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Abstract

A variety of injuries can lead to nerve damage, a condition which is largely untreatable and can result in loss of sensation, paralysis, or fatality. The ongoing challenge of developing therapies for nerve trauma is hindered by the poor healing capacity of these injuries; axonal extension through the wound area is often prevented by the formation of a mature scar, halting functional regeneration. Presently, there are few accurate and cost-effective methods for testing therapies aimed at healing mature neuronal scarring in the peripheral nervous system following injury. Animal models, the current testing standard, are expensive and provide minimally relevant preclinical data on the efficacy of new neuronal regeneration therapies. To provide more meaningful, less expensive neuronal regeneration therapy testing adaptable to a variety of scar types, a proof-of-concept *in-vitro* scar model was designed and prototyped to evaluate axonal extension and collagen alignment. With further development, the device could serve as a high-throughput system for evaluating axonal extension through various types of scar tissue.

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Chapter 1 Introduction

The purpose of this chapter is to introduce to the audience the connective scar tissue model for regenerative medicine applications. The topics discussed in this chapter are as follows: the problem the project is addressing and the general need supporting the problem, the overall goals of the project, and the project methodology.

1.1 Problem Statement

The control system of the human body is the central nervous system (CNS) and the system receiving the commands is the peripheral nervous system (PNS). The CNS is comprised of the brain and spinal cord, while the PNS consists of all other nerves in the body. The brain and spinal cord are comprised of neurons, nerves and glial cells (astrocytes and oligodendrocytes) that together enable communication between the CNS and PNS. The PNS is responsible for the motor, sensory and interneuron connections in the muscles, skin, organs, etc. Injury to any of these systems is often permanent. The healing process of the CNS and PNS primarily result in the production of fibrous connective tissue which permanently inhibits axonal extension by the nerves and neurons. In this process, white blood cells, proteins, and fluid quickly respond to the damaged tissue initiating the first step of the healing process, inflammation. The second phase is proliferation, which produces granulation tissue of fibroblasts and neovascularization. Fibroblasts are a critical cell type since they are in charge of the production of collagen, elastin, fibronectin, glycosaminoglycan and proteases. The last phase of the wound healing process is the maturation phase in which collagen remodeling occurs, a close cross-linking of type I collagen and removal of unnecessary vascularization. Finally, a fibrous scar made up of extracellular

matrix (ECM) forms. This ECM inhibits axonal regrowth and reconnection resulting a permanent disconnection and a lack of reinnervation. The scar’s function is to isolate, close, and protect the damaged area by promoting cell degeneration and creating a physical barrier to axonal extension, however, it also prevents the tissue from regenerating to its original, functional condition.

The most common CNS injuries are spinal cord injury (SCI) and traumatic brain injury (TBI). Approximately 1.7 million people in the US are affected from a TBI annually and 242,000 are hospitalized and suffer chronic consequences (CDC, 2006). TBI is caused by an external impact to the head, which can be classified from mild to severe. A severe TBI can be life threatening and a mild TBI is often a minor contusion. The consequences of this can vary from memory loss to altered body function or paralysis, all of these are caused by axonal disruption of glial scars. Paralysis is one of the most common results of these injuries due to the disruption of nerve connections during the injury. Spinal cord injuries alone cost approximately $40.5 billion annually and $158 billion in the United States in home care and nursing services combined (Christopher & Dana, 2013). Standard medical insurance is often unable to cover the complications linked to these conditions, posing a significant challenge to patients and their families. These injuries are primarily accidents occurring in the workplace during manual labor, reflecting the socioeconomic class of many patients; such patients are unable to readily afford insurance covering medical expenses associated with paralysis and other complications. (Reeve, 2012) PNS injuries are mostly musculoskeletal soft tissue and connective tissue injuries, which made up 77.4% of PNS injuries in the United States during 2011 (Pollak, 2013). Musculoskeletal soft tissue injuries (micro and macro traumas) and connective tissue injuries, the two main types

of injury to the PNS, both naturally lead to a wound healing process which alters the physiology of these tissues, often reducing the functionality of the remodeled tissue in most cases. In the healing process, the constitution of the ECM changes to promote rapid but imperfect repair. These changes in the ECM serve to create the scar tissue that obstructs the axon connection of the PNS neurons. Connective tissue injuries are the broadest because connective tissue covers most organs and muscles in the human body. More than 3.5 million children are injured playing sports yearly, with injuries including head traumas, sprains, strains and lacerations, 775,000 of whom must be hospitalized in the emergency room (Hopkins Medicine, 2009). Since 2011 the cost of children's ER diagnosed lacerations, sprains and other musculoskeletal injuries alone was over $935 million a year (Healey, 2013). Furthermore, in professional sports, more than 2,000 PNS injuries per 10,000 athletes are sustained every year, increased 21% from previous years (Fitzgerald, 2017). As mentioned previously, the causes of PNS injuries range from trauma cases of motor vehicle crashes, workplace accidents, and falls to homicides. In 2014 around 26.9 million people in the US were treated in the emergency room costing $671 million (CDC, 2016). The prevalence, cost, and impact of injuries to the PNS illustrates the importance of developing therapies to enable complete functional regeneration and innervation of damaged tissue.

Advances in medicine have yielded preventive practices for these injuries and some treatments which may improve patient outcomes to an extent. Mild TBIs do not require treatment, but moderate to severe cases usually require urgent care. Typical treatments to prevent further injury include providing adequate oxygen and blood supply and maintaining blood pressure at appropriate levels. Depending on the severity of the injury, patients may need rehabilitation to regain impaired functions (Mayo, 2014). Patients often cannot regain any loss of

function after treatment, such as memory loss or paralysis, often due to disconnection of the axon terminal in a specific location of the brain. The untreatable consequences of a TBI are the fibrous glial scars being modeled in this project. SCI is still an untreatable injury, however preventive actions such as immobilization avert further damage to the neck and cervical spine. SCI is treated with physiotherapy and medication until the inflammation dissipates and the scar on the spinal cord is formed, however, there is no further improvement after that point due to the axonal extension blockage. The PNS muscle injuries are usually treated with ice packs, compressive bands or slings and anti-inflammatory medication. For example if a runner strains a hamstring, the tear heals by creating an ECM scar that binds with the neighboring muscle sheath tissue. This muscle binding results in a loss of independent muscle movement, which may cause chronic pain and reduced muscle function. The probability that this area is re-injured secondary to the fibrous scar is fairly high (IAAF, 2012).

1.2 Project Goals

Our project aims to develop a system for creating a cell-based 3D model of connective scar tissue to facilitate the study and testing of regenerative medicine applications for scar tissue repair. This model will assist researchers, such as scientists and engineers, to improve their testing methods for scar tissue therapies in order to ultimately develop treatments to restore neuronal function after damage to the nervous system. The team aims to design a self-anchored, high-throughput 3D cellular device that accurately mimics the environment of scar tissue using biocompatible materials and necessary growth factors.

The team chose this project due to the fact that the issue at hand, regeneration of scar tissue, has no current *in-vitro* model representation which can be accurately used for research.

The lack of a solution is due to the complexity of the wound healing process, where the ECM produced by fibroblasts after injury prevents axonal extension and tissue regeneration, forming scar tissue in the peripheral nervous system. The team's areas of interest include extracellular matrix (ECM) composition and remodeling, the role of fibroblasts in scar formation, neuronal regeneration, tissue architecture (3D cell culture and ECM composition), the anchoring of connective tissue, and adhesion of cells in a hydrogel. These areas of interest are critical to assessing the efficacy of the device and ensuring that a meaningful model is designed.

Our hypothesis for our design of this product is that selective modification and control of critical variables in the connective scar tissue environment can enable the creation of an engineered *in-vitro* scar, promoting axon extension and resulting in accurate modeling of human *in-vivo* scar conditions.

1.3 Project Strategy

The project strategy is a high-level plan for how to proceed with a project in order to meet critical stakeholder needs and objectives in an efficient manner. In the context of this project, the project strategy will follow from an initial client statement, leading to revisions of the client statement, needs and objectives analysis, background and technology research, definition of functions and requirements, identification of constraints and metrics, development of design alternatives, and lastly, documentation of a final design. Establishing and adhering to this sequence ensures a methodological approach to the design process, while promoting successful and well-documented final deliverables.

First, the initial client statement was recorded and revised to arrive at a finalized client statement, "The purpose of this project is to develop a system to create an adaptable, realistic,

cell-based 3D model of connective scar tissue to enable the effective study and testing of regenerative medicine solutions in various scar tissues." From this information, the team ascertained client needs, wants, and project objectives; these were then clarified with background research into the problem context and similar state-of-the-art systems. With revision, the following need statement was established: "A way to address the lack of adequate connective scar tissue models in regenerative medicine research that enable the study of axon extension through the scar tissue." The primary goal of this project is to design a process for creating an engineered 3D cellular model of connective scar tissue which enables the study of axon extension through said tissue, with consideration for the adaptability of the model to varied scar types.

Following needs analysis and clarification of the client statement, several objectives and constraints became clear. Key objectives included safety, supporting axon extension, realistic representation of in-vivo scar conditions, robustness and reliability, adaptability, compatibility with laboratory and transportation systems, cost-effectiveness, ease-of-use and training, reproducibility, and disposability. Some critical constraints on this project include time, budget, patents, and regulatory approval. Throughout the design process, communication with stakeholders is critical to project success and meeting stakeholder needs; the process of developing the above criteria was iterative and incorporated stakeholder feedback.

With the needs, objectives, and constraints defined, the team broadened the design space to investigate technological alternatives and designs. Research into these areas is imperative for choosing the optimal design to meet the client's goals and generating deliverables that fill their real needs. However, before alternative design prototypes could be created, the team had to

establish which relevant variables were controllable in the context of the project, considering the constraints. Researching which variables were relevant and how they could be measured took place before prototyping because the final design must reflect these critical variables in order to emulate human connective scar tissue. Exploration of a wide variety of technologies and design alternatives is supported by extensive research, and it enables the team to choose the best possible final design.

In order to know when the final key objectives have been met and the project is complete, metrics must be established to track progress. Metrics follow from requirements rather than needs because needs are typically not measurable. The primary requirement for this project is for the 3D cellular scar model to promote axon extension; if neurons visibly, fully, and consistently extend across the model scar tissue, then this requirement has been met. Secondly, this model must be a realistic representation of human scar tissue. If this requirement is met, then the model and human scar tissues should have similar physical and biochemical measurable properties, in addition to promoting axon extension. Lastly, in order for this product to gain market acceptance, the cost of using and manufacturing it must be comparable to that of state-of-the-art models; assessing this metric involves maintaining a log of material, manufacturing, time, and usage expenses and finding similar data from other models for comparison. Metrics enable the team to quantitatively and qualitatively track progress throughout the design process, ensuring needs, objectives, and requirements are successfully met on time and on budget.

The following chapter summarizes the team's research into the societal and scientific context of the problem being addressed by this project, the current state-of-the-art, the shortcomings of current testing models, and the quantitative and qualitative definition of the scar

environment. Subsequent chapters will detail the team's design process, alternative designs, prototypes, and development of the final process and product from both biomedical and systems engineering perspectives. Finally, results, conclusions, an adaptable protocol, and manufacturing guidelines will be discussed, along with future design improvements.

Chapter 2 Literature Review

The purpose of this chapter is to provide the introductory information necessary to understand our project's objective in neuron regeneration. A thorough literature review was done on the anatomy and physiology of the cells used in our experiments, the importance of neuron regeneration and its current models, and finally neuron axon extension. This aims to inform the reader of any relevant information contextualizing the project's purpose.

2.1 Cell Anatomy and Physiology

2.1.1 Neuron Anatomy

The average brain is composed of over 100 billion neurons which have three basic functions: Receiving information, determining whether this information should be conveyed to the target cell, and communicating the appropriate information to the target cell. Neurons are able to communicate with each other through a well structured neuronal network. We can understand neuron communication by studying the synaptic connections and transmissions in said neuronal networks (Byrne, 2016). There are over 10,000 different specialized neurons, of which we will discuss three (Stufflebeam, 2008). These three types of neurons are classified into sensory neurons, motor neurons, and interneurons depending on their function. The cell bodies of some PNS neurons, for example sensory neurons, are located in clusters (ganglia) outside of the CNS and are responsible for transmitting information, such as touch, pain, and temperature, from both the inside and the outside of the body and bring that information to the inside of the CNS (Sadava, 2009). Motor neurons have long extensions known as axons with are located from the

CNS all the way to the muscles, organs, and glands which they then tell what to do. Lastly, interneurons connect one type of neuron, which can either be a sensory neuron or an interneuron, and transmit said information to either a motor neuron or an interneuron, thus conveying information in the CNS.

A neuron's nucleus is located in its soma (neuron cell body). Neuron proteins are synthesized in the soma, which is where dendrites and axons branch from. Dendrites have the task to receive and process signals that are classified as inhibitory (no generation of an electrical impulse) and excitatory (generation of an electrical impulse) (Byrne, 2016). Since a single neuron can have more than one dendrite and processes many action potentials, determining whether or not a neuron exhibits an inhibitory or excitatory response depends on the amount of signals it receives. If an incoming signal is inhibitory then the action potential is passed to the axon. Axons come from the axon hillock which comes from the cell body. For interneurons and motor neurons, this area is where the action potential commences. Unlike dendrites, axons are covered in myelin which works as a unique insulator and transmits nerve impulse rapidly. Connection from an axon to a target cell occurs at an axon's end, where it branches into axon terminals (Nicholls, 2009).

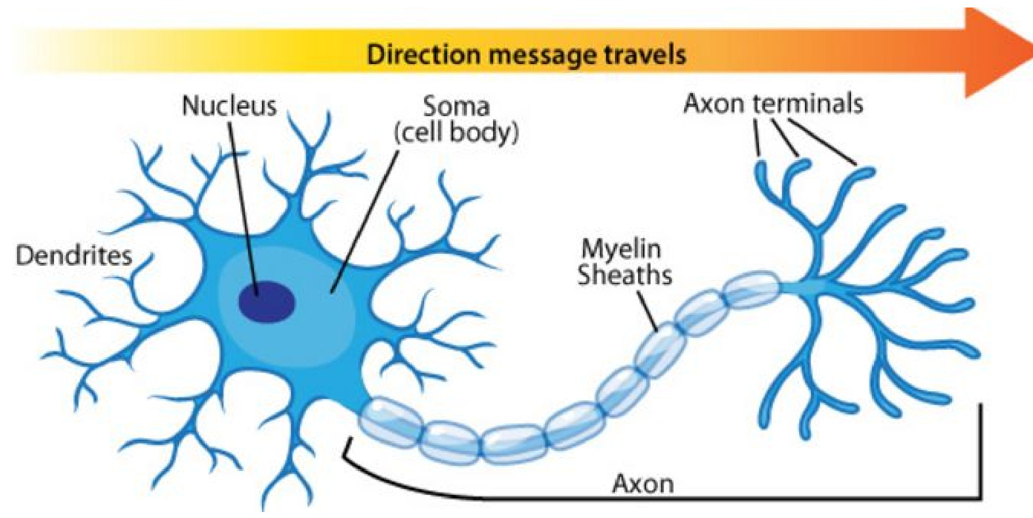


Fig. 1 Structure of a Typical Neuron

2.1.2 Neuron Signaling

Neuron signaling and the transmission of information from neuron to a target cell occurs through action potentials. The three stages of an action potential are as follows: a nerve action potential of a duration of 1 msec occurs, after that the action potential is classified as either inhibitory or excitatory, finally nerve cells assess the quantity of information with respect to the frequency of the action potentials. To understand how action potentials occur, we need to study the action potential difference present inside and outside the membrane potential using a microelectrode. Ions are distributed unevenly inside and outside of a nerve cell, and a cell's membrane is highly permeable to K^+ (Byrne, 2016). A microelectrode has a measurement of about -60 mV inside of a cell, this can vary from -80 mV to -40 mV depending on the nerve cell, and a resting potential of 0 mV on the outside. The diffusion of positive K^+ will leave negatively charged ions on the inner surface of the membrane. The eventual equilibrium of outwards K^+

will balance any electrical force occurring at an inwards direction. This balance in potential can be calculated using the Nernst Equilibrium Potential (Byrne, 2016).

