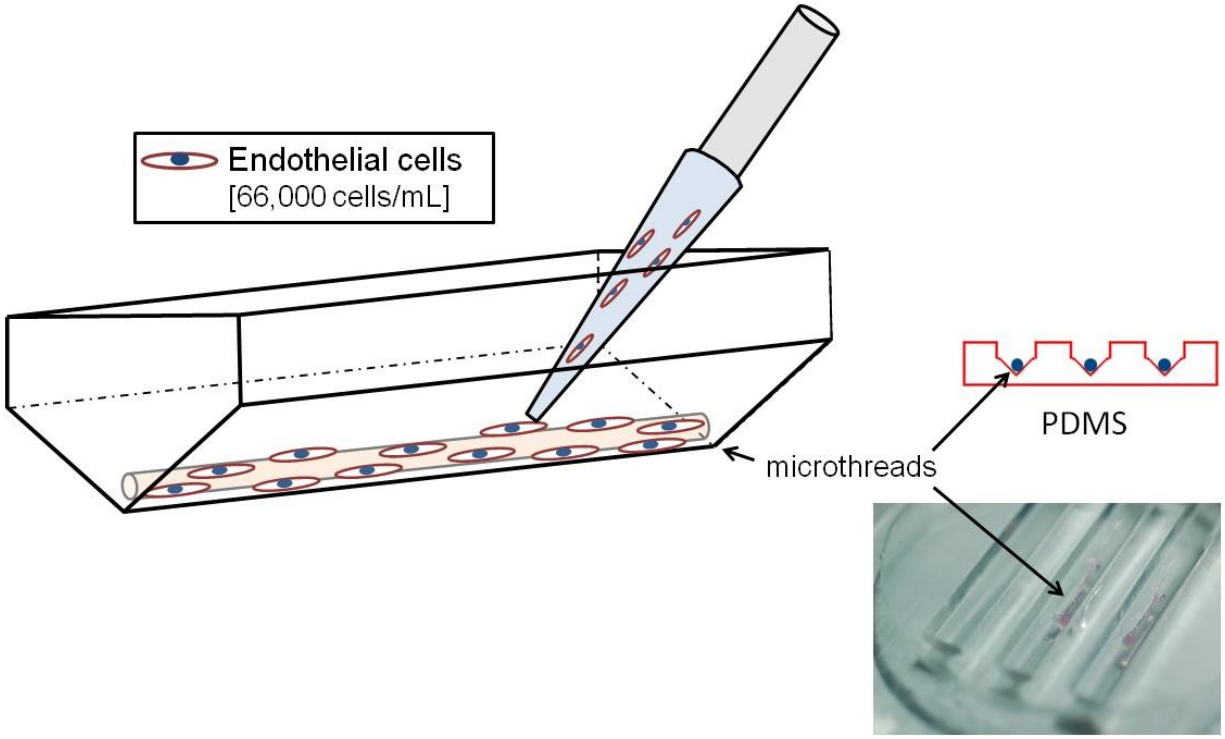

Figure 31: Schematics and Photograph of the HUVEC Surface Seeding Device.

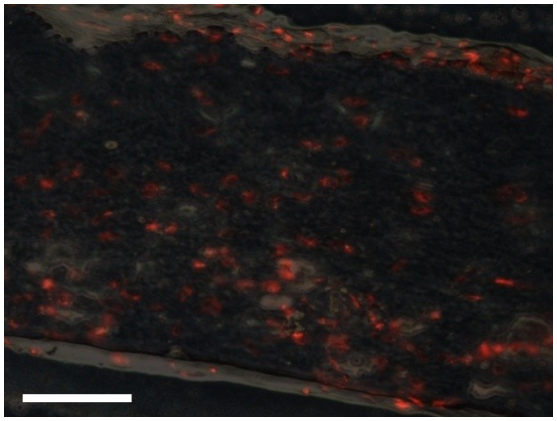

Figure 32: Fluorescence/Phase Contrast Overlay of HUVECs Seeded on the Surface of a Collagen Microthread. After seeding with HUVECs for 4 hours, threads were rinsed in PBS and inspected using fluorescence microscopy for cells. Orange fluorescence indicates presence of DiI-Ac-LDL positive HUVECs (left panel). Negative controls without HUVECs seeded on the microthread surface had no fluorescence in the orange channel (right panel). Scale bar = 100μm.

After developing methods to effectively embed fibroblasts within and seed endothelial cells on the surface of collagen microthreads, it was necessary to validate the utility of fluorescence microscopy as the chosen characterization method throughout this study. Previously,

microthreads have demonstrated autofluorescence that has impaired analysis by fluorescence microscopy. An unstained, acellular thread was analyzed for autofluorescence in the green and orange channels. Limited, if any, autofluorescence in the green and orange channels was found (as shown in Figure 33).

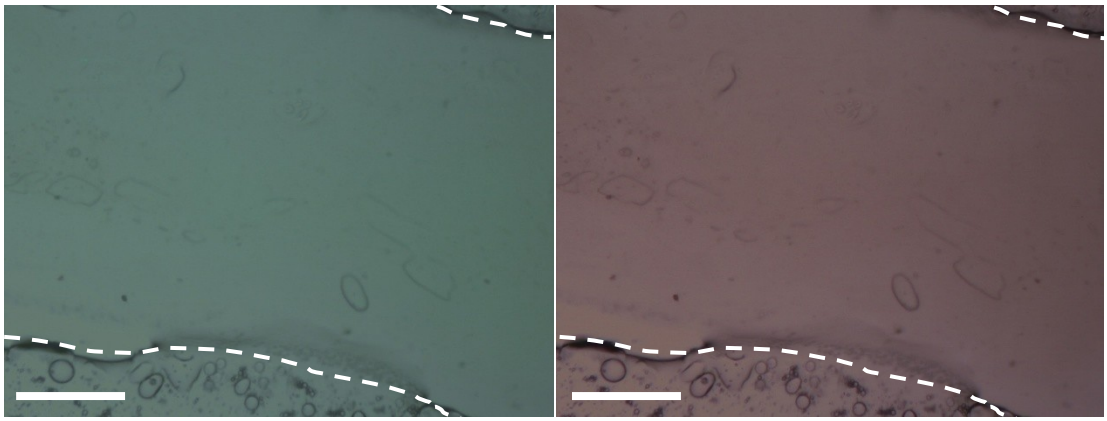

Figure 33: Acellular Control Microthread. Phase contrast/fluorescence overlaid images of an acellular thread. Limited fluorescence was observed in the green (left panel) and orange channel (right panel). Thread boundaries indicated by dashed lines. Scale bar = 100μm.

6. Final Design & Validation

6.1 Co-Culture Studies

The protocols developed through the pilot studies demonstrated the ability to extrude the HDF-populated thread scaffolds under sterile conditions, seed HUVECs on the surface of collagen threads, visualize cells both within and on the surface of the threads, and distinguish between the two cell types. Samples were collected at 24-hour time points for up to 3 days of co-culture, as this is the time point at which tubule formation has been observed in similar collagen scaffolds [46]. The methods described in this section detail the team's final protocols for creating and analyzing the collagen microthread-based co-culture system (as shown in Figure 18).

6.1.1 Cell Culture of HDFs and DiI-Ac-LDL-Labeled HUVECs

Human dermal fibroblasts (HDFs, primary isolates from neonatal foreskin tissue) at passage 12 and human umbilical vein endothelial cells (HUVECs, primary isolates, Lonza, Mapleton, IL) at passage 8 were used in this study. HDFs were cultured in DMEM (Mediatech, Herndon, VA) supplemented with 10% FBS (PAA, Ontario, Canada) and 1% penicillin/streptomycin (Mediatech, Herndon, VA). At 90% confluence, the HDFs were trypsinized and re-suspended at a concentration of 180,000 cells/mL. HUVECs were cultured with EGM media (Lonza, Mapleton, IL) at 37°C. At 80% confluence, the HUVECs were trypsinized and re-suspended at a concentration of 66,000 cells/mL. To differentiate between the two cell types, the HUVECs were pre-loaded with DiI-Ac-LDL prior to surface seeding. The HDFs were not to be labeled with MitoTracker in the co-culture system because it would appear in the same color channel as the phalloidin stain (described in 6.1.4 Fluorescent Labeling of Cell Cultures).

6.1.2 Fabrication of HDF-Populated Microthreads Using Cold-Mix Extrusion

The final design consisted of a multi-step approach; the first step was to create HDF-populated collagen microthreads (as shown in Figure 34).

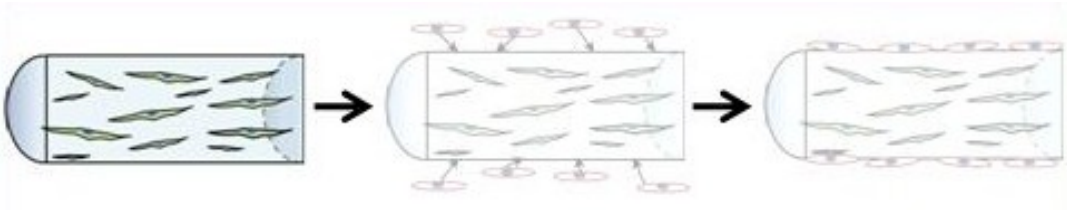

Figure 34: Step I – Embedding of HDFs within Collagen Microthreads

Fibroblast-populated self-assembled collagen threads were produced using the novel cold-mix extrusion method previously described and shown in Appendix C. Acid-soluble type I collagen was mixed with 5X DMEM and human dermal fibroblast cell suspension at a 4:1:3 ratio. The three solutions were mixed thoroughly and extruded through a polyethylene tube with inner diameter of 0.86 mm into fiber formation buffer (FFB; pH 7.42, 135 mM NaCl, 30 mM TrizmaBase, and 5 mM NaPO4 dibasic; Sigma, St. Louis, MO). The threads were incubated for 30 minutes in FFB at 37°C before transfer to DMEM culture media. These HDF-populated threads were cultured for 24 hours prior to seeding HUVECs on the thread surface. Acellular control threads were also extruded using the same method, substituting DMEM for the cell suspension. Samples were fixed 4 hours after extrusion and at 24 hour time points thereafter and processed for analysis (Figure 27). After 48h of incubation, samples were fixed and processed to visualize cell nuclei and actin as shown in Figure 35.