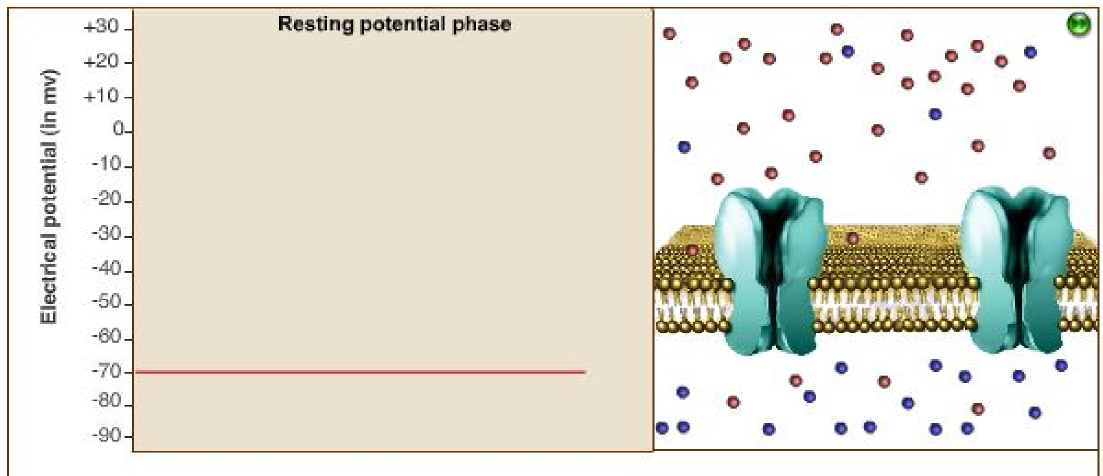


Fig. 2 Resting Potential Phase

2.2 Importance of Neuron Regeneration

Repair and replacement of damaged nerve cells is necessary after injury or disease have occurred in the nervous system in order for neuroregeneration to take place. This ability to regenerate nerve cells, however, is limited, posing a problem when treating patients. The ability to work with in vitro neuron cell culture has had an impact on the advancement of treatment related to neuroregeneration. Furthermore, it has created an extensive area in the development of in vitro models for testing and research (Steward, 2013). In vitro neuron cell culture is exceptionally demanding since mature neurons are unable to undergo cell division. Researchers have found a way around this by using an immortalized cell line which are fairly easier to work with than primary cell lines and can divide an unlimited amount of time. A disadvantage of using these derived cell lines, however, is that their physiology and neuronal phenotype differ from these mature neurons from

which they are derived of. For this reason, researchers tend to manipulate specific culture conditions to further mimic an *in-vivo* environment (Gordon, 2014).

Neuron cell lines have been used to study neuron behavior efficiently in cell culture, however, primary cell cultures are not tumor-derived, which make them a better model representation of *in-vivo* conditions and therefore a more advantageous testing method. The downside of using primary cells is the difficulty in culturing. While cell lines offer an unlimited amount of exponentially growing cells, primary cells are not immortal and their available numbers for research and testing are quite limited and take relatively longer to proliferate. Since animal tissue is composed of several types of cells *in-vivo*, it is important, when using primary cells, to separate the desired cell type from others and determining its purity. This can be done by immunocytochemistry (ICC) with cell lineage-specific markers.Another consideration when using primary cultures is obtaining ethical protocol approval from both the Institutional Animal Care and Use Committee for animal cells and from the Institutional Review Board for the use of human tissue. The separation of astrocytes and oligodendrocytes from your primary neuronal cell culture is also essential (Gordon, 2014). The last consideration when using primary cells is that they are more difficult to transfect than a cell line, although specific transfection protocols are to be used when introducing genetic material such as DNA or double stranded RNA.

2.2.1 Wound Healing

After tissue injury, repair is done by synthesizing of a fibrous extracellular matrix (ECM) which aims to replace any damaged tissue and ultimately re-model itself to mimic healthy tissue. The ECM is in charge of organizing the behavior of different cell types in order to repair the damaged tissue. The first steps of cell-ECM interactions are to quickly close the wound and minimize the risk of infection. Tissue repair occurs in steps which ultimately lead to scar tissue remodeling. The first step is platelet aggregation, this forms a provisional matrix by blood coagulation which results in a cross-linked network of fibrin and fibronectin that prevents blood loss. The excessive amount of platelet aggregation increases the growth factor and adhesive protein count which in turn stimulate the inflammatory response, promoting immediate cell migration into the wound site (Midwood, 2004). The next step is where neutrophils make soluble debris and monocytes differentiate into macrophage that release more growth factors and cytokines. Keratinocytes regenerate the epithelial barrier in superficial wounds while fibroblasts replace the temporary matrix with granulation tissue made of fibronectin and collagen. After revascularization occurs in the damaged area due to endothelial cell presence, fibroblasts differentiate into myofibroblasts which contract the ECM and attempt to contract and close the wound. Any present cells that are of no use undergo apoptosis, providing the wound its collagen-rich scar tissue (Midwood, 2004).

2.2.2 Scar Composition

After injury, the skin's composition and structure are affected by their directionality. Direction-dependent biomechanical behavior is a property of skin which is impacted by the alignment of its collagen-rich fibrous components and its amorphous gel-like matrix. Some factors that may affect the scarring response and therefore the biomechanical composition in wound healing are genetic influences and controlling mechanical forces to reduce scar development and maturation and aid wound healing. Healthy tissue and scar tissue both exhibit similar high-load stiffness, reduced resistance to failure, reduced low-load compliance, and material directionality. These properties and the reduced biomechanical ability encourages researchers to find clinical treatments for the restoration of the skin's viscoelastic behavior at the scar site. Clinical treatments such as scaffolds, grafts, and Tissue Engineered (TE) skin substitutes already exist and provide us with information of the directionally-dependent biomechanics of the skin.

Glial scar formation occurs after injury to the CNS in a process involving astrogliosis. Glial scarring is the body's natural mechanism to protect and begin healing the nervous system. Neuro-developemental inhibitory molecules secreted by the cells in the scar have been found to play a role in this mechanism as well as in neurodegenerative diseases. Glial scars are composed of the following: reactive astrocytes, microglia, endothelial cells and fibroblasts, and the basal lamina. Reactive astrocytes are the main component of glial scars. These are responsible for the increase synthesis of glial fibrillary acidic protein (GFAP), an essential protein which allows astrocytes to synthesize cytoskeletal structures. The outcome of this process is the formation of a dense plasma membrane which fills the voids of dead neuronal cells, this process is known as

astrogliosis. Astrogliosis modifies the ECM, as mentioned above, secreting laminin, fibronectin, tenascin C, and proteoglycans. The ultimate amount of secretion of these components determines the degree of inhibition of neuronal outgrowth and regeneration. Microglia is the second most abundant cell type in a glial scar. Microglia secrete cytokines, coagulation factors, neurotrophic factors, and bioactive lipids near the wound site. Another component of glial scars are the endothelial cells and fibroblasts, which secretes active molecules that stimulate/recruit fibroblasts and endothelial cells. These cells are in charge of stimulating angiogenesis and collagen secretion near the injury site. The final component of the glial scar is the basal membrane, a histopathological extracellular matrix which shields the astrocyte process. The basal membrane is made of glycoproteins and proteoglycans and consists of three layers, the basal lamina being the most important. Important components of the basal membrane are type IV collagen and laminin, which serves as a structure/support. The basal membrane and its components surround the blood vessels and nervous tissue near the injury site, which begins the wound healing process.

2.2.3 Materials for Cell Culture

This section aims to present the relevant materials that will be used during the project design and discuss why they are essential components to the team's project.

2.2.3.1 Molds

The molds used to create the cell-seeded collagen cylinders were comprised of nylon connectors (female luer 1/8" barb), 4mm inner diameter teflon tubing, and three sizes of silicone

tubing used as a mandrel for forming the collagen cylinders. This experimental approach was modified from K. Doshi's work on cell seeding onto similar molds for the creation of cell-derived tissue tubes.

2.2.3.2 Collagen

Culturing on a flat petri-dish will result in a two-dimensional layer of cells. When subculturing, it is always more precise to use a three-dimensional model in order to represent the surrounding cells and biological matter in which cells are in. Collagen gives these cells an extracellular matrix (ECM), or scaffold, which provides *in-vivo* like morphology and physiologically relevant environments that are more accurate for testing intercellular interactions. In a two-dimensional environment, cells can only interact chemically and mechanically along one axis, providing a poor representation of the complex cellular interactions occurring *in-vivo*. Creating a scaffold of collagen gel fibers mechanically supports the cells and induces more meaningful chemical signalling. In order to meet our project's needs, the team chose PureCol EZ Gel Type I Bovine Collagen as the desired collagen. PureCol EZ Gel is a ready-to-use collagen solution which can be easily manipulated at temperatures of about 37C.

2.2.3.3 NIH 3T3 Cell Line

This 3T3 cell line, first established by George Todaro and Howard Greene in the 1960s from tissue of an albino Swiss mouse (*Mus musculus*) embryo, is widely used in cell culture for laboratory research. These cells are widely used because of their inability to induce tumors when injected into animal models and their immortality, amongst other factors. Some specific characteristics of 3T3 cells are that they are inhibited bytemazepam and other benzodiazepines, the original cells are contact inhibited (unlike

the actual cell line), they are sensitive to leukemia virus as well as the sarcoma virus, and they are also receptive to transformation using polymavirus and Simian vacuolating virus 40 (SV40).

2.2.3.4 Neuroscreen-1

Neuroscreen-1 cells (NS-1) are a neuron-like subclonal line of PC12 cells, a cell line derived from rat pheochromocytoma cells which are widely used in different standard model systems for neurons. NS-1 are usually the prefered cell line due to their their strong and healthy growth properties with 50-80% less doubling time in relation to PC12 cells, their lack of aggregate formation in cell culture, their high affinity for nerve growth factor (NGF) which consequently produces neurite outgrowth in short periods of time (2-3 days), and their superb use in screening assays.

2.2.3.5 Ascorbic Acid

The use of ascorbic acid optimizes collagen secretion (in Balb 3T3 fibroblasts). When adding ascorbic acid to a 3T3 cell culture was proven to secrete collagen after a lag time of about 45 minutes, while using no ascorbic acid results in the accumulation of collagen in cells until secretion occurs after 1.5-2 hours (Peterkofsky, 2004).

2.2.3.6 Growth Factors

Basic fibroblast growth factor (bFGF/FGF-2) has been studied and used for fibroblasts to undergo cell division and stimulate cell proliferation. The mammalian Fibroblast Growth Factor (FGF) family is made up of 18 secreted proteins that interface with four signaling tyrosine kinase

FGF receptors. FGF has a major role in adult tissue, where it controls metabolic function and tissue repair and regeneration by the activation of signaling pathways. When there is FGF present but abnormal activity in these pathways *in-vivo*, defects in the organs and impairment in injury response can be found as well as metabolic problems (Ornitz, 2015).

The nerve growth factor (NGF) is a neurotrophic factor and neuropeptide which regulates a target neuron's growth, proliferation, and survival. NGF plays an important role in inducing axonal outgrowth in nearby nociceptive neurons, which is why we chose to use it in our NS-1 cell culture.

2.3 Background of Knowns and Unknowns (Current Models)

In recent times, the field of neuron regeneration has been growing thanks to developments in the use of substrates that allow the effective growing of otherwise dysfunctional neurons. By concentrating on the use of an effective neuron model in research and testing, scientists have found that investing their time in using the right substances for neuronal growth has paid off. A recently new technology in the neuroregeneration field is the use of carbon nanotubes (CNTs) which promote the desired neuronal growth. CNTs have been used to control neuronal activity, focusing on either a network of synapses or single cells (Fabbro, 2012). CNTs have multiple applications, ranging from scaffolds for nerve tissue engineering, neuronal surface interfaces in implants, and electrode coatings. This relatively new technology has many unknowns, which is why their experimental credibility is obtained by successful neuroregeneration. The reason why CNTs have been applied to this field is because they mimic in vivo axonal pathways that connect the brain and spinal cord, by replicating this pathway,

neurons now have an accessible route to reconnect and form new networks. CNTs have shown to affect neurite outgrowth and branching when exposed to biologically active charge-altering compounds. In a study of CNTs and regrowth of neurons, scanning electron microscopy was used to measure how much growth occurred within the neuronal membrane.

Another interesting use of this technology implies purified CNTs to promote the formation of nanotube-neuron hybrid networks that allow for neuron integration, synaptic transmissions, and network formation. Regrowth of neurons was then quantified using different methods that tracked the growth for about 3-4 days. To do this, the initial amount of tracks and branches were counted and the average elongation (per hour) for every branch carefully was measured with a scanning electron microscope (Chu, 2001).

Other technological approaches for solving neuroregeneration have emerged, one of them being a real time device useful for imaging neuronal activity. This lab's interest focuses on endolysosomal trafficking in neurons and how endosomes use different molecules acquired from non-polarized cells. They are researching neuronal-specific proteins and what roles they play in neuronal endosomes by studying their function and regulation in live time. Succeeding in this field using in-vivo models has proven to be difficult and time-consuming, which is why many researchers have been trying to acquire more information about neurons in-vitro. 3D cultures are a big part of this, since they offer a better understanding of the activity of neurons from a mechanical, chemical, and physiological standpoint. 3D cultures have adapted the use of scaffolds or other gel-like materials (agarose, hydrogels) to mimic in-vivo conditions and provide the axon with an assisted mechanical guidance to obtain results (Gingras, 2003). These 3D cultures can be manipulated to the user's satisfaction in order to meet their needs, as opposed

to using a 2D cell culture which, even though is widely used, does not provide the complex mechanical, chemical, or physiological conditions that a 3D environment can offer (Tibbitt, 2009). Of course 2D cell culture has its benefits, such being that the cells can be manipulated and imaged easily and that their proliferation and migration can be readily assessed. The difference in these 2D and 3D cell culturing techniques and the reason why 3D is much more difficult to carry out is the use of a hydrogel and the controls of environmental factors.

Many areas in neuroregeneration are still unknown, such as the specific brain-immune signaling that occurs after brain and spinal cord injury. This phenomena occurs after a brain injury, when the peripheral immune response is unable to process immunosuppression, which poses a threat to the whole immune system (Liesz, 2015). The risk of infection increases dramatically while the immune system is occupied with brain-immune signaling. The main focus of our project, axonal regeneration after damage to the peripheral nervous system is still a big unknown in the field as well. The signaling responsible for axonal regrowth after transverse dissection or damage to axons needs to be better studied and quantified. Researchers in the field speculate that this might be caused by loss of action potentials, membrane depolarization, and a change in the calcium ion signaling.

2.4 Background of Neuron Axon Extension

Axon and dendrite extension are really important in the transfer of information after injury to the nervous system. Neurons can polarize and form one single axon, multiple dendrites, and ultimately form functional synaptic contacts in relatively crude in vitro conditions. This testing for axon extension has become the dominant model for study of axon initiation and

growth and has helped researchers understand the roles of specific molecules in vitro (Dotti et. al. 1998). Axon initiation and growth in vitro are intrinsic properties of each neuron which occur when there is a lack of extracellular cues. This, unfortunately, is not the same as *in-vivo*, since these extracellular cues are relevant when studying axon extension (Barnes and Polleux 2009).

Glial scars are what prevent neuronal outgrowth and axon extension *in-vivo*. After injury to the CNS, axons sprout and aim to repair the damaged by extending across the injury site. However, the glial scar prevents this axon extension by creating physical and chemical barriers made of dense gap junctions. Another problem axons encounter is the secretion of different growth-inhibitory molecules by the astrocytes and more physical/chemical barriers generated from the basal membrane.

Axon initiation in vitro provides researchers with an experimental template to base their discoveries of neuronal polarity and the molecular identity of axons and dendrites on and build upon that foundation. There have been studies observing the transition of cultured hippocampal neurons through the early stages of immature neurites to axon and dendrite extension, dendritic spines, and functioning synapses (Dotti et. al. 1998).

Chapter 3 Project Strategy

This chapter aims to guide the reader through the design process for this project with detailed information about the objectives, constraints, functions, specifications, requirements, initial and final client statements, and the project management approach.

3.1 Initial Client Statement

The initial client statement was developed on the basis of a need for a more meaningful and refined model of a connective tissue scar which could be used to test therapies for encouraging neuron extension through human scar tissue. The purpose of this project is to develop a system to create a cellular model of connective scar tissue in either the peripheral or central nervous system to enable the effective study and testing of regenerative medicine solutions in these systems. Overall, this initial client statement provides broad insight into the purpose of this project and the medical research context of a proposed solution.

3.2 Design requirements (technical)

Following from the initial client statement and preliminary research, detailed lists of objectives, constraints, functions, specifications, and metrics to assess the efficacy of the design were established. These lists guide the process of meeting the technical requirements for the project and ensuring the final design meets meaningful stakeholder needs.

3.2.1 Objectives

The broad attributes of the final design are listed as follows, delineated as primary and secondary objectives:

3.2.1.1 Primary Objectives

Reproducibility: The final device should be repeatable and reproducible to be meaningful to research. It is critical that the protocol for creating this model can be executed readily by a

trained individual, and that the process consistently results in a standardized, usable model. Additionally, reproducibility is important because multiple simultaneous trials are desirable for meaningful experimentation and comparison of therapy effects on neuron progression through the scar model.

Functionality: The device is designed to be a testing apparatus for assessing the efficacy of therapies on a connective tissue scar which inherently prevents neuron progression. The scar portion of the model should prevent axon extension and it should not collapse (remain as a vertical cylinder). Neurons should be able to grow as expected in the inner ring of the model.

Reliability: The final device must be reliable and robust to be used in a pharmaceutical testing laboratory. The final iteration resulting from this project should produce meaningful, consistent results at least 85% of the time in standard use cases, and it should be evident, with training, when inconclusive data is produced as a result of failure or anomalies in that specific unit. The data the model yields should be consistent across identical trials, and error should not be a problem with a properly manufactured model.

Measurability: The device should yield meaningful data relating to the efficacy of a therapy in promoting axonal extension through the model scar. To accomplish this, standard equipment, and visualization technologies such as staining, fixation, and histology, should be usable for testing. Specifically, the device should be compatible with the following:

- Immunofluorescence staining: Hoechst 33342, Alexa Fluor 488 Phalloidin, DAPI
- Histological reagents: Picrosirius red and fast green and associated processing reagents
- Fixation: Paraformaldehyde

- Permeabilization Detergent: Triton-X100

Safety: When used as intended, the model should be safe for the user to work with. It should also meet environmental and personnel safety standards for disposal.

Table 1: Primary Objectives Pairwise Comparison Chart

Objectives	Reproducibility	Functionality	Reliability	Measurability	Safety	**Score:**	**Rank:**
Reproducibility	--	0	0	0.5	0	0.5	**5**
Functionality	1	--	1	1	0	3	**2**
Reliability	1	0	--	0	0	1	**4**
Measurability	0.5	0	1	--	0	1.5	**3**
Safety	1	1	1	1	--	4	**1**

3.2.1.2 Secondary Objectives

Versatility: The manufacturing protocol should be customizable and versatile to model various scar types, as needed. Creating a customizable protocol increases the impact of the design to multiple scar types, enabling the development of more specialized applications of the model for therapy research.

Cost-Effectiveness: The device's final cost should be minimized in order to be meaningful in a scientific environment where budget is a concern. Commercial viability cannot be attained if the device is very expensive, suggestions of how to further reduce the overall cost should be considered for future work. Additionally, the cost of time for personnel to create and use the model should be minimized.

Disposability: The device should be disposable to support ease-of-use and to reduce the risk of contamination or improper cleaning between trials. This objective places additional importance on reliability and reproducibility to yield meaningful and consistent data. The device must be in compliance with standard laboratory biohazard disposal protocols and techniques.

Biocompatibility: The device should be comprised of noncytotoxic materials which do not degrade, leech, or hinder cell growth and proliferation over the intended lifespan of the device in a normal use case. It should be biocompatible for both the neurons and the fibroblasts.

Usability: The device should be simple enough to create and use that it provides a preferable alternative to current models and testing approaches, resulting in a more competitive product. Ease-of-use also reduces the risk of human error in experimentation.

Table 2: Secondary Objectives Pairwise Comparison Chart

Objectives	Versatility	Cost-Effectiveness	Disposability	Biocompatibility	Usability	**Score:**	**Rank:**
Versatility	--	0	0.5	0	0.5	1	**4**
Cost-Effectiveness	1	--	1	0	0.5	2.5	**2**
Disposability	0.5	0	--	0	0.5	1	**4**
Biocompatibility	1	1	1	--	1	4	**1**
Usability	0.5	0.5	0.5	0	--	1.5	**3**

From the results Pairwise Comparison Charts for Primary and Secondary Objectives, the following list ranks the device objectives in order of priority:

Primary Objectives:

1. Functionality
2. Measurability
3. Reliability
4. Reproducibility

Secondary Objectives:

1. Biocompatibility
2. Cost-Effectiveness
3. Usability
4. Disposability and Versatility

3.2.2 Constraints

Constraints serve to limit feasible design alternatives and provide guidance on options for materials, components, and resources available for development of the final product. The process of identifying constraints led the team to further define the scope of the project and ultimately revise the initial client statement to result in feasible goals for this project. A list of constraints follows.

Time: This project spans from September 2016 to April 2017. The team is constrained by the final deadline, April 18, 2017, and Project Presentation Day, April 20, 2017.

Budget: The funds allotted to the team place clear limitations on the materials we have access to, the complexity of the final design, and how we allocate and prioritize features of the design. In total, the team has $750.00 in our budget, $250.00 per person. Use of the MQP Laboratory is a one-time expense which will cover basic equipment usage, basic materials, and use of the laboratory space.

Skills: While new skills will be acquired during the process of this project, the scope of the project and the complexity of the final design is limited by the team's current skillset and understanding of the subject area. Many ideal designs will not be feasible as a result of the team's experience as undergraduates.

Regulatory: Several FDA and ISO standards and regulations are pertinent to the design of this device, as detailed in the following section, Design Requirements (Standards). Without adherence to these legal constraints, the device will be irrelevant and unusable for testing, and could likely cause harm to personnel, equipment, or be cytotoxic to the cells in the model. These regulations ensure a standard of quality, consistency, and safety in new medical devices.

Dimensions: The dimensions of the device are limited by the non-vascularized nature of the engineered tissue being constructed. Nutrients, growth factors, and other chemicals are available to the cells in culture via diffusion, thus the thickness of the model scar and neuron cultures are limited. However, the size of the model must be appropriate for personnel to use for therapy testing and it must be measurable and appropriate for imaging.

Laboratory Resources: The resources available to the team in the MQP Laboratory and available for acquisition within the budget limit the feasible design choices. The lab is utilized by many MQP teams, and this will impact the availability of resources and equipment.

3.3 Design requirements (standards)

Adhering to standards associated with medical device design will ensure the fulfillment of safety requirements, consistency, manufacturability, repeatability, and compatibility of the final product. Relevant medical device design standards are listed below.

- ISO 11737-2:2009 - sterilization of medical devices
- ISO 10993 set of standards: biological evaluation of medical devices
    - Cytotoxicity in cell culture context
    - Biocompatibility in cell culture context
- ISO 13485:2003 - quality control of medical devices and *in vitro* testing
- ISO 14971 - procedure for risk analysis and management for medical devices, throughout product lifecycle
- ISO 15189:2012 - medical laboratory standards for quality and competence

The aforementioned standards are commonly referenced in the process of medical device development. Sterilization is critical to both safety and efficacy of this product; without a sterile testing environment, contaminants can impact the test results, spread to other cell cultures, experiments, or equipment in a shared laboratory, or harm the user. Sterilization standards are most readily met in disposable devices and models, because the product does not have to be sterilized in between uses; instead, the device is discarded and the risk of contamination is greatly reduced. A disposable device carries the added benefit of preventing unintended chemical, biological, or pharmaceutical interactions due to improper use or cleaning in between tests. Secondly, the ISO 10993 set of standards prescribes guidelines and approaches for

ensuring that a cellular device is bio-inert and does not have cytotoxic or non-biocompatible properties or materials. This is critical to the success and reliability of the model. For instance, if cytotoxic materials were incorporated into the model, then one or more cell types being used would be compromised or killed. Overall, this would severely impact the usefulness and accuracy of testing with this model.

Standards addressing quality control are important when considering long-term manufacturing processes and designing for scalability. Quality control involves the practice of engineering a standardized, repeatable process for replicating the model reliably in a manufacturing setting (process engineering). Since it is a goal that the final product will be brought to market, it is imperative to consider the capacity for the process to be standardized and scalable to meet demand throughout the product life cycle.

Poor risk analysis and risk management are primary reasons why late-stage products and projects fail. Risk management standards for medical devices help to ensure that usable, reliable products are brought to market which will benefit the medical community long-term. Our team intends to adhere to these standards in order to create a sustainable product which will fare well in the market and provide a genuine benefit to regenerative medicine research.

3.4 Revised Client Statement

Through research and feedback from the client, the team iteratively generated the following revised client statement:

“The purpose of this project is to develop a system to create an adaptable, realistic,

cell-based 3D model of human connective scar tissue to enable the effective study and testing of regenerative medicine solutions in the PNS."

Our initial client statement was refined, clarified, and made more specific after conducting research and narrowing down our feasible objectives and design alternatives, considering different factors and restraints. We decided to focus on a 3D cellular model due to its effectiveness and accuracy of representing *in-vivo* conditions of human connective scar tissue. We chose to change our model of interest from the central nervous system to a fibroblast-based connective scar tissue.

3.5 Financial Approach

The risk of completing a project over-budget can be mitigated by conducting a comprehensive analysis of the costs of materials, supplies, and other expenses. While additional, unanticipated expense may be incurred during the project for a multitude of reasons, it is imperative to estimate the base cost as accurately as possible. If the expected base cost exceeds or closely approaches the budget, the project may be risky to complete in the proposed way.

In the context of this project, many materials were required at a reduced cost or at no cost, as they were included in the team's use of the laboratory. The initial laboratory fee ($100) covered a number of basic supplies and consumables, and still other materials were given to us from other laboratories on campus. The following financial estimates are based on average prices for the materials from reputable companies, and are accurate as of the time of publication of this document. Our total budget for the project was $750.

Table 3. Material Costs

Material Name	*Cost of Material (in standard saleable quantity)*
Silicone mandrel tubing	$0.30 from a 50 ft reel ($76.00 per reel)
Nylon barb connectors (2 x 1/8" I.D.)	~$0.25 each
Teflon tubing (~1.5cm L x 4mm I.D.)	$1.37 per ft
Neuroscreen-1 cells	$0, cell line has been discontinued for purchase
NIH-3T3 Fibroblasts	$282.00 per 20uL
Neonatal Fibroblasts (ATCC® PCS-201-010™)	$427.00 per 1mL
Bovine Type I Collagen, PureColEZ	$320.00 per 35mL
DMEM	Free from university
Various cell culture disposables: pipette tips, centrifuge tubes, well plates, cell culture plates, hemacytometers, etc.	Accounted for in lab use fee
Ascorbic acid	~$3.93, commonly available
Neurobasal media with nerve growth factor	Free from university
Vacuum grease	$14.69, free from university
24-well cell culture treated plates	Free from university
DAPI stain	Free from university
Phalloidin 488	Free from university
Agar gel	$6.50 for 15 grams, free from university

3.6 Management Approach

The project management approach provides guidelines for completing various deliverables for the project. A Gantt Chart (Appendix B) was used to define ideal time

limitations for various activities over the course of the project, from formulating a client statement, to writing the paper, to planning a presentation, to the steps involved in the engineering design process. Several key milestones are as follows:

- Initial Client Statement by September 20
- Objectives, constraints by October 1
- Revised Client Statement by November 1
- Chapters 1, 3, and 4 complete by November 1, 2016
- Functions and specifications by November 18
- Functions-Means analysis by December 1
- Chapter 2 complete by February 1, 2017
- Chapters 5 and 7 by April 10
- Chapters 6 and 8 by April 18
- At least four alternative designs by February 1
- Evaluation of key design parameters and prototyping by February 20
- Final design selection by March 1
- Final design iterations and data March 1-April 10
- Presentation slides complete by April 13
- Presentation rehearsal from April 1-19
- Project Presentation Day: April 20
- Compile MQP paper by April 23
- Final edits by April 25
- Submit eCDR by April 27

Our team's approach to leadership over the course of this project is to allow all team members to lead the project for at least a term, to enable each of us to gain leadership, communication, and project management experience. The role of meeting secretary and recordkeeper was also delegated to alternating students each term.

Chapter 4 Design Process

The purpose of this chapter is to communicate the various design alternatives considered before the final design was selected. To provide a better understanding of why the final design

was chosen over the other designs, a comparison against each of the options was made to clear which one was the most suitable one for the client's needs.

4.1 Needs Analysis

Conducting a thorough needs analysis is a critical step in determining the strategic focus of the project, leading to the development of functions and specifications. Each of these factors shape the direction of the project and which criteria are given priority in the design process, considering constraints. There are a multitude of important attributes associated with each identified need; in addition to a clear description of each need, it must originate from a stakeholder or stakeholders so as to provide a rationale for why the need exists. Priority indicates how important meeting a specific need is to the overall success of the project. Compliance assessment is a form of metric to clearly indicate when the need has been met. Following discussion with the client and background research, we determined several critical needs relevant to this project, delineated in the Needs Table below.

Table 4. Needs Table

Title	Description	Traceability	Rank	Compliance Assessment
Deadline	Project should be completed by the end of April, 2017	WPI, client	1	Finished by deadline? yes/no
Safety	System developed should be safe to use as directed	Client, researchers, FDA/regulators	1	FDA standards, follows general safety guidelines
Operational	System should yield a testing model which accurately models human connective scar tissue	Client, researchers, patients, providers, animals used for testing	1	Test if product allows for study of axon extension through scar tissue

Training	Training needed to use system should be minimal	Researchers	2	Document training protocol; get feedback from researchers
Ease of use	System should yield a product less complicated to use than current state-of-the-art testing models	Researchers	2	Feedback from researchers
Reliability	System should produce a standardized, usable testing model as often as possible	Researchers, client	2	Testing, repetition
Compatibility	Use of the system should avoid the need for new or additional lab infrastructure	Researchers, facility/lab	1	Document equipment used, feedback from researchers
Scalability	Should be able to implement the system to manufacture the testing model to meet demand (repeatable/potential for automation)	Client, researchers	3	Make process repeatable; research manufacturing capability; consult experts
Maintenance	Product should be easy to maintain in a standard lab setting	Researchers, facility/lab, client	2	Feedback from researchers, document conditions used
Cost	Product should be cost-effective	Researchers, WPI, client	1	Assess average current model cost and compare

4.1.1 Design Functions

It is essential to determine the main functions that must be incorporated to ensure a successful final design. The functions listed below are in order of importance to the achievement of the client statement and completion of the design:

1. Obstruct axonal extension
2. Self-anchored
3. Prevent extraneous neuron migration
4. Enable observation and measurement of axon extension for therapy testing

Obstruct axonal extension: The design must meet the client's expectations of a fully functional device mainly in myofibers, but also other cell cultures, therefore the inhibition of the motor neuron and axonal extension are more important than anything else (the scar itself may inhibit axonal extension, but the application of known therapies should yield axonal extension results similar to those found in comparable scars *in-vivo*). The objective of this function is to demonstrate the axonal extension is obstructed by the scar matrix and that is a great model of a human scar tissue for applications of regenerative therapies that are currently being developed.

Self anchored: In order to emulate the scar environment, the 3D cellular model must be self-anchored. Tissue growth and proliferation cannot occur *in vitro* without proper self-anchoring. In the context of muscular scar tissue, self-anchoring occurs at certain locations in the tissue culture but not throughout. The scar tissue itself will be anchored to emulate healthy tissue on either side, but the scar itself will not exhibit axonal extension or normal function.

Prevent extraneous neuron migration: The model must prevent the migration of neurons and the extension of axons underneath or around the scar model. This is critical to reliable quantification of neuronal extension results; if the neurons migrate around the scar, they are not effectively being evaluated for axon extension through the scar. In order to meet this function, a form of seal must be established between the scar model and the substrate it is resting on.

Enable observation and measurement of axon extension for therapy testing: In order for this model to be useful for realistic drug and therapy testing, it is essential for it to be conducive to measuring axon extension in a laboratory, using current state-of-the-art imaging, staining, histology, and measurement technologies. Without this functionality, the model will not meet its core need of improving testing in regenerative medicine research.

4.1.2 Functions-Means Analysis

A functions-means analysis provides a basis for selecting methods, or means for implementing critical functions. The readable, tabular format is beneficial in clearly communicating what alternatives are being considered in fulfilling the critical functions, as stated.

Table 5. Functions-Means Analysis

Functions	***Means***		
Prevent axonal extension	Mechanical hostile environment	Ascorbic Acid to promote collagen synthesis	TGF-β to promote collagen synthesis
Self-Anchored	Promoting cell adhesion to the cell culture plate	PDMS mold and supports	Cylindrical, self-supporting model
Prevent extraneous neuron migration	Vacuum grease seal	Agar base support mold	Agarose base support mold
Enable testing of axonal extension with neurons	Tissue sheet with neuron on one side	Cylindrical model with neurons in center	Dual tissue sheets with neurons in between

Means Explanations:

Ascorbic Acid to promote collagen synthesis: The functionality of the device is contingent on

its ability to effectively prevent axon extension, to emulate *in-vivo* scar conditions which disrupt neuronal function. The collagen cylinders with the fibroblast seeded within will be treated with ascorbic acid. This is an essential cofactor in hydroxylation of prolines in collagen and helix formation and subsequently stimulates collagen synthesis, *in-vitro* (Zou, Y., et al., 2008). This collagen synthesis process with help develop a collagenous fibrous tissue mimicking a scar tissue. Other option were either a limitation due to our budget or it was very inefficient for the testing part of this project

Cylindrical, self-supporting model: The cylindrical shape of the scar tissue was to promote a more stable self-supporting device that can be manipulated and sectioned easily. The silicone mandrel in the cylindrical mold is what shapes the tube in the center of the cylinder. This mandrel is to aid the tissue form and hold the shape, but also for the cells to anchor to it. Other options for this function were unstable or not very reproducible.

Vacuum grease seal: The extraneous neuron migration was a concern for the axonal extension test, therefore a seal was prototyped and chosen for this concern. The best seal for this device and testing method was sterile vacuum grease applied carefully to the bottom of the cylinder circumference. This seal is to prevent any type of migration under or around the tube scar tissue to be certain that if there's axonal extension through the scar is because the device failed and not because of a leakage error. The other options were either prototyped and failed or due to similarities to the material the fails were ruled out as an option.