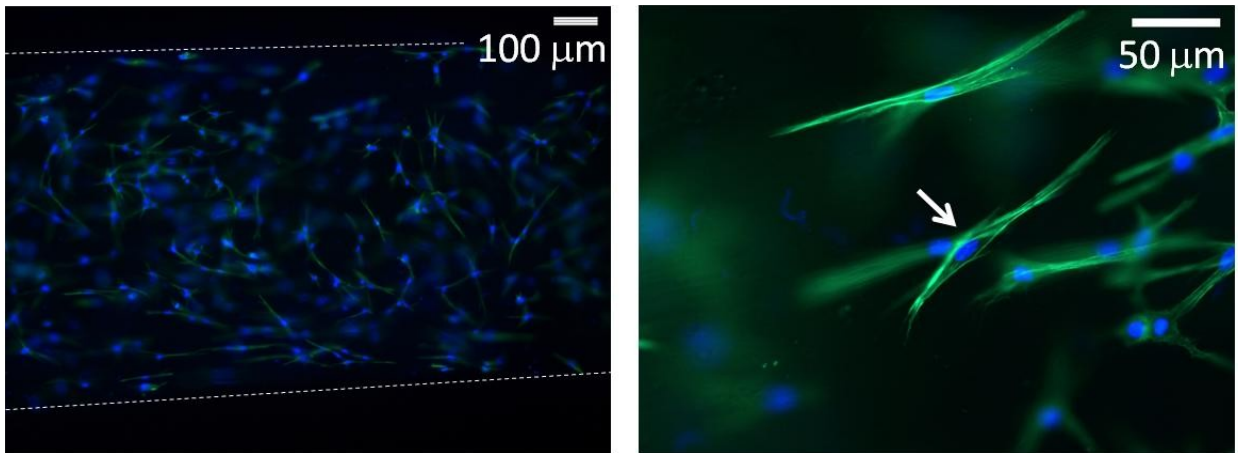

Figure 35: HDF Monoculture Images. HDF-seeded collagen threads after 48 hours of culture at low (left) and high (right) magnification. White arrow indicates an HDF in the plane of focus. Samples were stained with phalloidin (actin; green) and Hoechst (nuclei; blue). Dashed lines indicate thread boundaries.

6.1.3 HUVEC Surface Seeding

The second step was to seed endothelial cells on the surface of the fibroblast-populated collagen microthreads (as shown in Figure 36).

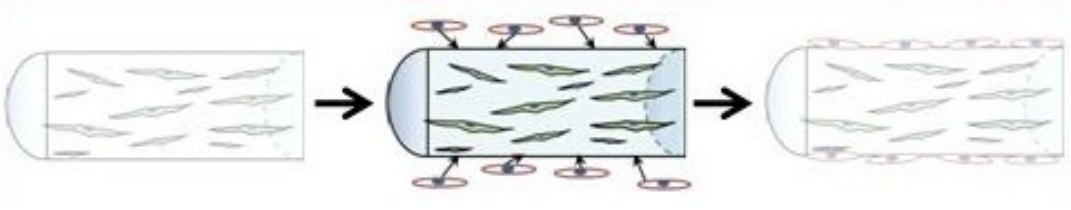

Figure 36: Step II – Surface-Seeding of HUVECs on Collagen Microthreads.

After 24 hours in culture in DMEM culture media, acellular control threads and HDF-populated threads were transferred to a custom-built PDMS seeding device for surface seeding. The device provided a constrained volume and geometry to facilitate cell contact and adhesion to the curved surface of the collagen microthreads. One hundred microliters of the HUVEC suspension were pipetted into each channel and the threads were incubated for 4 hours. The microthreads were then washed in PBS and transferred to EGM (CC3124, Lonza, Mapleton, IL) media for culture at 37°C. Cultures were fixed 48 hours after HUVEC seeding and processed for analysis (Figure 37).

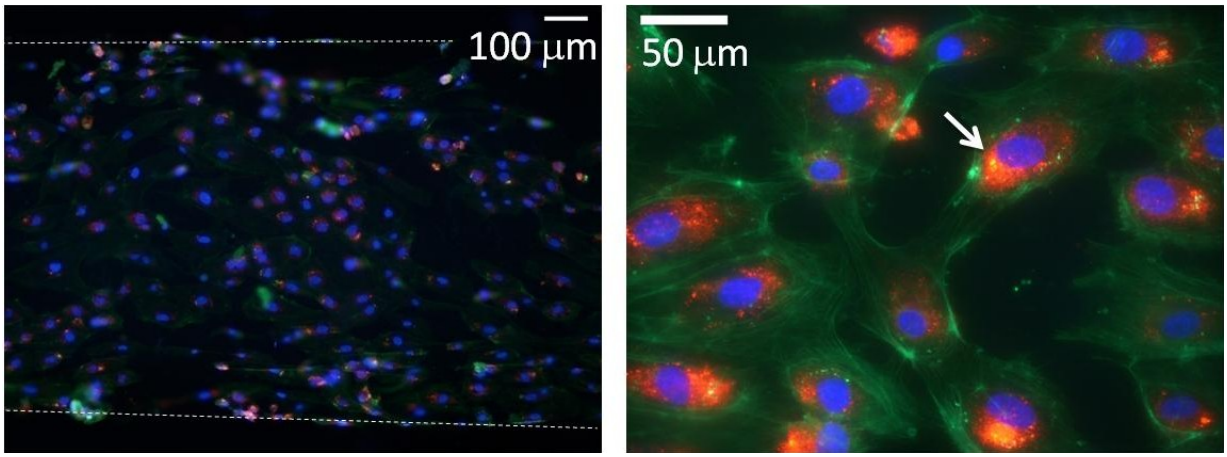

Figure 37: HUVEC Monoculture Images. HUVEC-seeded collagen threads after 48 hours of culture at low (left) and high (right) magnification. White arrow indicates a HUVEC in the plane of focus. Samples were stained with phalloidin (actin; green) and Hoechst (nuclei; blue). HUVECs were labeled with DiI-Ac-LDL (orange). Dashed lines indicate thread boundaries.

6.1.4 Fluorescent Labeling of Cell Cultures

Cell-seeded collagen microthreads were removed from culture every 24 hours for observation. Each sample was placed on a glass slide, fixed with 4% paraformaldehyde, and stained with phalloidin to visualize cell morphology and Hoechst to visualize cell nuclei. This staining is in addition to the DiI-Ac-LDL labeling of HUVECs.

6.1.5 Fluorescent Imaging

Standard fluorescence microscopy was initially used to visualize stained samples. Due to the 3D structure of the collagen threads, a more effective method of visualization was needed. To accomplish this, confocal fluorescence microscopy was also performed on the threads. This type of microscopy allows for single planes of the sample to be imaged with minimal interference from out-of-focus fluorescing regions of the sample.

Fluorescent imaging of the cell-seeded constructs confirmed embedding of HDFs and surface attachment of HUVECs at all time points. HDF presence was determined by the co-localization

of the actin (green) with a nucleus (blue). HUVECs were distinguished by the added presence of DiI (orange).

HDF and HUVEC mono-culture control experiments were performed to validate HDF embedding and HUVEC surface seeding methods. Figure 27 shows an HDF-seeded thread construct 4 hours and 24 hours after extrusion. The HDFs at 4 hours after extrusion exhibited very limited spreading, however an increasingly spread morphology was observed after 24 and 48 hours of culture (Figure 35). Standard fluorescent images of the HDF-seeded threads included fair amounts of unwanted signal from out-of-focus cells. This suggested that the HDFs were distributed at different depths within the thickness of the thread and motivated the use of confocal microscopy for clearer images. Figure 37 shows an acellular collagen thread 48 hours after HUVEC seeding. Unlike the HDF monoculture threads, the majority of HUVECs appeared in the same plane of focus.

Figure 38 shows an HDF- and HUVEC-seeded collagen thread after 48 hours of co-culture. Visualization throughout the thickness and along the length of the thread suggested uniform seeding of both embedded HDFs and surface-seeded HUVECs. Further characterization of the samples with confocal microscopy can be seen in Figure 39.