Cylindrical model with neurons in center: The main reason why the scar model is shaped as a hollow cylinder it is because of the testing purposes with neuron axonal extension. The cylindrical shape should assist in the self-support of the tube and also should allow for neurons to

be seeded in the center. The more collagen hydrogel or media were going to be in the center of the scar tissue (preferably collagen to support the cylinder shape). This is to test more efficiently the axonal extension from one side of the scar to the other. Emphasising that the neurons would have to extend outward only since there no other place to extend to, forcing them to try to go through the fibrotic tissue/device. Other option for the testing were going to take longer or might not give us an accurate result.

4.1.3 Design Specifications

The specifications for this device meet the client's need and expectation to ensure a successful scar model. After research and close examination of the feasible options the model's specifications are as follows:

1. Realistic presentation of a human connective tissue scar model
2. Scar size and shape (< 1mm in height, thickness of < 200 um to allow for diffusion of nutrients throughout tissue, diameter of <1.5 mm to self-support)
3. Collagen fiber alignment (collagen fibers must be aligned rather than randomly organized to mimic scar conditions)

Realistic presentation of a human connective tissue scar model: For the model to be successful there must be a scarred area on the connective tissue fibers. The cell culture and connective scar tissue matrix area must be three dimensional. The model will not recreate an entire granular fiber, only a scarred section of it. Fibroblasts are going to be used to produce the extracellular matrix. The model must represent the cross-linking collagen and elastin structure of a mature scar to inhibit nerve axon extension (1 to 20 µm) through the tissue fibers.

The scar size and shape: It is important that the device is adaptable in order for it to provide general value to the regenerative medicine community; the vast variety across scar types suggests the need for an adaptable manufacturing process designed to be broadly applicable to non-muscular scars. The type and size of the scar is important, in addition to the adaptability of the model to other scar types. Considering the scar cannot be vascularized the model has a thickness limit of 200µm. The shape of the model should follow the structure of a scar matrix and be useful for axonal extension testing procedure. The dimensions should not make the model excessively challenging to manipulate, while being small enough to allow for testing in a 96-well plate to support the ideal of high-throughput therapy testing.

Collagen fiber alignment: In order for the scar model to emulate the physical conditions of an *in-vivo* scar, collagen fibers within the scar must be aligned. This is a significant factor in preventing axonal extension and reducing the patient's quality of life. Random orientation of collagen fibers is representative of a healthy case rather than a scar environment.

4.2 Design Concept Prototyping, Feasibility Studies, Experimental Design

In order to determine what design alternatives are most appropriate for consideration in the final design, a variety of tests, models, and simulations must be conducted on these concepts and alternatives to ascertain which best meet the needs and functions required for the final design choice. This process can be broken down on a per-function basis; a variety of candidates can be developed and considered for how to meet each function.

This section details the cell culture techniques used, as well as hypotheses and the means by which these hypotheses were tested experimentally. In this way, key functionalities of the device were separated into discrete, testable units which could be validated and assessed

effectively.

4.2.1 Cell Culture Techniques and Protocols

NIH 3T3 fibroblasts were used to create a 3D cellular model of a connective tissue scar, and Neuroscreen-1 neurons (NS-1) were used to test the efficacy of the scar model in preventing axonal extension through the scar region, thereby determining if the model scar is representative of an *in-vivo* mature connective tissue scar in this way. NIH 3T3 cells are an immortalized fibroblast cell line, and Neuroscreen-1 cells are a neuron cell line.

The media formulation used for the NIH-3T3s was complete media consisting of 10% fetal bovine serum, 1% Glutamax, 1% penicillin/streptomycin, and 88% DMEM media. The NS-1 were cultured in 5% fetal bovine serum, 10% AHS, 1% Glutamax, 1% penicillin/streptomycin, and 83% RPMI. NGF was added to this media to create differentiation media for the NS-1 cells. NS-1 need collagen coated plates to facilitate the attachment to the plate, for this procedure we used 0.2 mg/ml Bovine Type I Collagen, 0.02M filtered acetic acid from 1M acetic acid, and sterile diH2O.

The routine used for subculturing both the NIH-3T3s and the NS-1 cells in 2D was as follows:

1. Inspect confluency of the cells under a light microscope.
2. Aspirate medium from plate.
3. Add 5ml DPBS(-) to rinse cells.
4. Aspirate DPBS(-).
5. Add 3ml 0.125% Trypsin-EDTA to the plate.
6. Incubate on a slide warmer for 5-10 minutes until cells are detached.
7. Verify cell detachment under light microscope.
8. Add 2ml complete medium to the plate to neutralize trypsin.
9. Disperse cells by repeated pipetting.
10. Transfer cell suspension to 15ml centrifuge tube.

11. Perform cell count if needed.
12. Centrifuge tube at 200G for 5 minutes.
13. Aspirate supernatant.
14. Resuspend cells in an appropriate amount of media of choice.
15. Plate desired number of cells, add media to a total of 10ml media and cells in plate.
16. Inspect plated cells under microscope.
17. Incubate plate.

The routine for the collagen coating for ten NS-1 plates was as follows:

1. Put the 3.125ml of 0.2 mg/ml of Bovine Type I Collagen in a 15ml conical tube on ice.
2. Add 1ml of 0.02M of filtered acetic acid into a 15ml conical tube, place the tube on ice.
3. Add 45.875ml of sterile diH2O in a 50ml conical tube and place the tube in ice.
4. Mix the acetic acid into the diH2O tube
5. Add the 3.215ml of collagen in the acetic acid and diH2O mix close and mix gently.
6. Add 5 ml of the collagen mixture into each 100 mm petri dish.
7. Let the plates sit for 1 hour.
8. Aspirate the collagen mixture from the plates.
9. Let the plates air dry for 20-40 minutes or until they are completely dry.
10. The plates can be stored in a 4 ◦ C refrigerator.

4.2.2 Experiments to Validate System Parameters

In order to test the device and testing design, experiments were run on the individual system parameters as follows.

4.2.2.1 Cylindrical Collagen Molds

The cylindrical collagen molds (Rolle, 2010?) were build with nylon connectors (female luer ⅛" barb), teflon tubing (3.96 mm) and silicone tubing (1.96mm, 1.19mm, and 0.94mm). The NIH3T3 fibroblasts were seeded onto two plates one with 100,000 and the other with 250,000cells each. Each plate was cultured in DMEM with 10% FBS and 1% Pen/Strep Stock (10,000 IU Penicillin and 10,000 µg/ml Streptomycin) at 37°C and 5% CO_2 in air in a

humidified atmosphere for 6 days. Cell density was around 3.2 and 8 million cells to in each of the 100mm petri dish to seed 2 million cells in each mold. The 1ml of 3.2mg/ml PureCol EZ Bovine Type I Collagen were separated into two 1.5ml microcentrifuge vials.

Hypothesis:

The smaller the silicone tubing and the higher the cell density the sturdier the collagen tubes are going to be. The small silicone tube will provide a thicker width and the large cell density will increase the contractile structure of the collagen tube.

Experimental Design:

The team used 8 autoclaved molds and manipulated them with autoclaved small forceps. Two of the molds had a 1.96mm OD silicone tube, three had a 1.19mm OD silicone tube and three had a 0.94mm OD silicone tube. Use 1ml of PureCol EZ Bovine Type I Collagen in a 3ml syringe and flicked the syringe gently to get rid of undesired bubbles. Then a 27 ½ G needle was used to inject 0.25ml of collagen in each mold.

Another 8 autoclaved molds were autoclaved with the 0.94mm OD silicone tube. The molds were arranged and placed 4 molds per 100 mm petri dish. The cells were counted and centrifuged to create a pellet of ~3.2 and another of ~8 million cells. The supernatant was aspirated and the pellets were left behind. Use 1ml collagen to resuspend the ~8 million cell pellet and place the 1 ml of collagen with resuspended cells in a 35 mm petri dish. The team collected the 1 ml of collagen with the 3T3 cells with a 3ml syringe and flicked the syringe gently to get rid of undesired bubbles. Then a 27 ½ G needle was used to inject 0.25ml of collagen with 2 million 3T3 cells in each mold. The same process was repeated for the ~3.2

million cell pellet two seed 0.25ml of collagen with 800,000 3T3 cells in each mold. The molds were incubated for 2.5 hours and then the collagen cylinders were removed from the molds and placed in culture media. Each mold was imaged and was observed for 5 days to see the cylindrical structure stability and strength. Collagen tubes that were treated were cultured for 21 days and were imaged with DAPI (blue) and Alexa Fluor-488 Phalloidin (green) to check cell density and cell attachment.

4.2.2.2 Axonal Extension Testing

The testing section has two different parts of prototyping to verify the axonal extension test.

4.2.2.2.1 Neuroscreen -1 Differentiation

Neuroscreen-1 (NS-1) were cultured in 5% fetal bovine serum, 10% AHS, 1% Glutamax, 1% penicillin/streptomycin, and 83% RPMI at 37°C and 5% CO2 in air in a humidified atmosphere. The neuronal growth factor was added to the culture media to a 0.1 µg /ml of concentration. Two 6-well plates were collagen coated following the standard protocol mentioned above.

Hypothesis:

If the neuroscreen-1 cells are culture in a differentiation media with NGF they will display axonal extension differentiation.

Experimental Design:

The team seeded NS-1 in 2 6-well plates to verify the axonal extension differentiation. In each of the 6-well plates, two wells were controls with 150,000 NS-1 cells cultured in cell culture media. Two of the wells had 150,000 NS-1 cells with 0.1 μg/ml of NGF and the last two wells had 300,000 NS-1 cells with 0.1 μg/ml of NGF. These cells were cultured for 72 hours and imaged with a light microscope at t= 0 hr and at t= 72 hr to observe any axonal extension differentiation

4.2.2.2.2 Leakage Testing

Two sizes of polyethylene tubing were selected for this prototyping test that were cut into 2cm tubes. The 3% agar was mixed with diH2O in a glass bottle, was autoclaved and kept at 60 ∘C in a water bath. The vacuum grease was autoclaved in a glass bottle and was left on the counter for 20-40 to cool down.

Hypothesis:

If the tubes are sealed with either vacuum grease or agar there won't be cell culture media leakage, diffusion or cell migration when the axonal extension test is being performed.

Experimental Design:

The team followed a standard 3% agar coating protocol for two 100mm petri dishes. The two different polyethylene were placed on the 100mm petri dishes either before adding the agar or after to create two conditions. Red food coloring dye was diluted with water and placed in the middle of the polyethylene sample tubing and observed for 24 hours. The plates were imaged with a regular camera to record the results. The vacuum grease was applied to the bottom circumference of the polyethylene sample tubing with a needle. The layers of the vacuum grease were either thin or thick to create two different conditions. Blue food coloring dye was diluted

with water and placed in the middle of the polyethylene sample tubing and observed for 72 hours. The plates were imaged with a regular camera to record the results.

4.3 Alternative Designs

Design 1:

3D cell culture of fibroblast on a 6-8 well plate anchored seeded in collagen gel. The cell culture is meant to be a straight (rectangle) sheet creating a scar matrix after being supplemented with the correct growth factors. The way this design is tested would be in 6 well plates, but instead vertically placed to test the axonal extension from one side to the other through the scar matrix.

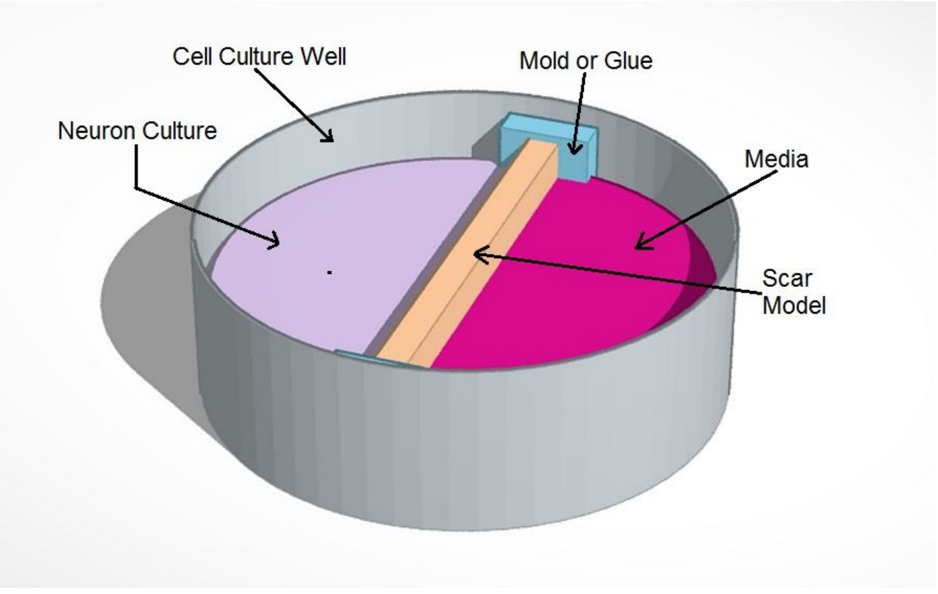


Fig. 3 Collagen sheet testing design.

Design 2:

2D cell culture of fibroblast on a 6-8 well plate to form a flat sheet of fibroblast matrix. The cells will received growth factors to secrete collagen and other supplements to promote cross-linking. Once the scar matrix is formed it will be anchored to collagen gel to start the process of rolling it over a silicone tube to create a cylinder. This cylinder will be sliced into appropriate sizes for the testing part of the project. For the testing part of the project neurons will be placed in the middle of the cylindrical scar to test the axonal extension to the outside of the scar.

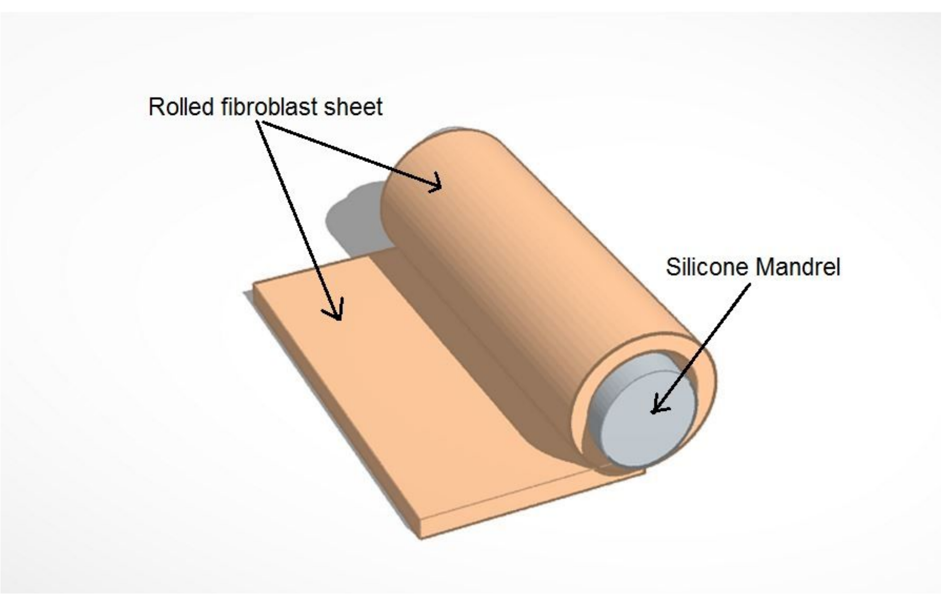


Fig. 4 Rolled fibroblast sheet on a mandrel.

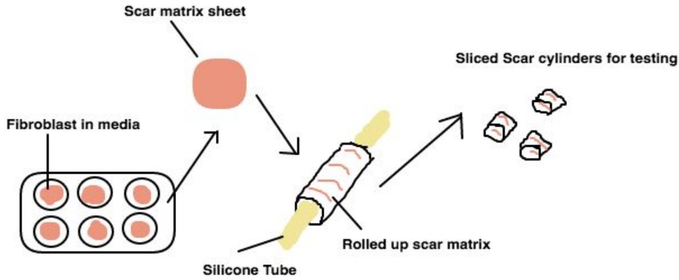


Fig. 5 Process of the culture and testing preparation of the design.

Design 3:

A PDMS cylindrical mold would be made to serve as a mold for the agarose model where the 3D culture would be paced. This mold would have the fibroblast and the collagen gel to serve as an anchor. The scar matrix will be formed with the appropriate supplements and growth factors. The cylindrical scar model will be sliced into smaller cylinders to test the axonal extension with the same procedure mentioned in design 2.