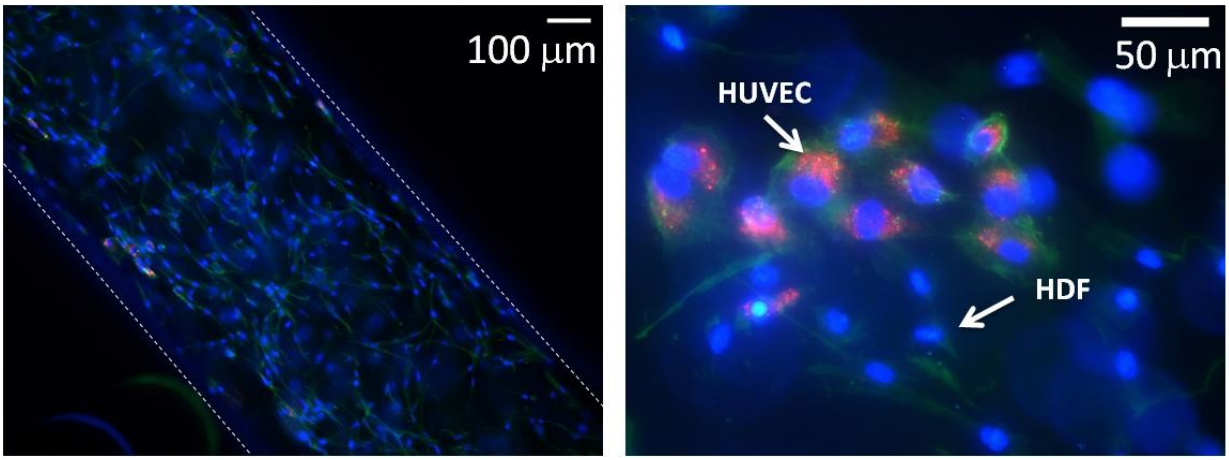

Figure 38: HUVEC- and HDF-Seeded Collagen Threads after 48 Hours of Co-Culture. Images were taken at low (left) and high (right) magnification. White arrows indicate HUVECs and HDFs in the plane of focus. Samples were stained with phalloidin (actin; green) and Hoechst (nuclei; blue). HUVECs were labeled with DiI-Ac-LDL (orange). Dashed lines indicate thread boundaries.

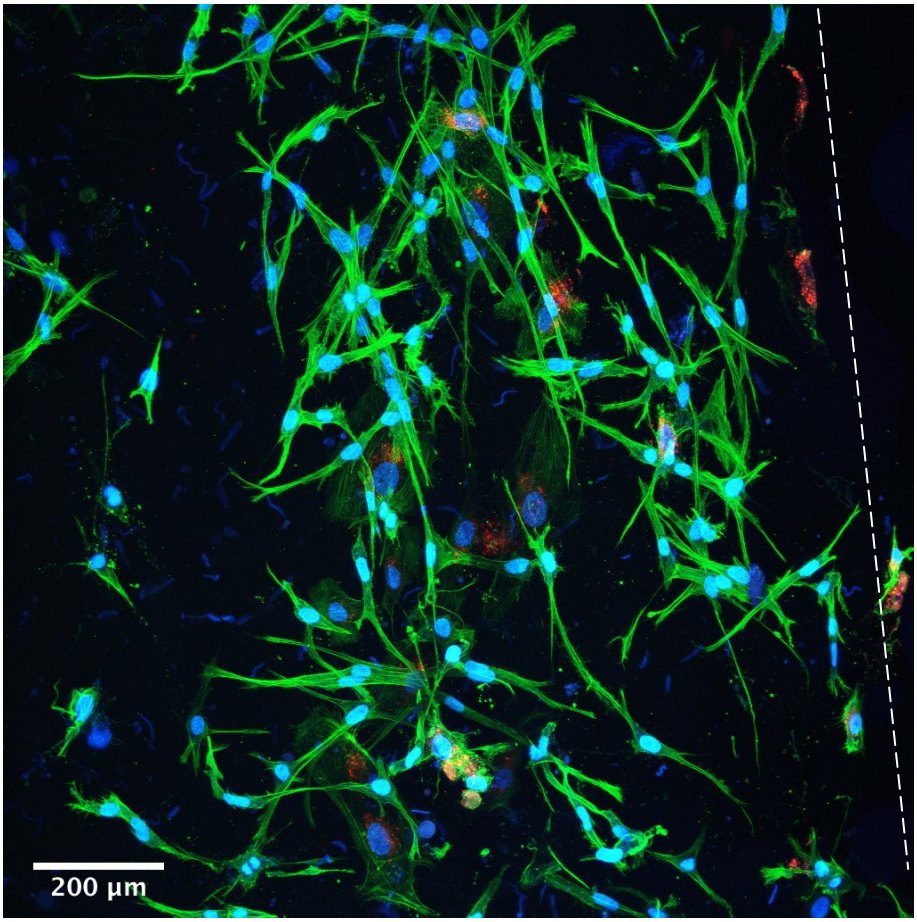

Figure 39: Confocal z-Stack Overlay of HUVEC- and HDF-Seeded Collagen Threads after 24 Hours of Co-Culture. Samples were stained with phalloidin (actin; green) and Hoechst (nuclei; blue). HUVECs were labeled with DiI-Ac-LDL (orange). Dashed line indicates approximate thread boundary.

6.2 Achieving Specific Aims

Specific Aim 1: **Provide a 3-D culture environment for therapeutic cell types by embedding one of the cell types in a collagen microthread.**

Achievement 1: The established approach for collagen microthread fabrication consists of extruding a collagen suspension through small diameter tubing in a non-sterile environment. Our group developed a process where we can incorporate cells within the collagen microthread. This method also allows microthreads to be fabricated in a sterile environment, which is crucial for long-term cell culture. It also preserved cell viability and thread integrity through control of the extrudants and extrusion rate, as well as the temperature, pH, and osmolality of the extrusion bath.

Specific Aim 2: **Seed second cell type on the surface of a collagen microthread. The objective of this aim is to develop a seeding technique that has the potential to more effectively seed a chosen cell type on the surface of the microthread.**

Achievement 2: A novel seeding device was designed, optimized, and manufactured by our group. This device allowed HUVECs to be seeded on the surface of hydrated collagen microthreads by providing a confined geometry and volume.

Specific Aim 3: **Demonstrate the ability to maintain a long-term cellular co-culture system.**

Achievement 3: Utilizing the novel microthread extrusion process and seeding device as described previously in Achievement 1 and 2, a streamlined process has been developed to fabricate collagen microthreads that function as co-culture systems with the chosen cell types. This novel fabrication method allows for construction of structurally-stable collagen microthreads containing embedded HDFs and surface-seeded HUVECs.

7. Discussion

7.1 Project Discussion

The main focus of this project was on the development of methods that would allow for the construction and subsequent characterization of a co-culture system on collagen microthreads to facilitate neovascularization. Methods of independently seeding each cell type were developed, validated, and then combined to create the final co-culture system. Analysis techniques based on fluorescent labeling of the samples were then used for system characterization.

All fluorescent images of the co-culture samples confirmed the presence of HDFs within, and HUVECs on the threads at all time points. Changes in HDF and HUVEC morphology were observed in both mono- and co-cultured threads as a function of time. These results were interpreted to also indicate the viability of both cell types even though no direct assays for cell viability were performed. This suggests that the media diffusion through the threads is sufficient enough to support cell growth and possibly proliferation. However, further studies involving more direct monitoring of cell viability and proliferation—such as a Live/Dead stain and Ki-67 expression respectively—could determine this. If both cell types were indeed proliferating and exchanging soluble factors via diffusion, pro-angiogenic co-culture effects, such as tubule formation and sprouting can be observed. Analysis of tubule formation would require detailed imaging techniques but has been performed before [6, 46].

Initial assessments of the co-cultured microthread constructs were performed using standard fluorescence imaging. However, due to the exceptional thickness of the samples, confocal microscopy was used. This type of microscopy allows for the imaging of one plane of focus at a time, greatly minimizing the signal received from out of focus regions of interest. The technique

also allows for a stack of images to be taken of the sample and then reconstructed into a 3D representation, played in sequence as a movie, or overlaid into a z-stack (Figure 39).

Confocal imaging of the co-culture samples confirmed the locations of specific cell types on the threads. Several z-stacks were taken at different locations along co-cultured threads and indicated the localization of HUVECs on the top, bottom, and sides of the thread. HDFs were localized within the thread at almost all depths. This confirmed the successful independent seeding of both cell types. The HDFs appeared to be distributed throughout the body of the thread in a fairly uniform manner, while the HUVEC surface-seeding exhibited limited uniformity with cells often localized on one or a few sides of the microthread in a monolayer configuration. This was possibly due to the geometry of the seeding device. While the device provides an efficient way to get a high concentration of cells onto the surface of the thread, their distribution on the thread surface is limited by the fact that the threads are cylindrical structures statically located at the bottom of a V-shaped channel under a cell suspension. The most likely explanation for this deficiency is that the topside of the thread was exposed to the cell suspension and received a monolayer of HUVECs while the other side received fewer cells because it faced the bottom of the channel. This could be eliminated by seeding both sides of the thread independently or by somehow making the seeding environment dynamic (rotating the thread, gently agitating the constructs, introducing media flow, etc.).

To properly gauge the angiogenic potential of these constructs, analysis of tubule formation or endothelial sprouting must be performed. This could be done by utilizing UEA-I lectin [46] to stain the membranes of the HUVECs prior to imaging. Reliable tubule formation assays coupled with effective control of seeding efficiency would pave the way for studies on HUVEC dose-response analysis to HDF seeding concentrations to determine which seeding ratios produce

constructs with the greatest angiogenic potential. Also related to this potential would be the degree to which the seeded cells alter the mechanical properties and composition of the microthread scaffold. Angiogenesis involves the remodeling of surrounding tissue through the secretion of MMPs, TIMPs, and other matrix-altering proteins. Future quantification of these changes would be invaluable for advancing our understanding of angiogenesis as well as determining how effective the co-cultured thread constructs would be at promoting angiogenesis and related processes *in vivo*. In addition, identifying and possibly being able to modify the mechanical properties of co-cultured microthreads would enable further development of implantation procedures.