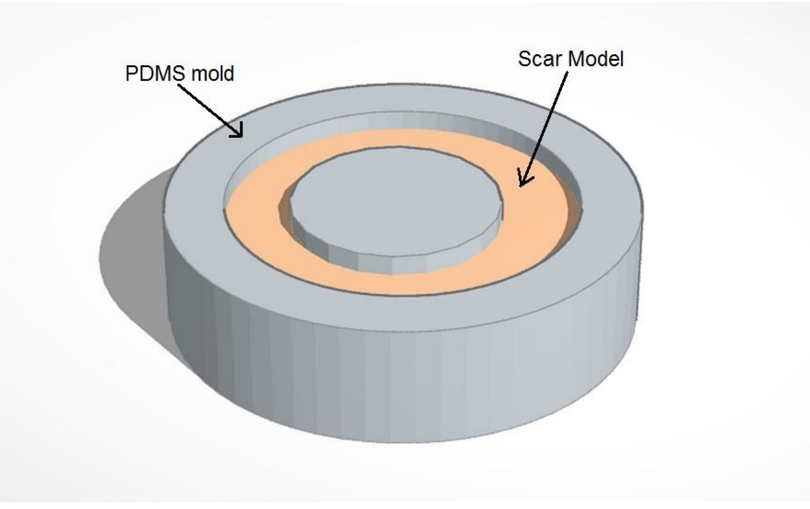


Fig. 6 PDMS cylindrical mold to shape the scar.

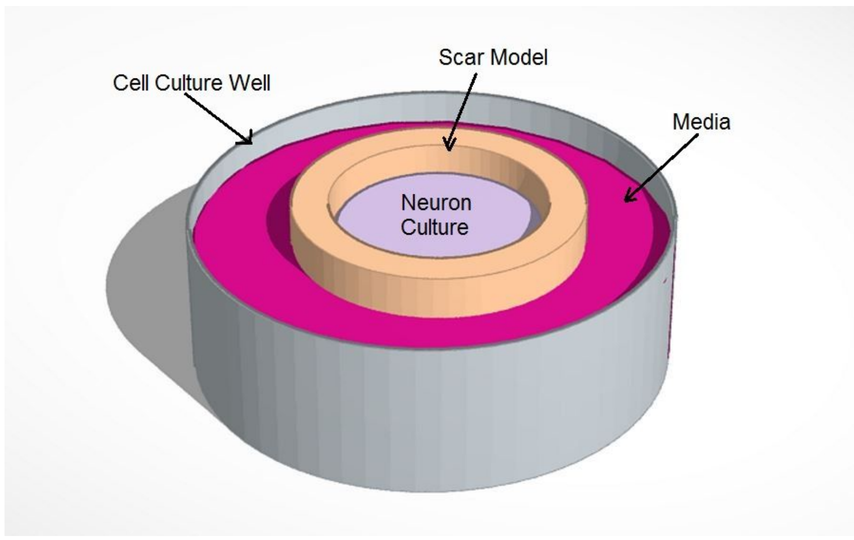


Fig. 7 Testing of the cylindrical scar.

Design 4:

The 3D culture will be placed in a collagen mold build in a teflon tube, nylon connectors and a silicon tube to create the cylindrical shape of 200 microliters of volume. The mold will be slice for testing purposes for axonal extension.

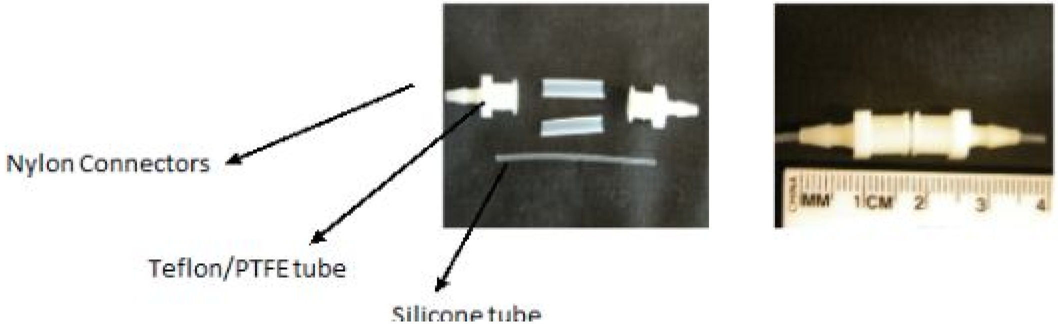


Fig.8 The molds for the the collagen cylinders.

4.4 Final Design Selection

After analysing the different design alternatives, a final decision was made that meet most of the client need and functions. A cylindrical shape scar was prefered over the straight rectangular one for testing purposed. A cost and benefit analysis was made for each design to finalize the final design selection.

4.4.1 Evaluation of the alternative designs

We created a table of analysis of the design alternatives based on the client needs and objectives from Chapter 3. The previously established designs are compared to each other in a scoring table based on a 0-5 scale with 5 being the best. The highest total scores are going to be analyse in greater detail to reach a final decision. The result of this decision-making process was Design 4, which had the highest score based on these selection criteria.

Table 6. Analysis of design alternatives

	Functionality	**Reproducibility**	**Reliability**	**Adaptability**	**Ease of Use**	**Measurability**	**Disposability**	**Cost-effectiveness**	**Total**
Design 1	1	2	4	2	2	2	4	3	20
Design 2	2	2	1	2	1	3	4	4	19
Design 3	3	3	4	2	2	4	4	2	24
Design 4	3	3	3	2	4	4	4	4	**27**

Chapter 5 Design Verification

This chapter presents the team's results from the experiments performed and mentioned in the previous chapter. These results help to narrow down the design components as well as support final conclusions about the project. The results are written in order of their completion, starting with testing axonal extension test prototyping, to collagen tubes with cells, to ascorbic acid treatment of the collagen tubes, to axonal migration testing and ending with collagen structure testing.

5.1 Axonal Extension Test Prototyping

The axonal extension test had two parts that were prototyped to minimize the testing result error. The first prototype was the neuroscreen-1 (NS-1) cells' axonal extension differentiation using neuronal growth factor (NGF). The second prototype was the leakage test to prevent cell migration and/or axonal extension underneath the collagen tube. For this test four gelatin, agarose, agar, and vacuum grease were considered, but only agar and vacuum grease were tested.

5.1.1 Neuroscreen-1 Differentiation

To test the axonal extension differentiation of the neuroscreen-1 cell line, the cells were placed in two 6-well collagen-coated plates. In each 6-well plate there were two controls that only contained the neuroscreen-1 cells with RPMI culture media (CM). Neuronal growth factor (NGF) was added into wells with two different cell densities, 150 and 300 thousand cells. The concentration of NGF added to each of the four wells was 0.1 μg /ml. The cells were observed for 72 hours to detect differentiation for axonal extension. Below are images of the wells from the start of testing (t = 0 hours) and after three days (t = 72 hours).

Table 7. Neuroscreen-1 Axonal Extension Differentiation Experiment 6-Well plate 1.

Time	Control NS-1 with CM 150,000-300,000 cells	NGF NS-1 with CM 150,000 cells	NGF NS-1 with CM 300,000 cells

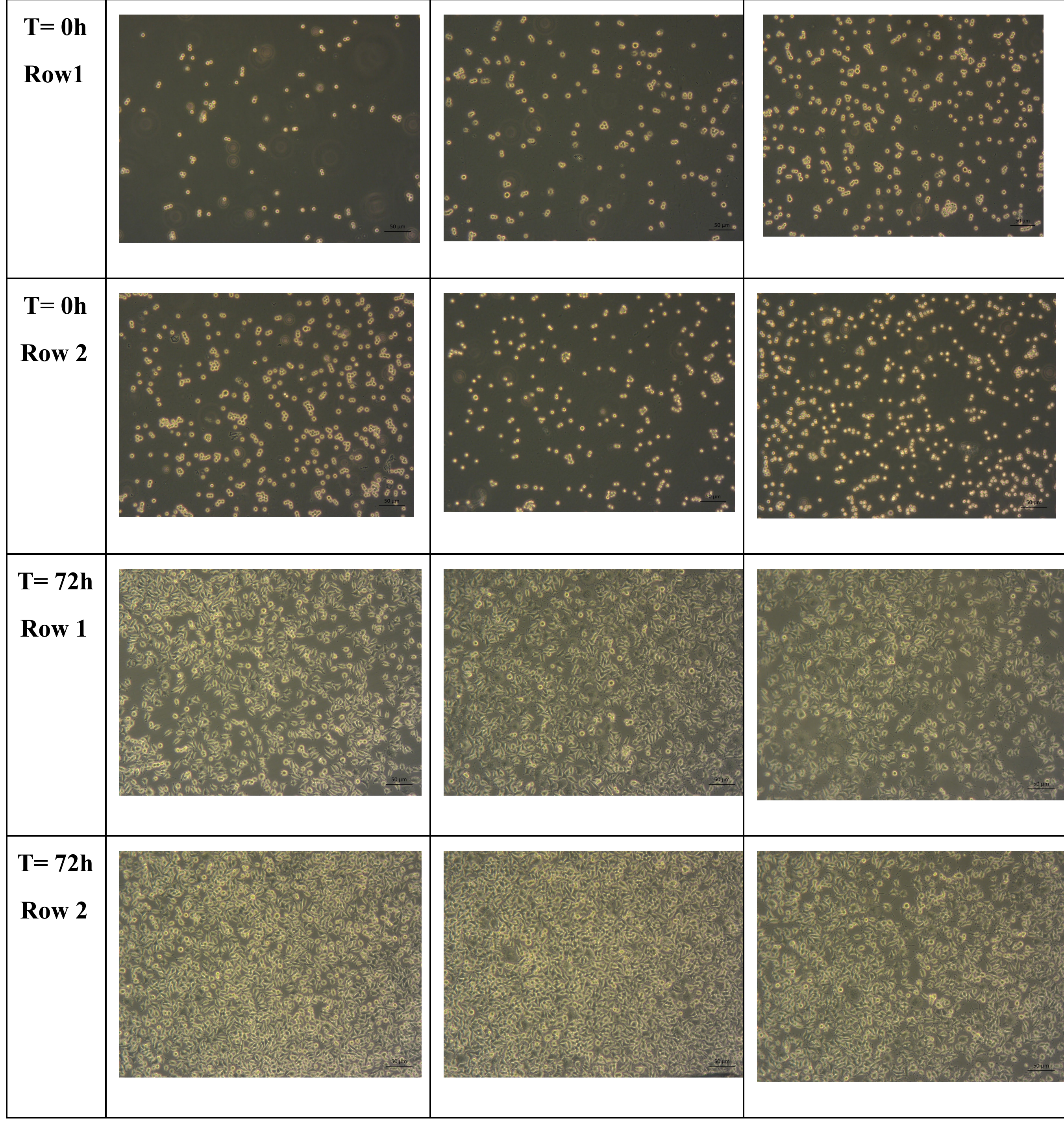
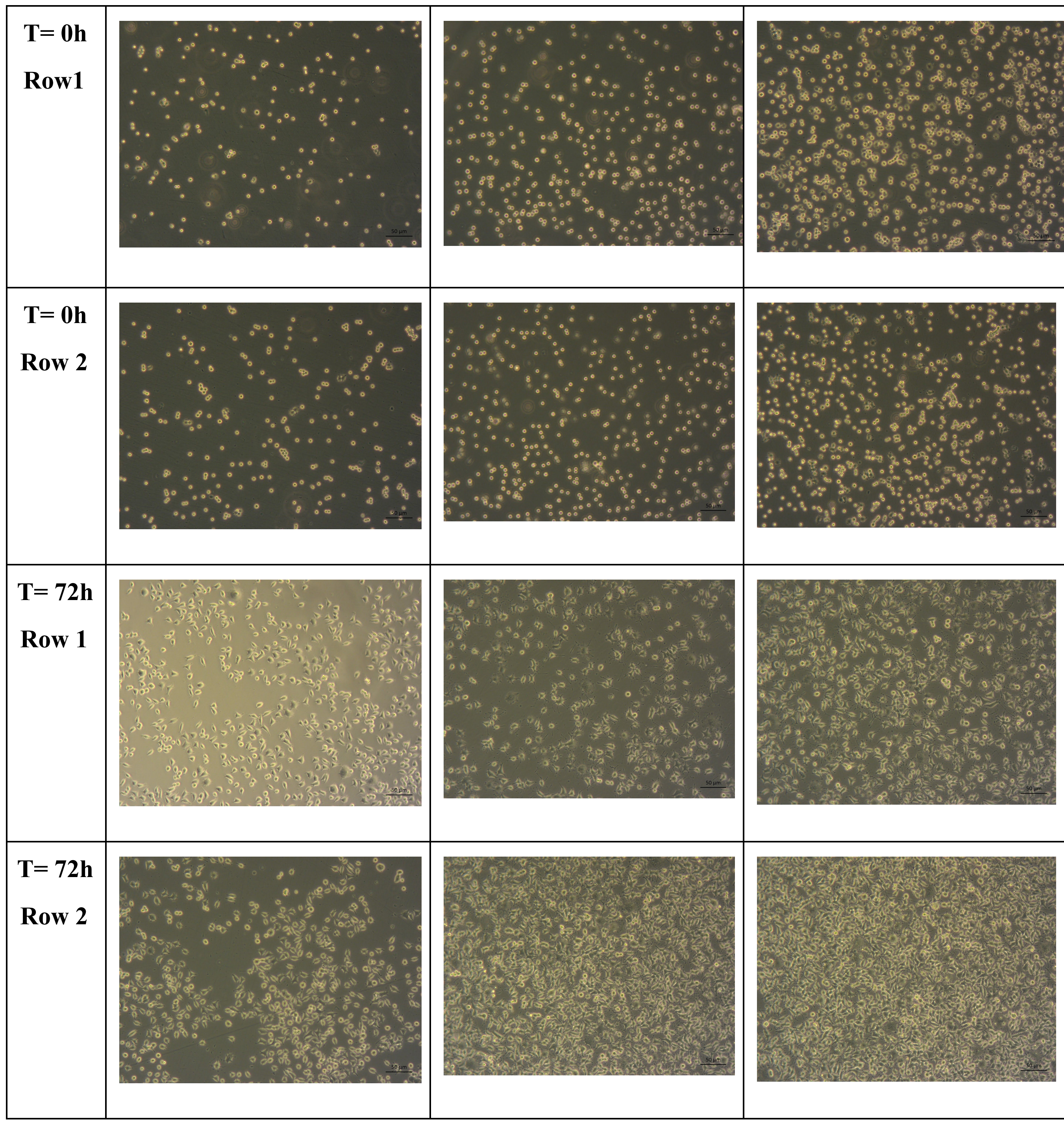


Table 8. Neuroscreen-1 Axonal Extension Differentiation Experiment 6-Well Plate 2 at t = 0.

Time	Control NS-1 with CM 150,000-300,000 cells	NGF NS-1 with CM 150,000 cells	NGF NS-1 with CM 300,000 cells


T= 0h
Row1
T= 0h
Row 2
T= 72h
Row 1
T= 72h
Row 2

5.1.2 Leakage Testing

The leakage test was performed with agar and silicone high-vacuum grease to prevent the cells from migrating or extending axons under the scar tissue instead of through it. This is to prevent any false negative results for the axonal extension test of the scar tissue. The agar and vacuum grease were tested using polyethylene tubing samples, water, and red and blue dye to readily visualize the leakage. The 3% agar coating was tested by placing the tubes upright in the agar before it gelled, so that the bottom portion of the tubes were encased in agar, and in a second case, before the agar was poured. ~75 ul red dye diluted with water was placed in the center of the polyethylene (PE) sample tubes. The vacuum grease was researched for autoclave tolerance and a volume of it was autoclaved successfully to be used as a sterile seal around the base of tubes. The vacuum grease was then tested with a thin and thick layer applied to the bottom circumference of the PE tubing. Below table 9 and table 10 display images that show the results of these tests. The 3% agar coating seal test failed after 24 hours of observation, the red dye leaked through by diffusion or leaking; in either case, agar failed testing for an effective seal against medium leakage and, therefore, cell migration. The vacuum grease passed the seal test for both the thin and thick layers, with a thin layer confined to the bottom edge of the tubing being more suited to this application, as it would not prevent cell attachment to the bottom of the cell culture plate. The tubes were observed for 72 hours for this experiment and they did not exhibit leakage. The tubes were evaluated for a week before they were disposed of safely, still showing no signs of blue dye leakage (Table 10). To more closely verify that cells would not migrate through the vacuum grease, 3T3s in complete media were pipetted into the center of

another set of PE tubes, which were sterilized using isopropyl alcohol and UV light, and sterile vacuum grease. No cell migration was observed after 24 hours.

Table 9. Agar Coating Seal Test.