Several limitations of this technology do exist however. In the context of a cellular therapy to promote angiogenesis *in vivo*, the threads lack in that they can only deliver a relatively small volume of cells to the area of interest. This may be offset by delivery of multiple threads but only marginally. Another limitation arises from cell and material sourcing. To eliminate any chances of rejection, the cells and collagen used for the therapy must be autologously sourced. Fibroblasts may be easily obtained from the patient's skin but endothelial cells may be harder to obtain, and autologous HUVECs are not readily available for the majority of patients. In terms of a bench top model for angiogenesis, this technology encounters a different set of limitations. First, even though the co-culture system utilized is pro-angiogenic, angiogenesis is defined as the formation of new blood vessels from existing ones, thus the eventual formation of an actual blood vessel would be unlikely. Also, the system by itself lacks any analogue for flowing blood which is a component that is probably essential to proper angiogenesis *in vivo*.

The localization and changes in morphology of both cell types after 48 hours of co-culture demonstrate that the HDF/HUVEC co-culture system was effectively translated to a collagen

microthread scaffold. This technology, in particular methods to independently seed two cell types, could also be utilized to construct threads for housing and studying other co-culture systems, not just pro-angiogenic ones. This opens up countless possibilities for creating easily deliverable cellular therapies and studying other *in vivo* physiologic mechanisms.

One point of note, though not related to the goal of the project, is the fluorescent properties of the collagen microthreads. While thread autofluorescence was minimal and did not significantly interfere with observing the various fluorescent labels used to image cells, interesting fluorescent patterns were apparent in unseeded threads that were stained as controls. An example of this is shown in Figure 40.

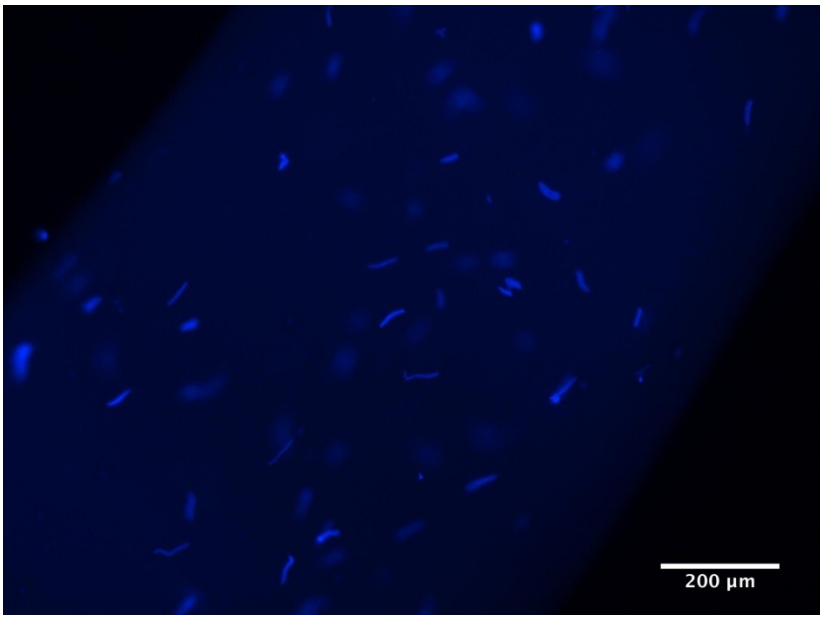

Figure 40: Fluorescent Image of an Unseeded Collagen Microthread Stained with Hoechst.

Small filaments of about 50 µm in length appeared to be dispersed throughout the thickness of the thread, and appeared in the blue channel of threads stained with Hoechst. These filaments also appeared in the red channel when threads were stained with ethidium bromide (the DEAD stain from the LIVE/DEAD assay by Invitrogen). Appearance of these filaments was attributed to non-specific binding of the stains used; however their presence under Hoechst and ethidium

bromide staining suggests that they may be some kind of nucleic acid. Fluorescent analysis of the cell cultures was minimally affected by this unwanted noise because Hoechst-stained cell nuclei have a very distinct round shape and were generally brighter. A cell was defined as the co-localization of a blue Hoechst-stained nucleus with green f-actin fibers stained with phalloidin.

7.2 Impact Analysis

In addition to our experiments, an impact analysis was conducted to relate this project to global concerns. In this analysis, we address economics, environmental impact, societal influence, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability.

Economics

The patient's perceived "willingness to pay" figures greatly into the overall economic value of this technology. The behavioral economic prospective theory shows that if one were to gage a person's reaction to the resultant lose or gain from the same reference point; loss is far more emotionally devastating than the emotional reward from the gain. This is relevant to the co-culture technology because the devastation of losing tissue function will, invariably, outweigh the emotional loss associated with losing a small sum of money, increasing the patient's willingness to pay for a desirable solution.

Environmental Impact

Co-culture will have a minimal environmental impact. The major environmental impacts can be found within the sustainability section of this report. Other environmental impacts can include the fact that if this device makes it to market, it would improve the lifespan of the patients. With more people living longer, the environment could be negatively impacted due to the waste that

humans produce on a daily basis. This could also adversely affect the scarce resources such as oil and create a strain on the economy.

Societal Influence

The co-culture system has the potential to greatly affect the ordinary person. By regenerating damaged tissue in patients, their quality of life will improve as will the lives of all who care about them. The emotional and social ramifications of this device could have strong potential if it goes to market.

Political Ramifications

This device has minimal political ramifications currently. It is possible that one day, when the manufacturing process is streamlined and perfected, that this could make a significant difference in countries around the world. Once it is streamlined, not only would European countries be able to utilize this device, but third-world countries maybe be able to use this technology as well.

Ethical Concerns

There are minimal ethical concerns that can be associated with this device. With the recent change in the policy on embryonic stem cell research, it is possible that this co-culture system could be called into question due to the fact that it has potential to utilize these cells. If this avenue is not pursued, then that concern will be nullified. The other ethical concern is dependent upon potential patient's religions. This is due to the fact that taking cells from a patient and utilizing them in a biomedical capacity can conflict with their particular religion's belief system.

Health and Safety Issues

The co-culture system was designed with the improvement of a patient's overall quality of life in mind. This system in theory should greatly improve a patient's health by repairing ischemic or infarcted tissues. With regards to the safety of this product, that will be deduced through extensive animal and clinical trials run in conjunction with the FDA. This will ensure that the invention is safe for patients and allow for the product to receive an HCPCS code, which allows for the system to be covered by insurance companies.

Manufacturability

Standard collagen microthreads are currently manufactured in a 3-day process, by hand and in small batches, using an extrusion system developed by Professor Pins of WPI. Microthreads are manufactured on location; the threads are not prefabricated at this point due to concerns with maintaining the threads in a sterile environment. While the novel process described in this report to create the co-cultured collagen microthreads takes only two days, it is unclear as of now how long co-cultured threads would need to be incubated before being implanted. This uncertainty is due to the early stages of the production of the threads and will improve as more research is conducted as the project moves forward.

When clinical trials occur, the microthreads will have to be manufactured in a contained, aseptic environment using collagen and reagents that are approved for clinical use. It is hoped that a bioreactor will ultimately be designed to hold microthreads and seed them with a patient's own cells.

Sustainability

There are multiple definitions for sustainability depending on which aspect of the business sector one is looking at. For the scope of this project, the definition that was chosen was “using methods, systems and materials that won't deplete resources or harm natural cycles”. Using this description, the co-culture system can be analyzed for its conformance to sustainability. Since the collagen and the cells are both naturally occurring and can be derived from patients themselves or bovine specimens, these resources are renewable and will not be depleted. Cells are capable of regenerating and the collagen can be collected in a multitude of eco-friendly ways. In addition to this, the 1mL syringes that are used once and then discarded can be sent to a reprocessing plant and recycled. This increases the sustainability of the extrusion system and is an added benefit to the product.

8. Conclusions & Recommendations

The main objective of this project was to develop a co-culture system for cell delivery using biopolymer microthreads as scaffolds. After extensive research, the team determined that HUVECs and HDFs were the best candidates for co-culture due to their well documented pro-angiogenic potential. Combining this system with a novel approach to microthread extrusion, the team demonstrated the ability to fabricate structurally stable collagen microthreads while retaining cell viability. The team was also able to design and implement an effective surface-seeding device. This device allowed HUVECs to be seeded on the surface of hydrated collagen microthreads by providing a confined geometry and volume. A set of characterization protocols were also developed to assess cell viability, morphology, and migration within the co-culture system.

Future work on improving the co-culture system may include *in vitro* analysis of tubule formation by extending culture time and developing a UEA-1 lectin staining protocol. This type of lectin binds specifically to the surface of endothelial cells, which would allow for visualization of tubule formation [46]. Seeding efficiency of both methods should also be analyzed, as it is vital for future development of the co-culture system. The ability to control seeding density would allow for the study of HUVEC tubule formation as a dosed-response to HDF seeding concentrations. Cell-cell and cell-matrix interactions should also be conducted in the future to characterize the extent to which angiogenesis occurs in the system. Finally, mechanical tests such as uniaxial testing, and degradation studies should be performed.

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Appendix A: Glossary

3T3-J2 – A standard fibroblast line derived from mouse embryo tissue

Alginate – A viscous gum widely used for cell immobilization and encapsulation

Angiogenesis – The growth of new blood vessels from pre-existing vessels

Angiotensin Converting Enzyme Inhibitor (ACE Inhibitor) – Any of a group of drugs that relaxes arteries by hindering the activity of certain enzymes

Anticoagulant – A drug that prevents the clotting of blood

Anti-platelet – A drug that inhibits or destroys blood platelets

Atherosclerosis – A disease of the arteries characterized by deposits of fatty substances along artery walls, resulting in the narrowing of the vessels

Autofluorescence – Naturally occurring fluorescent signal of a substance, such as collagen

Biomaterial – A natural or synthetic material suitable for introduction into living tissue

Cardiac – Pertaining to the heart

Cardiac Myocyte – Heart muscle cell

Cardiovascular Disease (CVD) – Any of a category of diseases that affect the heart or arteries

Cerebral – Pertaining to the brain

Chitosan – A polymer formed by chitin, a polysaccharide

Collagen – A strong fibrous protein that provides an extracellular matrix for tissues and cells within the body

Confocal Microscopy – An optical imaging technique used to reconstruct microscopic three-dimensional structures

Cutaneous – Pertaining to the skin

Cytokine – Any of several protein growth factors that stimulate proliferation, especially of endothelial cells, and that promote angiogenesis

Cytotoxic – Cell-killing; toxic to cells

DiI-ac-LDL – An orange fluorescent stain specific to endothelial cells

Dulbecco's Modified Eagle's Medium (DMEM) – A concentrated solution of amino acids, vitamins and supplementary components, such as salts

Endothelial Cell– A specialized type of cell that lines the circulatory system

Extracellular Matrix – The part of a tissue that provides structural support to cells, in addition to other important functions

Fibrin – A fibrous protein that assists with blood clotting

Fibroblast – A type of cell that synthesizes the extracellular matrix and plays a critical role in wound healing

Hoechst – A blue fluorescent dye that stains cell nuclei, to visualize cell location

Human Mesenchymal Stem Cell (hMSC) – A cell that can differentiate into many cell types, that is isolated from bone marrow

Hydrogel – A network of highly absorbant natural or synthetic polymer chains that are water-insoluble

Induced Pluripotent Cell (iPSC) – A type of stem cell artificially derived, usually from an adult somatic cell

Infarction – An area of tissue that is dead due to lack of blood flow

Ischemia – The restriction in blood supply that causes damage or death to tissue

Keratinocyte – A type of cell found in the epidermis (skin); also called "basal cell"

Mesenteric – Pertaining to the small intestines

MitoTracker – A green fluorescent dye utilized to facilitate non-terminal visualization of cells

Morphology – The shape of a cell; evaluation of morphology can indicate cell viability

Myocardial Infarction – A heart attack; death of cardiac tissue

Necrosis – Death of cells or living tissue

Neovascularization – The formation of functional microvascular networks

Perfusion – The process of blood flow to biological tissue

Phalloidin – A green fluorescent dye that stains actin to facilitate visualization of cell morphology

Phenotype – An observable characteristic or trait of an organism or cell

Polycarbonate – A thermoplastic polymer that is often used in medical applications as it is not cytotoxic, and is easy to manufacture, machine and sterilize

Polymerization – The process by which individual monomers link into chained polymers

Protease – Any of a group of enzymes that assists in the creation of proteins by hydrolysis of peptide bonds

Regenerative Therapy – A method of treatment that focuses on the restoration of tissue

Scaffold – A temporary framework that provides support

Sickle-cell Disease – A blood disorder characterized by abnormal, rigid red blood cells that results in a lack of perfusion

Skeletal Muscle Cell – A cell found in the skeletal muscle

Smooth Muscle Cell – A cell found in muscle tissue that performs functions not under direct voluntary control

Stent – A tube inserted into a blood vessel to prevent or counteract a localized blood flow constriction by holding the vessel open

Tachycardia – A condition in which the resting heart rate exceeds the normal range, leading to insufficient blood supply

Thrombosis – The formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system

Ulcer – A discontinuity in the skin; most prevalent types are diabetic ulcers (localized slowing or stopping of blood flow), pressure ulcers (lesions to the skin caused by unrelieved pressure; "bedsore"), and venous ulcers (occurs due to improperly functioning valves in veins, usually in the legs)

Vascular – Relating to the blood vessels of the body

Vasculogenesis – The process of new blood vessel formation where no pre-existing vessels occur; compare to "angiogenesis"

Vasodilator – A type of drug that widens blood vessels by relaxing the smooth muscle cells within the vessel walls

Ventricular Fibrillation – A condition in which the contraction of cardiac muscle in the ventricles of the heart is uncoordinated, resulting in trembling rather than effective contraction

Appendix B: Client Feedback

November 6, 2008

Dermal Fibroblasts

- HUVECS can be cultured in defined media,
    - Supplements
- Angiogenesis: Dermal fibroblasts cultured with HUVECs
    - Big correlation between increase in profusion and increase in function

hMSCs

- Common media
- Previous co-culture studies with many different cell types
- Lots done with hMSCs
- What is the enhanced benefit of doing this – why go through with co-culturing?

Smooth muscle cells

- All kinds of media
- Mature fibroblasts: what is the benefit of that co-culture?
    - What are we measuring, who cares
- Markers are specific – hard to distinguish between smooth muscle cells and fibroblasts in a co-culture system

Keratinocytes

- Co-culture with fibroblasts, lots of reciprocal expression mechanisms
- Differentiates fibroblasts to myofibroblasts with keratinocytes
- Increased tension generated by cells in differentiation

Skeletal myoblasts

- Desmin – all muscle cells, way to show difference between muscle cells and fibroblasts
- When co-cultured with cardiomyocytes → Cx43 (measurable outcome)

Endothelial cells

- Previous co-culture studies with many different types of cells
- Vasculogenesis
- Induce MSCs to differentiate into SMCs

3T3-J2s

- Primary mouse embryonic fibroblast
- Secrete growth factors

Cardiac myocytes

- Hypertrophic – cells get really big, or they make more cells, or they're upsetting matrix
    - Hypertrophy – heart gets enlarged

iPSC

- Cultured on a feeder layer
- Similar to embryonic stem cells

Embryonic stem cells

- Grown on mouse fibroblast feeder layer

<u>Criteria to think about when picking particular cells</u>

- Culture time
- Cost
- Availability
- Measurable outcome
    - Matrix: cell-type one, cell-type two, quantitative outcomes
- Purposeful (will do something in the heart)
    - All these things help the heart
    - Rank the ways they help, what does the most/best
- Rationale for using the combinations
- Growth factors will lose productivity, but cells will keep making useful/useable products
- In vivo, there's never *just* one cell type

November 13, 2008

Cell types

- Assume cost is a constraint (also objective)
    - Which best meets criteria?
- Endothelial cells and dermal fibroblasts meet that criteria
    - Check different proliferation rates in 2D versus 3D, collagen vs. synthetic
        - Contract lattice (directionality)
        - Can you control it?
    - (HUVECs)
    - Also ease of co-culture, etc.
        - Fibroblasts grow in just about everything
        - Grow fast, easy
        - Have GFP modified dermal fibroblasts
            - Will make images, assays easier
        - Acetylated LDL
    - Can look at a lot of stuff to compare (assays)
    - Neovascularization (good!)

Quantitatively assess project objectives/constraints (refer to Cell Adhesion MQP)

- 5 point scale (ex.)
    - Cost effective: 0 = scale (+$1000), 1 = ($900-1000), etc
- Time efficient
- User friendly
- (second tier objectives)
- If satisfies all 2nd tier objectives, then it gets highest score, all but one gets 2nd highest score, etc
- Create scoring matrix for cell types

November 20, 2008

Cell combinations:

- Redo filter system?
    - Maybe weight constraints for chart
    - Did we turn all objectives into constraints?
    - Work on design rubric

Design alternatives:

- Used the same constraints, categorized broadly and eliminated based on constraints, condensed
- Best of Class chart
- For report:
    - Pros and cons
    - Must be justified objectively
        - Steer away from "intuition driven" decisions

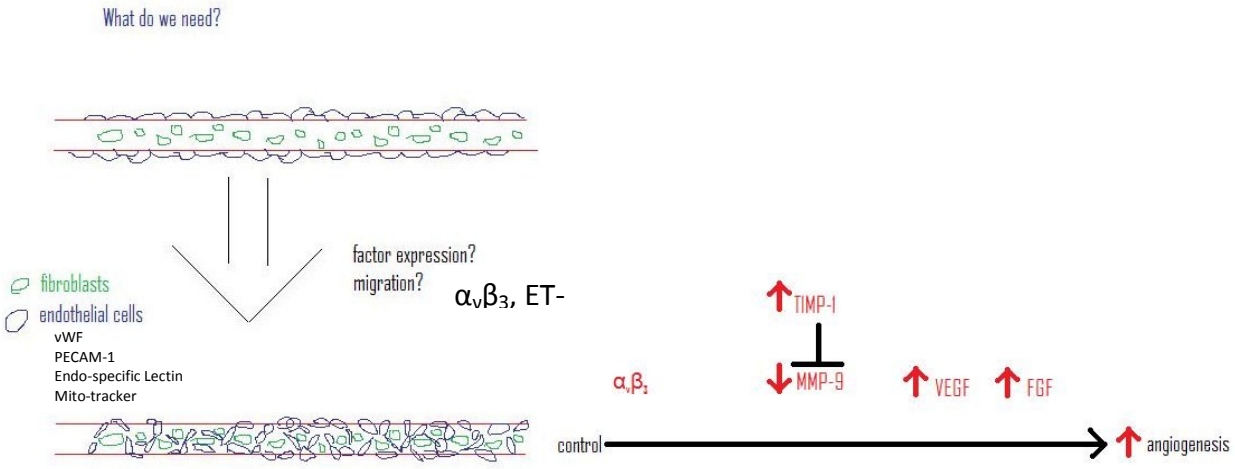
Report documentation

- Functions/means
- Design-driven changeable system
- Bench-top model
    - User requirement
        - Stable culture system
            - Culture cells in and/or on threads and make numerous measurements
    - Important benchmarks:
        - One cell type inside the thread
            - Shearing?
            - Embedding cells inside threads (similar to spheres?)
            - More important than co-culture!
                - Can co-extrude both cell types
                - Extrude cells in 2 threads
                    - Bundling/twisting
        - One cell type outside the thread
- Characterization?
    - Angiogenesis
    - Readout will depend on design configuration
    - Have to consider desired endpoint (therapeutic)