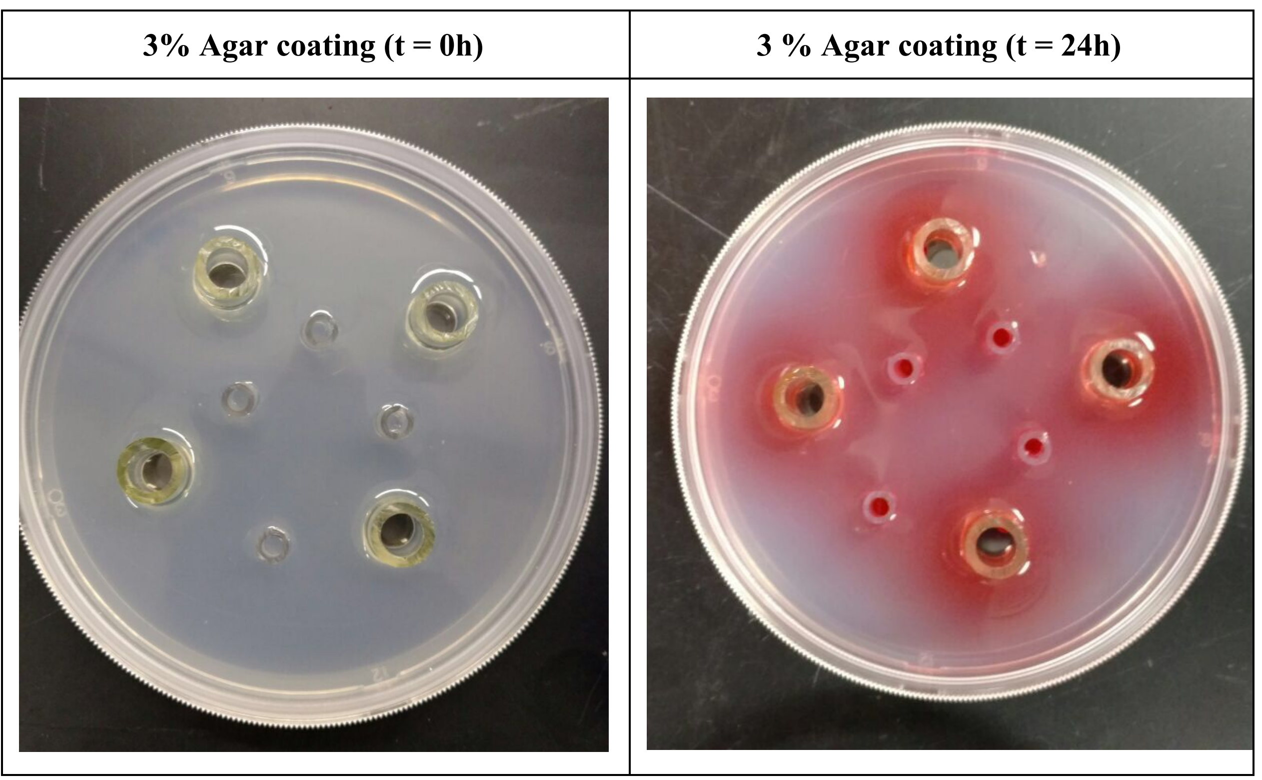
3% Agar coating (t = 0h)	3 % Agar coating (t = 24h)
	

Table 10. Vacuum Grease Coating Seal Test.

Vacuum Grease (t = 0h)	Vacuum Grease (t = 72h)

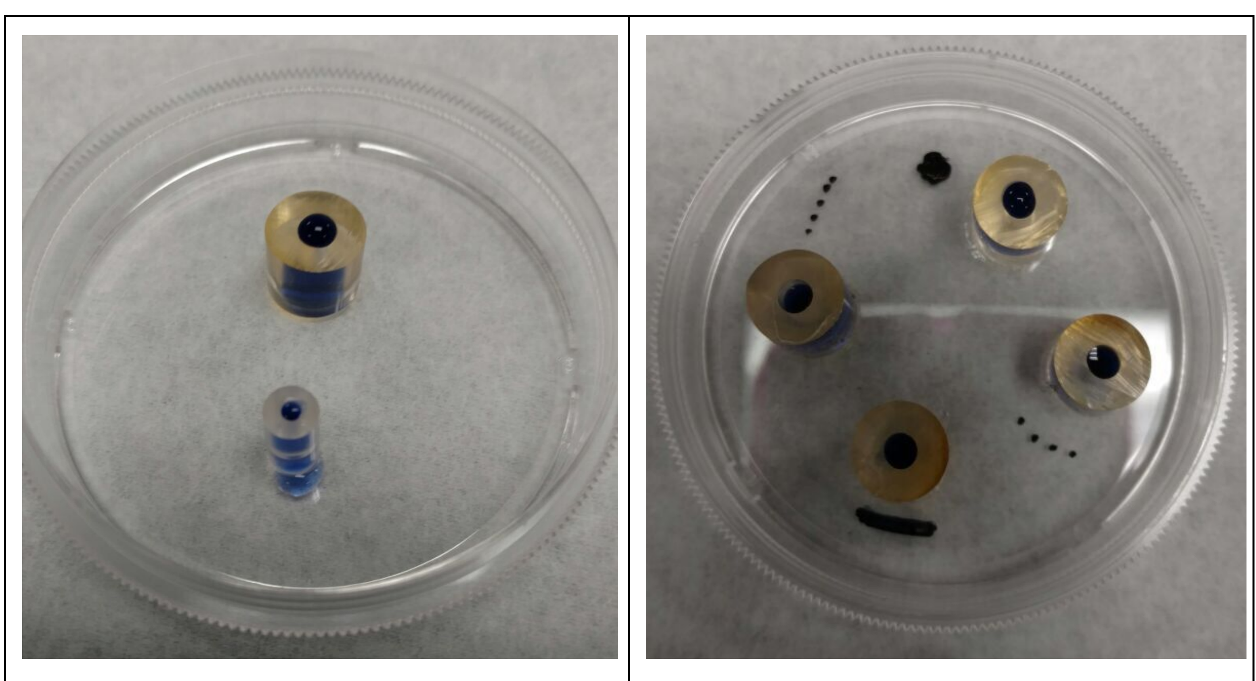
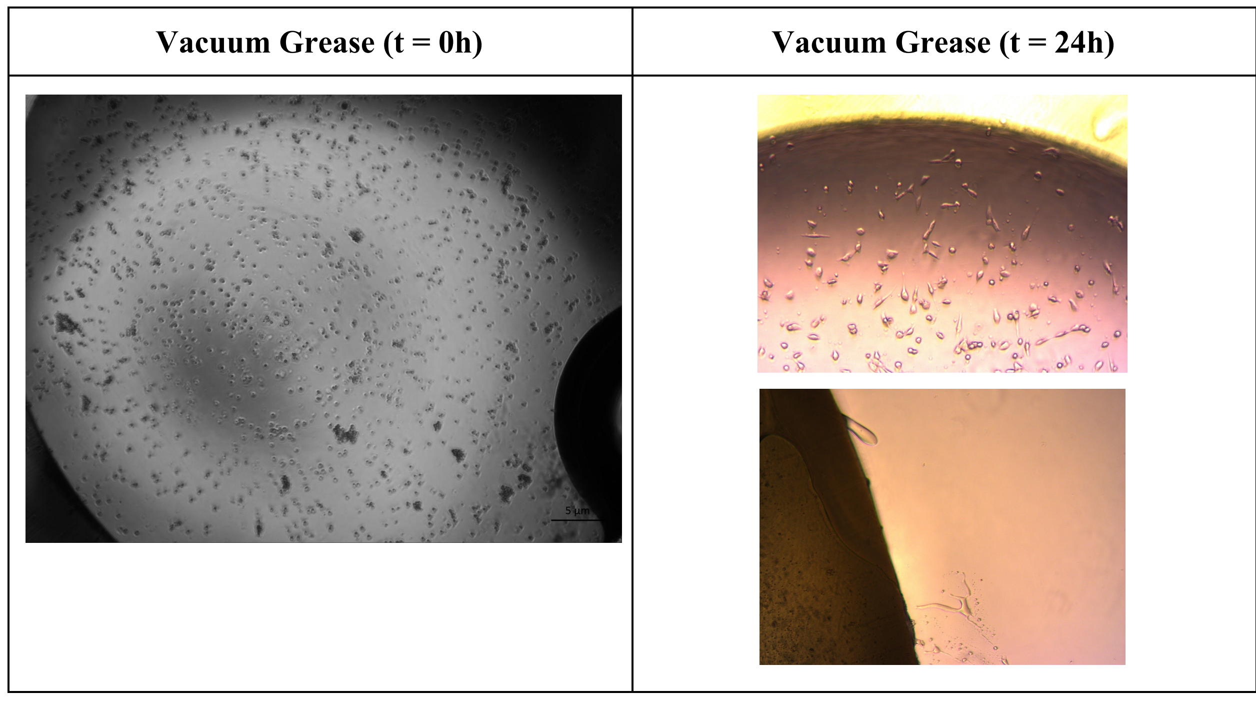


Table 11. Vacuum Grease Coating Seal Test with NIH-3T3 cells.



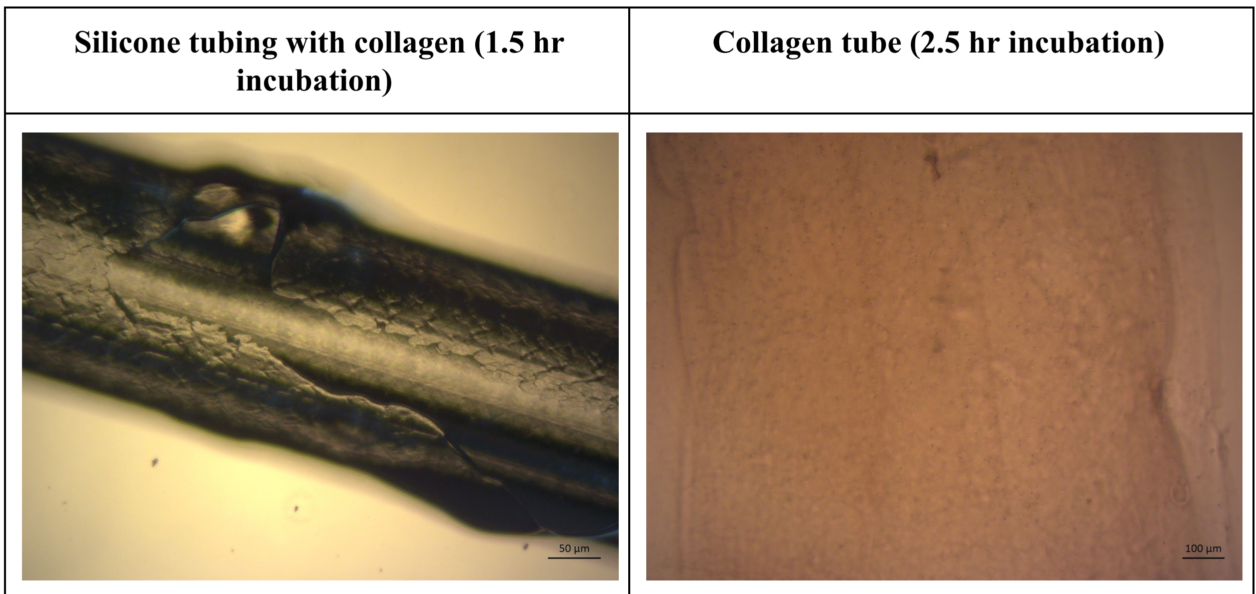
5.2 Collagen Tube Testing

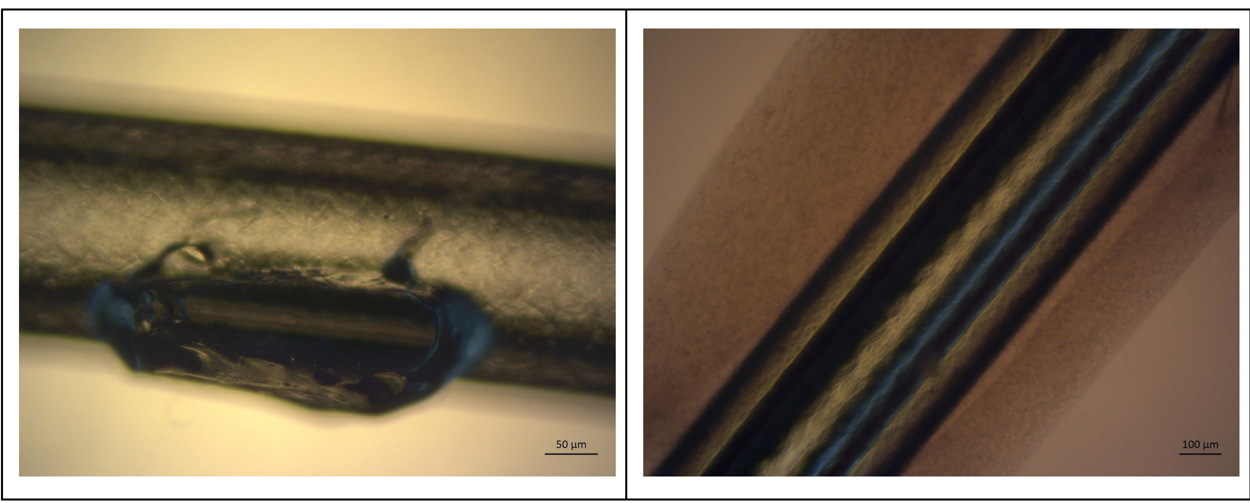
NIH-3T3 were resuspended in PureColEZ type I bovine collagen to form tubes using cylindrical molds, then cultured to develop a scar-like tissue. There were several steps to achieve this, beginning with prototyping and customizing the protocol, silicone tubing size, and the cell density seeded in each cylindrical tube.

5.2.1 Protocol Crosslinking Gelation Time Testing

To verify the collagen tube protocol and the structure of the tube verification tests were done with 3.2 mg/ml PureCol EZ Bovine Type I collagen. 1 ml of PureCol EZ was injected into five molds and followed a protocol to make the collagen tubes. Below are images of the collagen tube prototyping and variation of crosslinking time. The tubes were able to solidify after increasing the incubation time to 2.5 hours.

Table 12. Images of the Difference in the Collagen Gelification Due to the Crosslinking Time.

Silicone tubing with collagen (1.5 hr incubation)	**Collagen tube (2.5 hr incubation)**
	



5.2.2 Silicon Tubing Testing

The silicone tubing is part of the cylindrical mold to make the collagen tubes. The smaller the outer diameter of the silicone tube the thicker the collagen tube is going to be. Three different sizes of silicone tubing were tested to verify which one had the best structure and was easier to manipulate rated from 1 to 5, 5 being the best and 1 the worst.

Table 13. Silicone Tube Size Test for the Collagen Tubes.

Silicone Tube Size Outer Diameter (mm)	**Thickness of the Collagen Tube (μm)**	**Quality of Structure and Manipulation**
1.96	100	2
1.19	139	3
0.94	151	4

5.2.3 Cell Density

The collagen tubes were tested with NIH-3T3 cells to verify cell viability and longevity in the PureCol EZ collagen, and to qualitatively assess tube degradation over time due to fibroblast consumption of media within the collagen. The next test focused on the collagen tube cell seeding density necessary to achieve a strong structure, while ensuring that there were few enough cells to receive media and other additives via diffusion through the thickness of this nonvascularized tissue. This was also to track the cell migration off of the collagen tubes into the surrounding media and attachment to the plate. The first molds had 500,000 3T3s each (per 200 uL collagen), and the tubes were weak, poorly formed, difficult to manipulate, and did not retain their shape; they could not be worked with as intended for this project. Therefore the second cell density tested was 1 million 3T3s per tube, this case resulted in noticeably less fragile tubes, although they were still very challenging to manipulate without tearing or leave on the silicone mandrels. The third and final cell density was a gradient of 1.5 and 2 million 3T3s seeded in the collagen cylinders. Tubes we more structured and the this cell density did not appear to inhibit the diffusion of nutrients throughout the tissue.

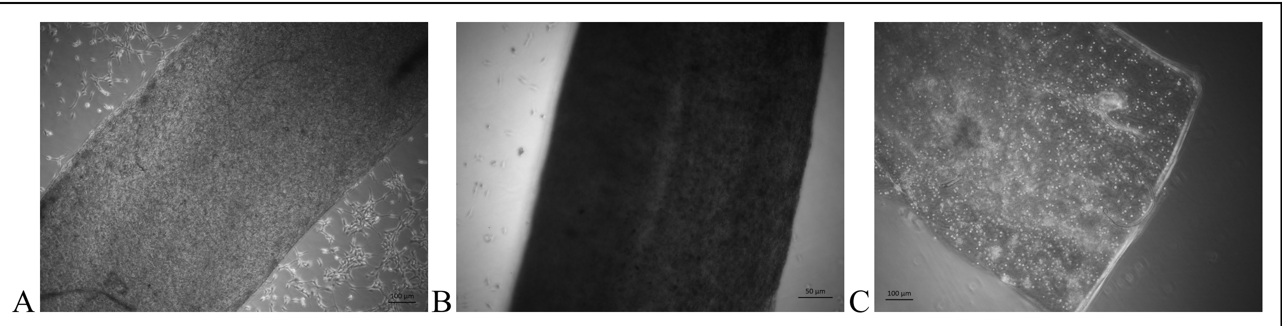


Fig. 9. A. Collagen tube with ~1.5 million 3T3s after 2.5hrs of incubation it was taken out of the mold and observed some cells didn't attached to the collagen. B. Collagen tubes cultured for one week to observed there's no cell migration. C. Cell attachment to the collagen in cylinder structure. All Imaged at 4X magnification.