December 2, 2008

Input:	Output:	What to look for:	How?
$Cell_f$-$cell_e$ interactions	• Angiogenesis (and inhibition)	• Vessel formation: ○ Inhibition: MMP-9, $\alpha_v\beta_3$ ○ Enhancement: VEGF, TIMP-1, bFGF	• Microscopy image analysis • Cell-specific markers • $\alpha_v\beta_3$ expression
	• Cell-cell contact		• FGF assay from media
	• Transdifferentiation	• Fibroblasts → myofibroblasts	• Microscopy ○ ED-A fibronectin expression

- Benchmarks:
  - Fibroblasts inside
  - Endothelial cells outside
  - Combined
    - Enhanced angiogenesis – VEGF, TIMP-1, bFGF
    - Impaired angiogenesis - MMP-9, $\alpha_v\beta_3$ Ab,
- Validate benefits of co-culture
  - Look at endothelial cell migration without fibroblasts?
  - Future work



December 11, 2008

- Configuration
    - Cell migration/diffusion model
        - ECs migrate extensively
        - ECs embed themselves
    - Bruce Albert's *Molecular Biology of the Cell*
    - VEGF is good
    - Soluble factors at work (combined model – Liu *et al.*)
        - Fibroblast conditioned media
    - TIMP-1 – increases angiogenesis via inhibition of MMP-9
- Benchmarks
    - Fibroblasts inside
    - ECs outside
    - Then both
        - Proof of concept
- Need to distinguish between the cells – ideas?
    - vWF (ECs) - red
    - α-SMA (fibroblasts) – green
    - DiI-ac-LDL – orange
    - Terminal? Any way to distinguish by non-terminal means?
    - Random sampling
    - Autofluorescence of the threads, want to check what they are to see what will work best
    - MitoTracker allows for lots less time
        - How they interact with each other
        - Day by day, how long it takes them to reach the center
- Angiogenesis vs. vasculogenesis
- What should we look for:
    - MitoTracker
        - Both on, both growing
    - Lectin
        - Can be injected into live animals
        - Use it to tag cells
        - Can add before seeding threads
        - Easier to get this stuff in while in tissue culture than on threads
- Getting cells on – alternatives:
    - Meaghan's method
    - Migration
- Bundle of threads, partially seeded partially not
    - Bundle of 4, 2 with endos, 2 with fibroblasts

January 21, 2009

- Written document – clients
  - What we think: didn't really keep up with the background – part of it was lack of feedback
    - Critical decisions made at the end of the term, so writing was caught at weird time
  - Expectations for document?
    - Should fully represent efforts
    - Table of figures/tables
    - Project approach (theoretically)
    - Introduction
    - Bibliography
- C-term Plan (us)
  - Finished extrusion with fibroblasts
    - Some confusion with images
      - Noise, thread interference
  - Optimized extrusion protocol
    - Done, but needs to be verified
  - Thread integrity, properties
    - Cold, neutral extrusion (mix all together first then extrude)
    - Double-check variables – temp, etc.
    - Collagen to total solution = 1:1
  - Extrudes better into FFB
    - 135mM NaCl, 30mM Tris, 5mM $NaPO_4$
    - Collagen gels better than straight into media
      - Waviness (accordion-ness)
      - Try to figure out how to get the kinks/coils out
    - Can cells remain viable in FFB?
      - Experiment from B-term said that cells can stay in such solutions for short times without dying
      - Checking cytotoxic effects
        - Experiments
        - Composition
      - Find balance of cell activity and polymerization
      - FFB to DMEM/FFB to 100% DMEM
  - Culture time
    - 7 days? Too long
    - 48-72 hours on/in threads instead
    - Create container to extrude threads into/seed ECs outside
      - PDMS gel with troughs
      - Autoclave?
  - Monitoring cell location
    - MitoTracker and Live/Dead (CytoTracker?)
      - Color – green MitoTracker, green Live/Dead
      - Need them to be alive, and need to know where they are

- Need them to be loaded with dye to track over time
- What else can we look for?
    - How is it going to change?
        - Expect them to spread (good indicator)
        - Contracting collagen gel
- Go to website for providers or call company
    - See if proteins express in dead cells
    - Methanol experiment to see if proteins express when cells are dead – methanol will fix cells
- Metabolic dyes only express with metabolic processes
    - Short term (5 days?)
    - Can cells be re-dosed? At least with CytoTracker (2-3 days otherwise)
        - Also experiment with this?
- Fluorescent reporter inside cell?
    - Plasmid?
    - Transducing fibroblasts (1-2 weeks)
    - Must have the right reporter, or they're just pretty cells
    - If cells are dead, do they still express?
- Culture in parallel
    - Some to be Live/Dead, some for long-term monitoring
- Co-culture assays
    - Different colors, etc.
    - Different kinds of dyes

- Inside
    - Prelim tests (controls)
- Outside
    - Prelim tests (controls)
- Combined

- Effects of co-culture
- Project objectives
    - Would rather have things like how to move it, culture, etc.
    - Instead of long-term culture
    - Focus on all objectives for 2-3 days instead of some for 7

January 23, 2009

- Methods
    - Hard because it's a process/series of procedures, not really a device
- In, out, and together
    - In
        - Modify extrusion process for our application and optimize
            - Cold-mix
                - Came about from trying other methods first
                - Show process, data, etc.
            - What we extrude into
            - Rate
        - Cell culture
            - Time
        - Threads
            - Integrity
            - Time for culturing
    - Out
        - How to seed cells
        - Culture device
- Experiment: Testing of HDFs
    - Ethanol
        - Kill cells, see if MitoTracker still expressed
            - Just because we see MitoTracker, doesn't mean cells are alive
            - Also stain with Dead component of Live/Dead
    - PBS
        - Control
            - To check MitoTracker and Dead
    - FFB
        - Checking cytotoxicity
            - Important for optimization of thread extrusion process

February 11, 2009

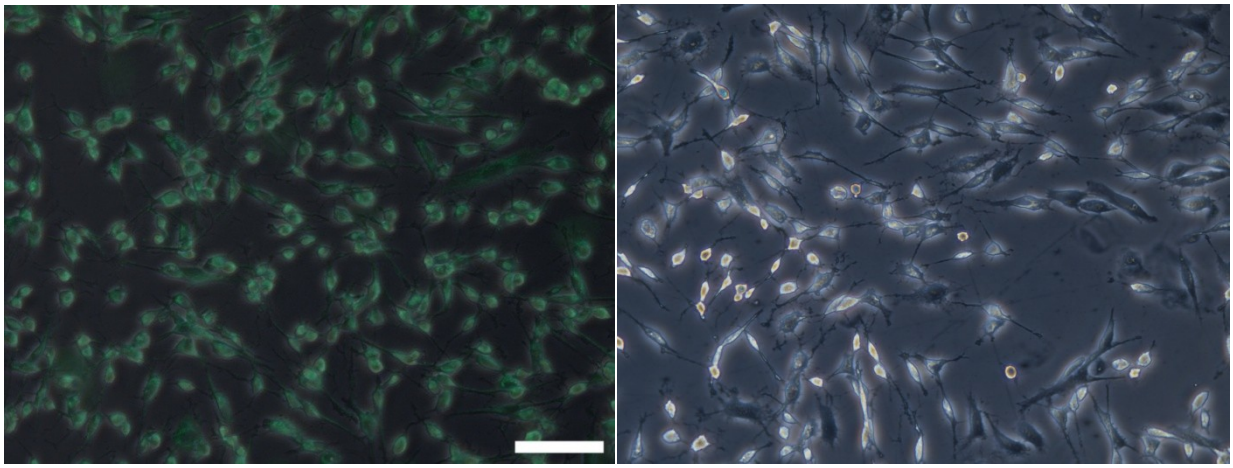
Future work:

- Measurable outcome: tubule function
    - Everybody does it, so there's lots of ways to do it, stuff to look at, etc.
    - Replicate something else to benchmark, also make sure we know that what we're doing works
    - Separate fibroblasts from endos
    - On a gel or something
    - Collagen gel out of same material we're extruding, let it set then add endos on top
        - With and without fibroblasts
        - Good control image
    - Methods:
        - # of pixels occupied by tubules
        - Sprout length
        - **Relative area and combined length of ECs involved in tubule formation**
            - How much better than the control
            - Lots of detail in paper
        - Length of tubule lines – on planar surface
    - How to adapt to thread?
    - How do you know the cells aren't growing down? How do you know the spreading is planar?
        - Good question, appreciate the issue, next group should use con-focal scope
    - **Dose response of fibroblasts**
        - Hypothesize that fibroblasts are helping endos, if we can show it = awesome
        - Actual exact perfect numbers aren't really necessary at this point (optimization, etc)
        - Try with lots of different fibroblast concentrations, and kind of assume ECs are the same every time – if experiments don't come out similarly, *then* go back and tweak the cell-seeding stuff
    - Endos do this, and then *all* the cells do this other thing

Appendix C: Protocols

Pre-loading of HDFs with MitoTracker

1. Rinse cell culture 3X with sterile PBS
2. Dilute MitoTracker with DMEM media to 0.05% (V:V)
3. Deliver the MitoTracker solution to the dish and incubate in 37C for 2 hr
4. Remove MitoTracker solution and rinse cell culture 2X with sterile PBS
5. Resume culture in DMEM (10%FBS)



Phase contrast/fluorescence overlay image of MitoTracker Green-loaded human dermal fibroblasts (left). Unloaded fibroblasts showed no autofluorescence in green channel (right). Scale bar = 100 μm.