After evaluating cell density, the tubes were observed and imaged as soon as they were taken out of the molds after 2.5 hours of incubation. Some cells did not attach or implant into the collagen cylinder and when they were taken out of the tube, these cells were visibly detached around the tube (Fig. 9. A). The tubes were collected into new 100 mm cell culture dishes to observe cell migration out of the cylinder. The tubes did not show any cell migration away from the tube at any point during the experiment (Fig. 9. B). The tubes had an evenly resuspended amount of 3T3s and cells attached and thrived in the PureCol EZ collagen (Fig. 9. C and Fig.10).

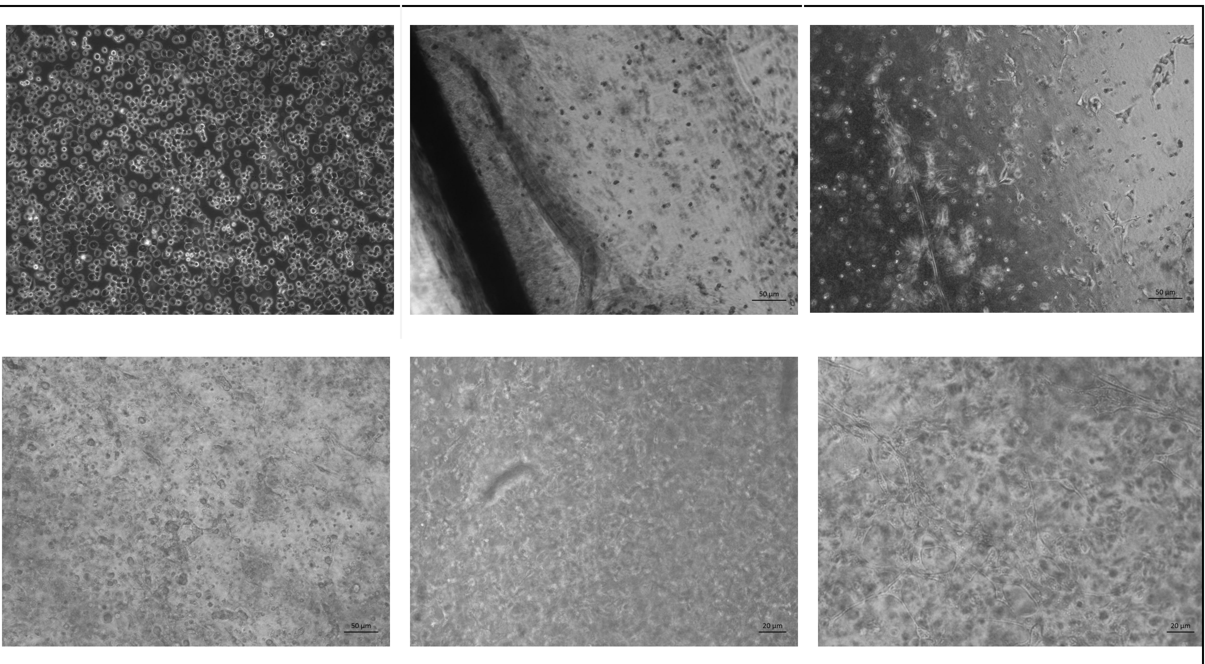


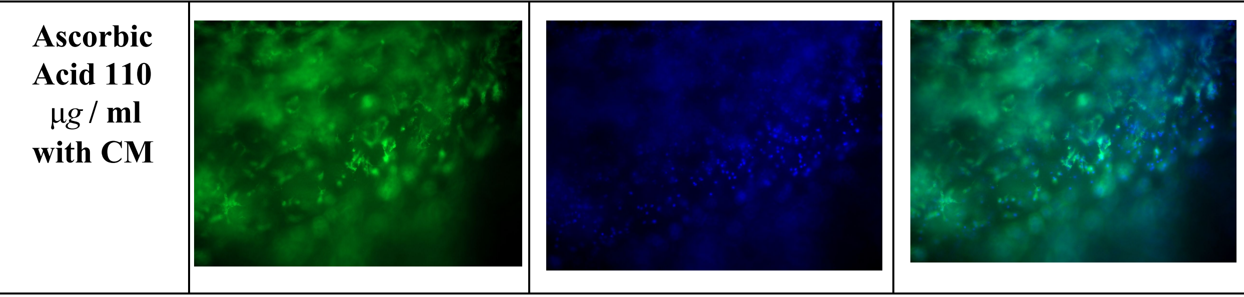
Fig. 10. The timelapse of a tube from t= 0 hours to 5 days to track cell attachment to the collagen with 10X of magnification

5.3 Ascorbic Acid Treatment

The collagen tubes were tested with ascorbic acid in two different concentrations 50 and 110 μg/ml. The tubes were cultured for three weeks (21 days) with either just culture media (control) or with culture media supplemented with ascorbic acid. Each week the culture media and the culture media with the ascorbic acid were changed accordingly. The cells were stained after the 21 days of culture with immunocytochemistry. Tubes were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA blocking solution and stained before being imaged. To differentiated the cell bodies (cytoskeleton) and the nuclei of the NIH-3T3 cells within the tubes, 200 ng/ml DAPI (blue) and Alexa Fluor-488 Phalloidin (green) actin staining were used. Below are images of the three different treatments the ~1.5-2 million cells/tube cylinders were tested on.

Table 14. Ascorbic Acid Treatment Test Immunocytochemistry Imaging.

Treatment	Cytoskeleton (Phalloidin)	Nuclei (DAPI)	Merged (DAPI and Phalloidin)
Control only with CM			
Ascorbic Acid 50 μg/ ml with CM			



5.4 Axonal Extension Testing

The collagen tubes were sectioned to smaller cylinders with a height of 1 mm. This height was not short enough for the tube to self- support, therefore the tube were sectioned to a smaller height of 0.5 mm. This height did not facilitate standing the tubes upright vertically because the tubes were very delicate and challenging to manipulate. The results for the axonal extension testing were inconclusive due to limitations on the vertical stability of the tubes.

5.5 Collagen Structure

The Tubes were fixed with 4% paraformaldehyde and hydrated in DPBS (+). For a 3D construct analysis for immunohistochemistry the tissues were processed through alcohols, cleared in Xylenes, embedded in paraffin wax, sectioned into 6μm slices vertically and horizontally, and mounted onto charged microscope slides. After the slides were dried the paraffin was removed with three incubations in Xylene, followed by hydration in alcohols gradient and rinsed in running water. For the histology staining, standard protocols were followed for Picro Sirius Red/ Green (Non-Polarizing). The slides were stained with Harris Hematoxylin, then rinsed with water. The slides were dipped quickly in acid alcohol and rinsed

in water, then the slides were dipped in Ammonia water then washed in running water. The slides were stained with the Picro Sirius Red fast green dye, followed by dehydration in graded alcohols, and cleared in three incubations in Xylene. The slides finally were permanently coverslipped with Cytoseal 60 and dried in a slide warmer to $60 \circ C$ prior to imaging on a bright field microscope. Picro Sirius Red was used to stain the collagenous fibers with red and and other tissue fibers with green. The collagen fiber alignment varies between the three different samples. The least collagen alignment is displayed in the control sample, which was cultured with complete media only. The samples that were treated with either 50 μg/ml or 110 μg/ml of ascorbic acid had more collagen fiber uniaxial alignments. In Fig. 11. and Fig. 12. the tissue resembles dense, irregularly distributed/aligned connective tissue with different amount of fibers according to the treatment. A degree of collagen fiber alignment appears to be visible along the intimal layer of ascorbic-acid treated tubes when sectioned vertically, even in the cases of incomplete cylindrical tubes which tore off the silicone mandrels in culture. Ascorbic acid treated collagen tubes had more and larger collagen fibers than the control tubes as shown in the images below.

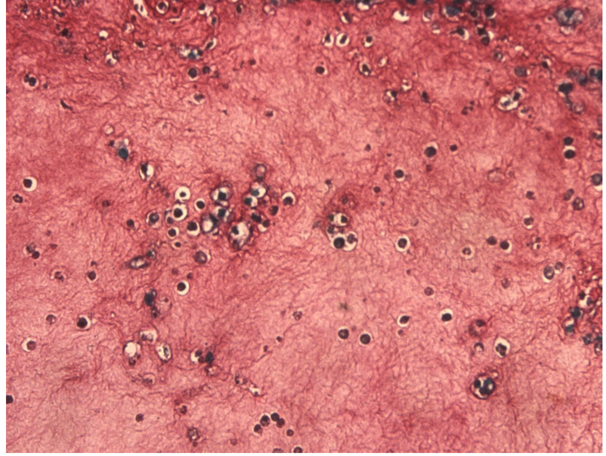


Fig. 11. Collagen tubes cultured in complete media

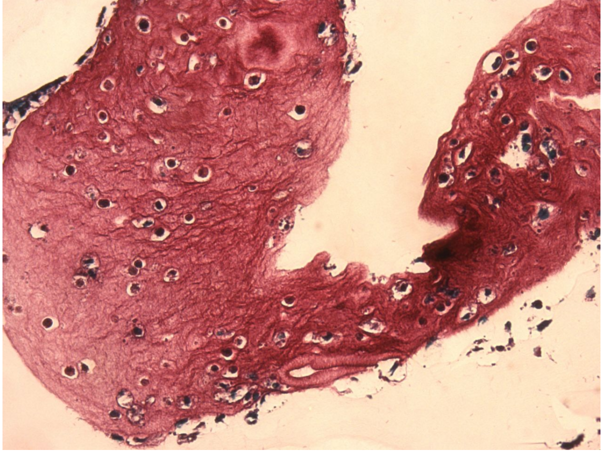


Fig. 12. Collagen tubes cultured in complete media

with 50 μg /ml of ascorbic acid. Imaged in the horizontal axis with 20 X magnification in a brightfield microscope.

with 50 μg /ml of ascorbic acid. Imaged in the vertical axis with 20 X magnification in a brightfield microscope.

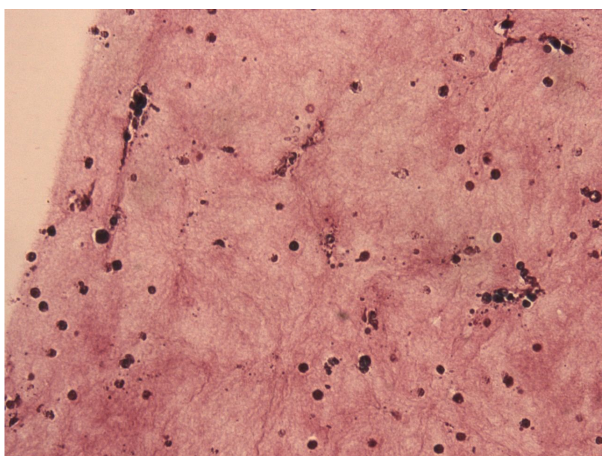


Fig. 13. Collagen tubes cultured in complete media. Imaged in the horizontal axis with 20 X magnification in a brightfield microscope.

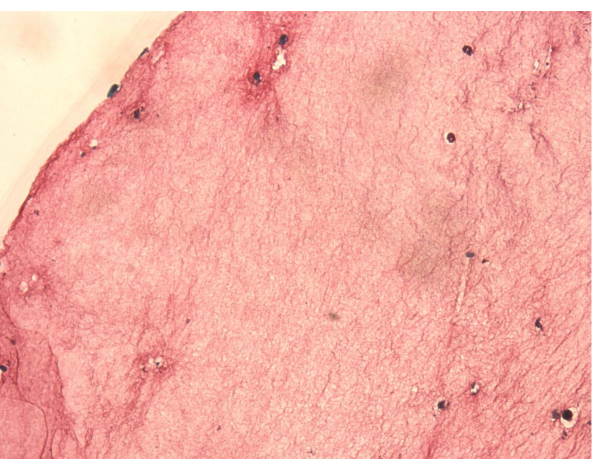


Fig. 14. Collagen tubes cultured in complete media. Imaged in the vertical axis with 20 X magnification in a brightfield microscope.

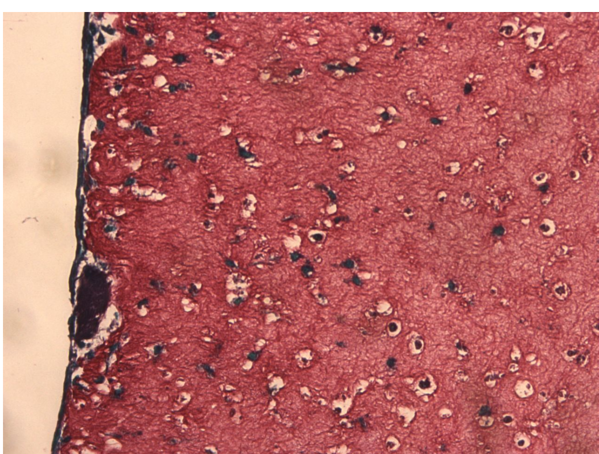


Fig. 15. Collagen tubes cultured in complete media with 110 μg /ml of ascorbic acid. Imaged in the horizontal axis with 20 X magnification in a brightfield microscope.

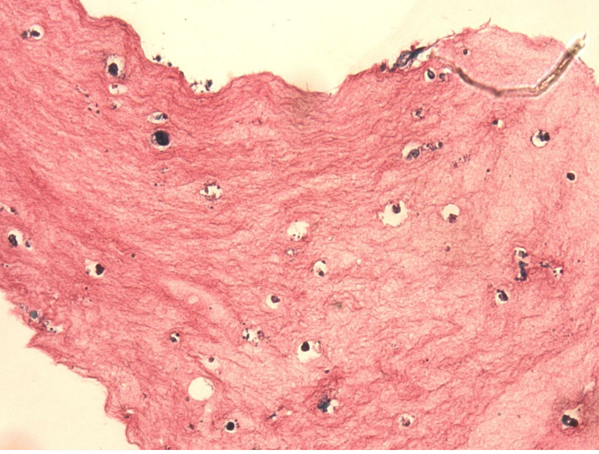


Fig. 16. Collagen tubes cultured in complete media with 110 μg /ml of ascorbic acid. Imaged in the vertical axis with 20 X magnification in a brightfield microscope.

Chapter 6 Final Design and Validation

This chapter is dedicated to detailing the final design and assessing its adherence to the objectives set forth by the team and associated stakeholders in previous chapters. The process of reproducing the device is emphasized to aid future researchers in replicating and expanding upon current work.

6.1 Objectives Verification

6.1.1 Functionality

The requirements entail that the device must prevent axonal extension, remain anchored and upright, prevent neuron migration around the scar model, and enable measurement of axonal extension. These functional requirements were not met due to the limitations of the model, particularly with regards to its durability. The cell-seeded collagen hydrogel cylinders were too fragile to test axonal extension, remain anchored and upright, or prevent neuron migration around the scar model.

6.1.2 Cost-Effectiveness

The final cost of the device, including all aspects of the manufacturing process, is reasonably low, thereby meeting the objective of cost effectiveness and illustrating potential as a proof-of-concept for a high-throughput drug testing system if developed further. Additionally, the nylon connectors can be reused ten to fifteen times before being affected by autoclaving,

further reducing the cost. The materials required to produce one unit of the device and their associated costs are delineated as follows:

Table 15. Cost of One Unit

Associated Component	***Materials***	***Cost***
Molds	Nylon barb connectors (2 x 1/8" I.D.)	~$0.25 each x 2
	Teflon tubing (~1.5cm L x 4mm I.D.)	~$0.06 from a 10 ft reel ($1.37 per ft)
	Silicone mandrel tubing (~6 cm L x 0.94mm O.D.)	$0.30 from a 50 ft reel ($76.00 per reel)
Cylinders	PureCol EZ bovine Type I collagen hydrogel (200uL)	$1.83 from a 35 mL bottle ($320 per bottle)
	NIH-3T3 cell line (1.5-2 million cells)	$282 per 20 uL, readily available in most labs; received at no cost from university - *$0*
Supplements	Ascorbic acid (~110 ug/mL; 40 uL)	~$3.93, commonly available
	Total:	**$6.62** *(assuming 3T3 availability)*

6.1.3 Reproducibility

The final device can be readily reproduced using the guides, product information, and protocols found in this report. The process for making a fibroblast-seeded cylinder supplemented with ascorbic acid by the prescribed methods could be followed by anyone with basic 3D tissue

culture skills, with the process of removing intact cylinders from the molds being the main area requiring skill or practice. Multiple cylinders (7 out of 10) treated with ascorbic acid exhibited collagen alignment in the intimal layer of the cylinder, illustrating repeatability of these results with ascorbic acid. The results of the tests done on the cylinders could likely be replicated by following the given procedures.

6.1.4 Adaptability

The final device and the protocol for recreating it are readily adaptable to various mold and tissue dimensions, and the supplements chosen to encourage scar-like tissue development are easily modified. There is additionally potential for mechanical stimulation of the tissue, particularly with further development of the process for leaving the cylinders on the silicone tubing. The model designed here meets the objective of adaptability to various dimensions, and stimuli, suggesting its versatility to multiple scar types with further development.