Preliminary HDF-Collagen Co-Extrusion

Materials:

- 10 mg/mL RTT collagen (0.4 mL)
- 5X DMEM

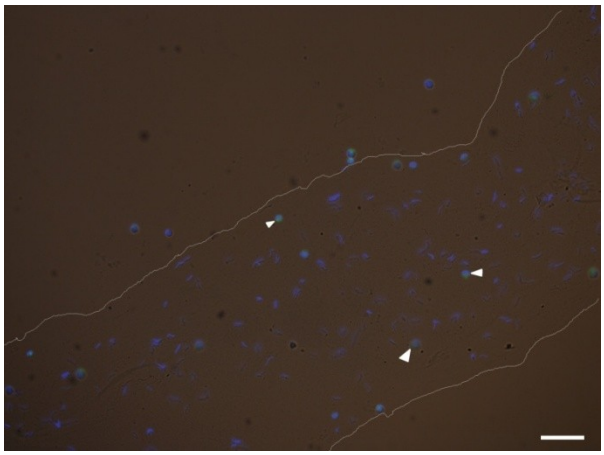
  To make:

DMEM Powder	13.48 g
H_2O	200 mL
$NaHCO_3$	3.7 g

- 1X DMEM (10% FBS)
- Dermal Fibroblast suspension at 180,000 cells/mL
- Fiber Formation Buffer
  - 135 mM NaCl
  - 30 mM TrizmaBase
  - 5 mM $NaPO_4$ dibasic
- Sterile 1 mL syringes
- Sterile 0.83 mm PE tubing
- Syringe pump

Methods:

1. In a 4°C cold room, place 0.4 mL of 10 mg/mL RTT collagen in 1mL syringe
2. Aliquot 0.1 mL of 5X DMEM
3. Mix 0.1mL 5X DMEM and 0.3mL of cell suspension in second 1mL syringe
4. Co-extrude at 0.25mL/min into 37°C 1X DMEM
5. Incubate for 15 minutes, PBS rinse for 15 minutes
6. Sacrifice for analysis



Bright field/fluorescence overlay image of a thread containing MitoTracker-loaded HDFs (green) and stained with Hoechst (nuclei; blue); HDF presence was determined by the co-localization of MitoTracker-loaded cells with a nucleus. Smaller blue particles not associated with a MitoTracker-positive HDF were identified as the result of non-specific binding of Hoechst stain to impurities in the collagen. HDFs are indicated by white arrow heads and thread boundaries are highlighted by white lines. Scale bar = 100 μm

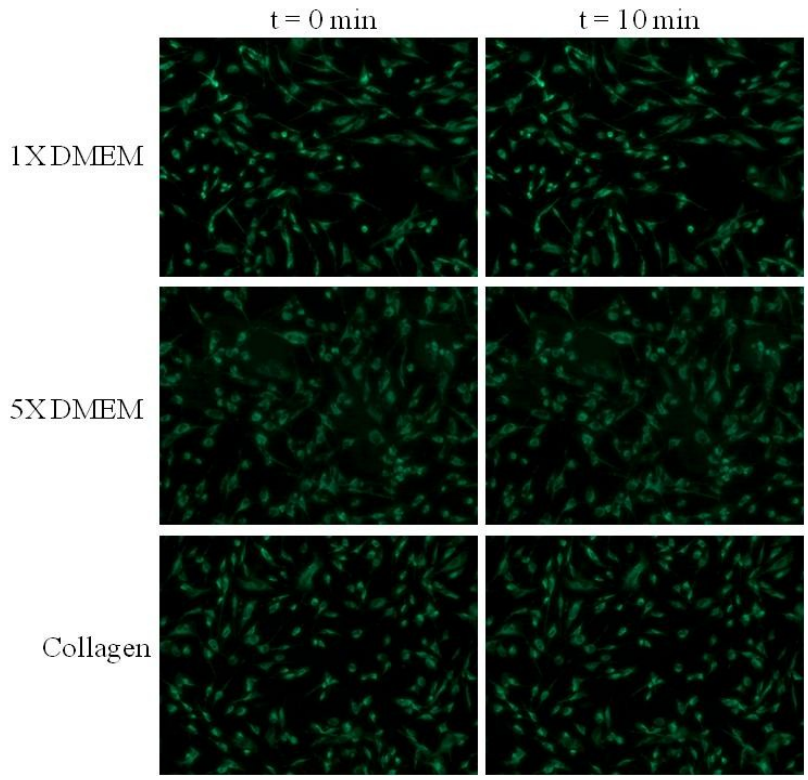
Co-Extrusion Components Cytotoxicity Study

Materials:

- Dermal Fibroblast suspension at 180,000 cells/mL (Pre-loaded with MitoTracker)
- 10 mg/mL type I collagen
- 5X DMEM
- 1X DMEM
- 6-well plate

Procedure:

1. Put 1mL of cell suspension into each well
2. Allow cells to adhere to plate surface for 4 hours
3. Remove media from well and replace with 3mL one of the co-extrusion components (collagen, 5X DMEM, or 1X DMEM)
4. Take observation using fluorescence microscopy after 10 minutes and compare morphology to initial morphology.



Fluorescence images of MitoTracker-positive human dermal fibroblasts before (left column) and after (right column) 10 minutes of exposure to the co-extrusion components. No changes in morphology was observed.

Acellular Co-Extrusion into FFB

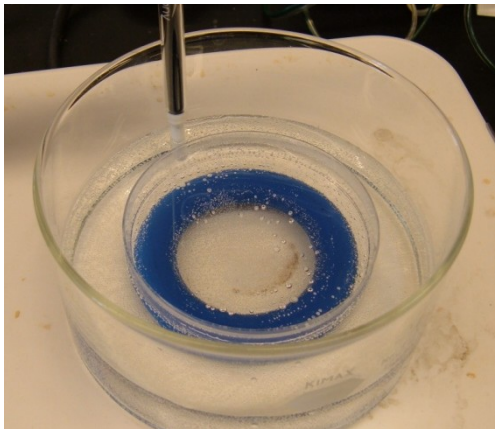
Objective:
To develop extrusion protocol that produces stable threads with long-term integrity. Extruding into FFB will provide time for the collagen thread to gel before being transferred to DMEM for culture.

Measurable Outcomes:
- Visual inspection for thread integrity
- Ability of thread to be handled with forceps
- Ability of thread to support its own weight when hung

Materials:
- (2) 1mL syringes
- Fiber Formation Buffer (135mM NaCl, 30 mM Tris, 5mM $NaPO_4$)
- 0.86 mm (ID) PE tubing
- 20G needle, with tip removed
- Blending connector tip
- 37°C water bath
- Syringe pump

Proportions	*Actual*
1 part 5X DMEM (on ice)	0.05 mL
3 parts 1X DMEM (without cells)	0.15 mL
4 parts 10 mg/mL collagen (on ice)	0.2 mL

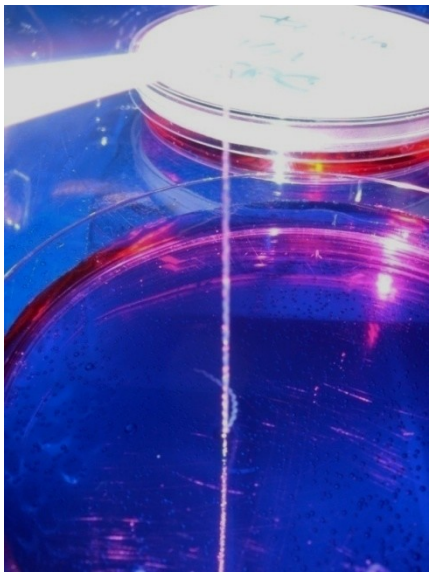
Methods:
1. Aliquot extrudants as shown above, keep at 4°C
2. Mix 5X DMEM and 1X DMEM and place in 1 mL syringe; place collagen in another 1 mL syringe
3. Heat FFB in Petri dish to 37°C in water bath
4. Assemble extrusion apparatus and extrude threads at 0.25mL/min



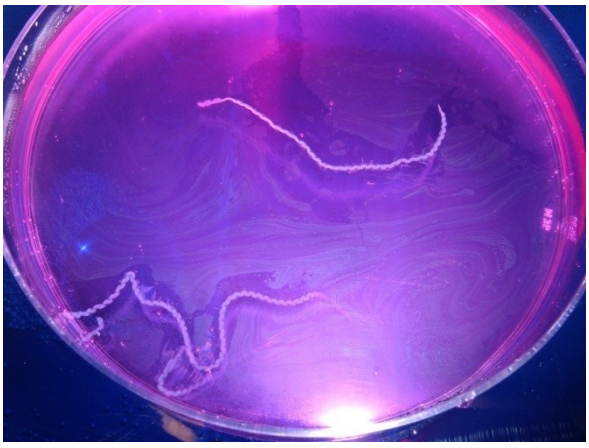
5. Remove samples to 37°C 1X DMEM at designated time points

Results:

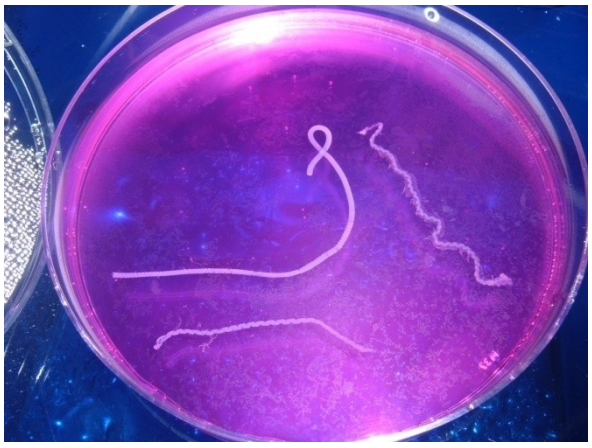
time →	0 min	30 min	60 min	6 hr
Batch1	*extruded*	*transfer to media* - stable threads	- stable threads - supports own weight	- visible loss of integrity - "frills" around thread - collagen film developing on surface of media - able to be handled - supports own weight
Batch2	*extruded*	---	*transfer to media* - stable threads	



t = 60 min, Batch1



t = 6 hr, Batch1 (30 min in FFB)



t = 6hr, Batch2 (60 min in FFB)

FFB Cytotoxicity Study

Materials:

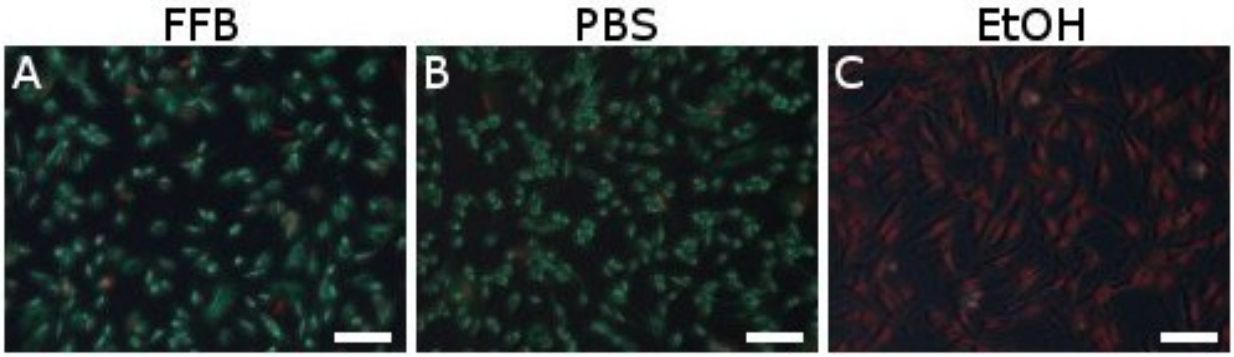
- Dermal Fibroblast suspension at 180,000 cells/mL (Pre-loaded with MitoTracker)
- FFB (3mL)
    - 135 mM NaCl
    - 30 mM TrizmaBase
    - 5 mM $NaPO_4$ dibasic
- PBS (3mL)
- EtOH (3mL)
- PBS (3mL)
- 6-well plate

Procedure:

1. Put 1mL of cell suspension into each well
2. Allow cells to adhere to plate surface for 4 hours
3. Deliver 3mL of reagent into individual wells:

FFB	PBS	EtOH
FFB	PBS	EtOH

4. Incubate at 37°C
5. Make observations under the microscope after 60 min
6. Stain cultures with 'Dead' component of Live/Dead stain (ethidium homodimer, EthD-1, red)



Phase contrast/fluorescence overlay of MitoTracker Green-positive HDFs on tissue culture plastic exposed to FFB, PBS, or EtOH for 60 minutes (*A*, *B*, *C*, respectively). Samples were stained with 'Dead' component of Live/Dead stain (ethidium homodimer, EthD-1, red) following incubation. Cells incubated in FFB and PBS exhibited no discernable difference in viability, whereas all cells exposed to EtOH were dead (with no MitoTracker signal present). Scale bar = 100 μm

Acellular and HDF-Collagen Cold-Mix Extrusion

Materials:

- 10 mg/mL RTT collagen (0.4 mL)
- 5X DMEM

  To make:

DMEM Powder	13.48 g
H_2O	200 mL
$NaHCO_3$	3.7 g

- 1X DMEM (10% FBS)
- Dermal Fibroblast suspension at 180,000 cells/mL
- Fiber Formation Buffer
  - 135 mM NaCl
  - 30 mM TrizmaBase
  - 5 mM $NaPO_4$ dibasic
- Sterile 1 mL syringes
- Sterile 22G blunt-tip needle
- Sterile 1.8 mL Eppendorf tube
- Sterile 0.83 mm PE tubing (sterilized by rinsing 3X in 70% ethanol, then 3X in sterile PBS)
- Syringe pump

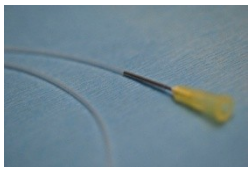
Procedure:

1. Place 0.4 mL of 10 mg/mL type I RTT collagen (on ice) in sterile 1.8 mL Eppendorf tube
2. Add 0.1 mL of 5X DMEM (on ice) to tube

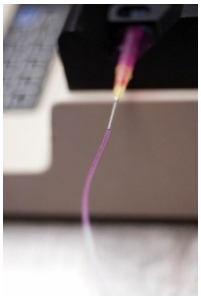
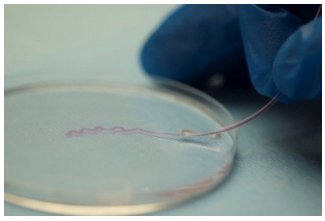
3. Add 0.3mL of DFs at 180,000 cells/mL in 1X DMEM (10%FBS) to tube
4. Mix solutions thoroughly by pipette, careful to minimize any bubbles

5. Leaving 0.2mL of air space (to accommodate length of PE tubing), draw entire solution into 1 mL syringe


6. Assemble extrusion system by inserting blunt-tip needle into PE tubing



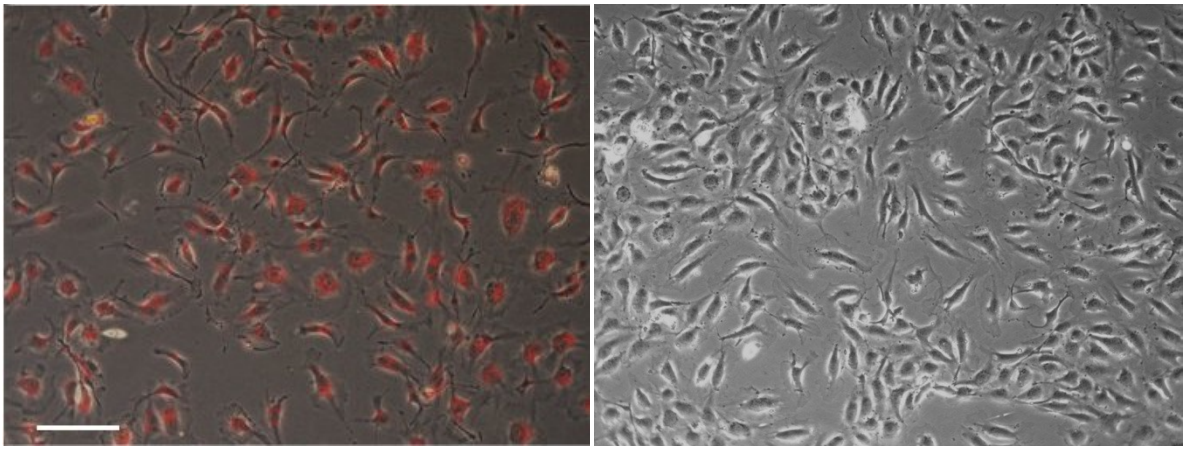
7. Attach syringe to extrusion system and, using a syringe pump, extrude at 0.25mL/min into 20mL of 37°C FFB in Petri dish

8. Incubate for 30 minutes at 37°C, then transfer threads to 1X DMEM (10% FBS) and continue to incubate

Pre-loading of HUVECs with DiI-ac-LDL

1. Rinse culture 3X with sterile PBS
2. Mix 150uL of DiI-Ac-LDL with 6mL of EGM media (for each culture plate)
3. Deliver DiI-Ac-LDL into culture
4. Incubate for 4 hours
5. Rinse 3X with PBS
6. Culture HUVECs in EGM culture media



Phase contrast/fluorescence overlaid image of human umbilical vein endothelial cells following DiI-ac-LD uptake (left panel). Unlabeled human umbilical vein endothelial cells exhibit no fluorescence in the orange channel (right panel). Scale bar = 100μm.

HUVEC Surface Seeding

Materials:

- HDF-populated microthreads
- Surface seeding device
- HUVECs (66,000 cells/mL)
- EGM media
- Sterile forceps

Procedure:

1. Using sterile forceps, remove 3 cm of HDF-collagen thread from incubation to surface seeding device

2. Pipette 100 µL of HUVEC cell suspension in each seeding channel to cover entire length of thread

3. Incubate surface seeding device at 37°C for 4 hours
4. Carefully rinse threads with sterile PBS
5. Using sterile forceps, transfer to EGM media for long-term culture

Thread Fixation and Fluorescent Staining Protocol

1. Place threads on glass slides and fix in 4% Paraformaldehyde in PBS for 10 minutes.
2. Rinse threads in PBS for 5 minutes. (3 times)
3. Permeabolize samples with 0.25% Triton-X in PBS for 10 minutes.
4. Rinse threads in PBS for 5 minutes. (2 times)
5. Incubate threads in phalloidin stain (Alexa Fluor 488 phalloidin, Invitrogen Corp., A12379) for 30 minutes. se 2.5 µL of stock solution in 200 µL of PBS for each sample. Keep samples out of direct light from this point on.
6. Rinse threads in PBS for 5 minutes. (3 times)
7. Incubate threads in Hoechst dye (1:6000 dilution with dH_2O) for 20 minutes.
8. Rinse threads in PBS for 5 minutes. (2 times)
9. Mount slides with aqueous mounting media or dehydrate samples and use Cytoseal (or another anhydrous mounting media).