6.1.5 Usability

The final device does not meet the objective of functionality, so it cannot be properly evaluated for usability. While the protocols provided in this document are reasonably easy to follow, the final device itself could not be used as intended and therefore only partially fulfills the objective of usability.

6.1.6 Disposability

The final device is sufficiently inexpensive to be considered single-use and disposable to prevent unintended therapy interactions with successive uses. Components of the design can be readily cleaned, sterilized, and reused (namely, the nylon connectors) if the user chooses to do so. All components of the device and mold are safely disposable following standard biohazard disposal procedures. There are no sharp components to consider in disposal. The objective of disposability and single-use design is important in generating a low-maintenance product that is appealingly easy to test with.

6.1.7 Safety

The final device is safe for any user with basic cell culture laboratory experience to use, according to the provided protocols and guides. The main safety risks associated with the device are that it is biohazardous due to the fact that it is a cellular model, and the fact that a needle is used in injecting fibroblast-seeded collagen into the molds. These acceptable safety risks can be minimized by using standard personal protective equipment and using caution in handling and disposing of biohazards and sharps.

6.1.8 Measurability

The final device could not be used to directly measure axonal extension due to the fragility of the collagen cylinders and their inability to self-support vertically. However, while the current

design is not measurable as anticipated, it can be evaluated for collagen fiber, cell density, and integrity over time alignment with histology and light microscopy. Later iterations could enable the collagen cylinder to self-support, and with the use of different species of mammalian cells, immunohistochemistry could likely be used to evaluate axon extension. Presently, the device partially meets the measurability objective, but does not readily support quantitative data collection.

6.2 Industry Standard Verification

Industry standards serve to enable safe and effective manufacturing practices by ensuring that devices adhere to a variety of safety, sterilization, toxicity, biocompatibility, and quality control standards in order to manage risks associated with the development of novel medical devices. The final device must comply with these standards to be manufactured.

Firstly, ISO 11737-2:2009 is the primary standard governing the sterilization of medical devices. As a testing platform for drugs, this device must meet this standard in order to be manufactured. The final device is in compliance with this standard, as illustrated by all molds and forceps being autoclaved, sterile needles and syringes being used, filter-sterilization of ascorbic acid, and sterile medium and supplements being used for cell culture. Aseptic technique and a level II biosafety cabinet were used to maintain the sterility of all materials used.

ISO 10993-1 and ISO 10993-5 detail standards for biocompatibility and cytotoxicity of medical devices, respectively. The final product design gave consideration to these factors in selecting

materials and supplements which are not cytotoxic to the cells being cultured and are biocompatible.

ISO 13485:2016 provides standard for the quality control of medical devices and *in-vitro* testing. This standard is too manufacturing-oriented for a proof-of-concept product such as this, but it should be considered in future, more complete iterations. It focuses on the quality management process for devices, specifically in clauses 7 and 8 concerning product planning and design, purchasing, production, measurement, data analysis of a complete product, and subsequent improvement of the design.

ISO 14971 is the primary product lifecycle risk management standard worldwide for safe medical devices. Considerations about user safety when working with this device were considered from this standard, and the theme of reducing and mitigating risks through iterative device design. The main risk associated with this device during normal and erroneous operation is its bio-hazardous components, namely the 3T3 fibroblast cells. The use of a needle for injection of the collagen-fibroblast cell suspension into the mold increases this risk; this risk can be reduced by using a blunt-tip needle. In choosing the final design amongst the design alternatives, consideration was given to minimizing the risk of biohazard contamination to the user. The final design chosen is one of the most self-contained and minimally hazardous alternatives.

6.3 Final Device: Fibroblast-Seeded Collagen Cylinder with Mold

First, the molds were assembled using the selected dimensions of silicone tubing (here, 0.94mm O.D., but can be varied for alternative model dimensions), 1/8" nylon barb connectors, and ~4 mm O.D. teflon tubing. A section of approximately 1.5 cm of teflon tubing was cut in half lengthwise with a razor blade and both halves are slipped into the wider end of one nylon barb connector. A second nylon barb connector was fitted onto the other end of the teflon tube and pressure was applied to the two nylon connectors at either end to ensure secure placement of the teflon tubing. Then a 6 cm length of silicone tubing of the desired diameter was inserted into the nylon connectors and Teflon assembly to create a fully assembled mold. The assembled molds were then autoclaved in groups of four to five (four to five collagen cylinders can be made using 1 ml of collagen suspension with 3T3 cells). Two pairs of straight, fine, textured grip forceps (#5 forceps ideal, larger tip is acceptable for this process) were also autoclaved.
Before proceeding, a stock solution of at least 1 ml of 0.125 M ascorbic acid should be made and filter-sterilized using a 0.45 um syringe filter in a sterilized container wrapped in aluminum foil because ascorbic acid is sensitive to ultraviolet light. A sterile 27 ½ gauge needle and 3 ml syringe were also brought into in the level-II biosafety cabinet (BSC) where the procedure was performed. 1 mL PureCol EZ Type 1 bovine collagen was put on ice in the BSC at this time. 1.5-2 million 3T3 cells were centrifuged, and the supernatant was aspirated as completely as possible to avoid dilution. Practicing antiseptic technique, the user should wear a labcoat and gloves, spraying the gloves with isopropyl alcohol before entering the BSC. The 27 ½ gauge needle can then be removed from its sterile wrapper and the Luer lock cap loosened, but do not

attach it to the syringe. If possible, use a blunt-tip 27 ½ gauge needle for safety. The autoclaved bag of forceps was then opened, followed by the autoclaved bag of molds. If necessary, the silicone tubes can be realigned within the molds using the sterile forceps if they have been displaced during autoclaving. Two 100 mm cell culture plates should be set out in the BSC at this time.

Next, working quickly to avoid rapid gelation of the collagen while avoiding introducing air bubbles, the pellet of 1.5-2 million 3T3s was resuspended in the 1 ml of collagen using a 5 ml serological pipette by repeated pipetting 3 to 4 times. The cell suspension was then ejected into a single region along the periphery of the cell culture plate. Next, take the cell suspension up into the 3 mL syringe. At this time, the 27 ½ needle can be attached to the syringe. Next, a single mold can be picked up with a sterile pair of forceps by grasping a nylon connector, and the cell suspension can be injected into the region between the silicone tubing and the teflon tubing, inside the nylon connectors, being careful not to puncture the silicone tubing. Once the mold was full and collagen can be seen seeping out the other end of the mold, remove the needle and place the complete mold gently into the second, empty cell culture plate. Repeat this process for filling the remaining four molds quickly, before the collagen gels.

Once the five molds are filled, allow them to dry in a cell culture incubator in their cell culture plate with the lid on for 2.5 hours or until crosslinked, firm gelation. At this point, the cell-seeded collagen cylinders can be removed from the molds. Begin this process by bringing the plate of molds into the BSC and practicing aseptic technique with gloves and isopropyl alcohol. Prepare a cell culture plate with 10 ml complete media and 40 µl filtered stock ascorbic acid solution. Next, grasp a mold with the two sterile forceps, one on each nylon connector, and

gently twist and pull apart the nylon collectors. The collagen cylinder will be inside of the Teflon tubing, around the silicone, and gradually extract it (ideally still around the silicone tube) and place it in the cell culture plate containing complete media and ascorbic acid. Repeat for the remaining four molds.

The procedure is now complete and the molds can be washed, outfitted with new silicone and Teflon tubing, and re-autoclaved.

After the cylinders have been created and cultured for a desired period of time, standard protocols can be followed for fixation, immunocytochemistry with DAPI and phalloidin or other stains, and histochemistry with picrosirius red and fast green or other reagents.

6.4 Impact Analysis

This section of the document details the impacts of the final device on the broader societal context in which it exists. Areas considered include economics, environmental impact, societal influence, political ramifications, ethical concerns, health and safety issues, manufacturability, and sustainability.

6.4.1 Economics

This device is unlikely to impact the economics everyday living for the average person. Since it is targeted towards pharmaceutical research and development of therapies for patients suffering from functional impairment as a result of scarring and peripheral nerve damage, the

majority of the population will not be economically impacted by this device. However, with further development and the success of a subsequent product in providing a high-throughput testing platform for new drugs aiding axonal extension, a related device leading to the discovery of new, effective therapies could have far-reaching indirect economic impact on patients, families, medical practitioners, insurance companies, pharmacological companies, and generally decrease the costs associated with a variety of scar-based peripheral nerve conditions.

6.4.2 Environmental Impact

This device is anticipated to have a low environmental impact with proper use and disposal. Beyond the significant environmental cost of operating a biomedical engineering lab stemming from electricity usage, a high amount of disposable plastic and paper products, and biological and chemical hazard disposal, minimal additional environmental impact is created by the creation of this device. However, improper use and disposal could lead to biohazard release into the environment.

6.4.3 Societal Influence

The device as it currently exists will not have meaningful societal influence. With further development, this proof-of-concept could ultimately improve the lives of thousands of individuals suffering from loss of peripheral nerve function from scarring and their families by enabling researchers to more effectively test therapies and obtain clinically-meaningful results. Health practitioners, insurance companies, and pharmacological companies could also experience benefits such as increased patient success rates, increased profits, and many other benefits of medical innovation.

6.4.4 Political Ramifications

This device is unlikely to produce political ramifications, even if improved upon and manufactured at scale, it will not impact the political climate or market of the United States or other countries. If future iterations of the device are more successful, they are unlikely to impact the global economy. Culturally, the development of therapies which cure a variety of distressing peripheral nerve diseases and injuries could potentially have meaningful cultural impact on many countries where the use of animal cell lines is admissible.

6.4.5 Ethical Concerns

The ethics of this device are fairly straightforward and unobjectionable beyond the use of animal cell lines in the device itself. However, when appreciated in a broader context, a key motivation for this device is to reduce the need for and use of animal models of scar, injury, and disease conditions. Considering the fact that this model in its current iteration uses a mouse cell line rather than primary cells, it is reasonably ethically permissible. With further development, the device may use human and rat primary cells, which are more ethically suspect than cell lines, but a more ethical alternative to animal models.

6.4.6 Health and Safety Issues

The primary health and safety risks of this model are associated with improper use and disposal. Making the device following the protocols we have suggested in this document presents minimal health and safety concerns if proper biohazard and cell culture handling techniques, a minimum level II biosafety cabinet, and personal protective equipment are used to prevent

contact with cells in culture and reagents. There is potential for infection and proliferation of the cells used onto the user at many points in the procedure, which can be minimized with standard aseptic technique protocols. A single needle in contact with cells is used in the procedure, posing a biohazardous safety risk to the user. Additionally, some histological agents used are caustic or carcinogenic and much of the equipment used in this process is dangerous, so efforts should be taken when performing histological processing to restrict reagent use to a chemical fume hood, wear personal protective equipment, and use caution and training to prevent physical injury when sectioning the tissues using the microtome.

In a broader sense, this device, with future work, has the potential to enable more effective drug testing and administration for patients with peripheral nerve conditions, affording them and their families a better quality of life. The impact of a related and improved high-throughput drug testing system could be far-reaching in improving drug development and patient success.

6.4.7 Manufacturability

The final design can be readily replicated by anyone competent with general techniques in cell culture by following the provided protocols. However, the process of removing the delicate fibroblast-seeded collagen cylinders from the molds is fairly skill-intensive and relies on the steady hand and careful work of the user. This aspect of the process would be very challenging to automate, and therefore may prevent mass-manufacturing of a similar device without process improvement or highly sensitive and responsive robotic assembly equipment. Aside from this, the majority of the process could be automated and a standardized manufacturing process would significantly reduce wasted Teflon tubing in making the molds,

collagen gel, and time. It would be ideal to automate the process of manufacturing the molds to standard dimensions, resuspending the fibroblasts in collagen without bubbles, and injecting the collagen into the molds in more consistent and efficient volumes. In its current state, the final device is partially conducive to manufacturing and process automation.

6.4.8 Sustainability

The majority of this design is disposable, leading to relatively minor sustainability concerns relative to the normal operation of a cell culture laboratory. Efforts were made to use reusable components for the molds to reduce cost and waste, however the scar model itself certainly cannot be reused. The presence of biohazards in the product could impact ecology with improper disposal. The laboratory in which the final product was made and the equipment associated with cell culture, such as incubators, slide warmers, microscopes, centrifuges, and biosafety cabinets consume a significant amount of electricity combined, resulting in a less-sustainably produced product. Additionally, the inevitable nonrenewable-fuel-intensive transport and shipping of all materials used creates another unsustainable impact. The use of biohazardous materials and sharps presents a sustainability issue in disposal. A variety of disposable laboratory materials were used in creating and designing the product, all of which were disposed of properly according to Environmental Protection Agency guidelines. Nevertheless, in the context of a standard cell culture laboratory, these disposal and sustainability concerns are relatively insignificant.

Chapter 7 Discussion

This chapter serves as an explanation of the significance of the data collected in Chapter 5. It also discusses the limitation of experiments and equipment used and the impact they had on the results.

7.1 Axonal Extension Test Prototyping

The axonal extension test had two main prototyping testing procedures. One of them was the NS-1 axonal extension differentiation. The NS-1 cells that were tested in two 6-well plates with NGF and were able to differentiate and express axonal extension within 72 hours. The concentration of NGF used was 0.1 μg /ml and this was added to the two different cell densities, except for the control wells. The group containing ~ 150,000 cells displayed more axonal extension differentiation in the images than the group containing 300,000 cell in the four wells in 2 separate 6-well plates. As a team we can conclude that a lower cell density of NS-1 has better results of axonal extension, with this information we can state that for future reference, experiments for axonal extension should rely on the information that was found. The other main prototyping test was the leakage test for axonal extension. The leakage test was performed separately with 3% agar coating and vacuum grease. The two 3% agar coating condition tests failed within the 24 hours of observation, this is possibly due to the diffusion of the red dye under the polyethylene sample tubes, therefore this test had leakage under/around the sample tubes. The agar was very hard to work with, since it created a very thick layer of coating in the plate. This was an issue for the collagen tube because the height was planned to be 500 microns.

Agarose was not even tested for this because it has a similar protocol than agar and the coating results would have been the same, causing the same difficulties the agar did. The thick and thin layers of vacuum grease passed the test. After the 72 hours of observation neither the thin or thick layer condition failed, there was no visible leakage recorded. The vacuum grease was selected to be the sealing method for the axonal extension test. The vacuum grease can also be applied using a more accurate method that will spread the vacuum grease exactly where it has to be.

7.2 Collagen Tube Testing

The collagen tubes were tested in three different stages to achieve the best model possible within the timeframe of this project. The first stage of the cylindrical collagen tubes testing procedures was the incubation time adaptation for our design goal. The incubation time in the collagen mold protocol was 1.5 hours for the rat tail type I collagen. This incubation time was not working properly for the PureCol EZ Bovine Type I Collagen gelification process, therefore, two different times were tested, 2.5 hours and 3.5 hours. The final incubation time that was decided was 2.5 hours because it was enough for the collagen to gelify, but not too long that it would begin to dehydrate the hydrogel and cells. The second stage was the size selection of the silicone tubing to be used inside the mold to create a cylinder 3D shape. The team tested a 1.96mm outer diameter tube, a 1.19mm outer diameter tube and a 0.94mm outer diameter tube for the molds. The molds with the three different tube sizes were injected with PureCol EZ Bovine Type I collagen only and incubated for 2.5 hours. The chosen silicone tubing size was 0.94mm outer diameter because it provided the team with the thickest (150 μm thick) cylinders.

The thickness of the cylinder helped with the structure of the tube and the collagen tubes, making the tubes easier to manipulate and section. The third stage was the cell density testing for the collagen cylinders. The tubes were initially injected with 800,000 3T3 cells within the PureCol EZ Bovine Type I collagen following the protocol and the 2.5 hours of incubation time. The tubes were imaged with a light microscope and we found there were too litlle cells in the tubes to create create any type of fibrous tissue. When the 3T3s or any other type of cell are seeded in a 3D cell culture the proliferation speed slows down exponentially and the cell function is not the same as a 2D cell culture. Therefore 2 million 3T3 cells were seeded per collagen cylinder to increase the possibility of a high cell density after 3 week of cell culture. The images taken with the light microscope showed that the cells were evenly distributed and attached to the collagen hydrogel. The cells were observed for any cell migration and/or cell death, the images taken showed that there was no cell migration from the collagen cylinders to the 100mm cell culture plates. The imaged cells looked healthy and had minimal actin stress fibers.

7.3 Ascorbic Acid Treatment

Ascorbic acid was used to stimulate collagen secretion from the fibroblasts to attempt to create a scar/fibrous tissue. The collagen cylinders were cultured in two different ascorbic acid concentrations, 50 μg/ml and 110 μg/ml mixed in culture media (usually 10mL). There was a control treatment where the collagen tubes were cultured in cell culture media only. These cells were cultured for 21 days and the culture media and ascorbic acid were replenished every week accordingly. The collagen tubes were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA blocking solution and stained before being imaged. To

differentiated the cell body (cytoskeleton) and the nuclei of the NIH3T3 cells within the tube, 200 ng/ml DAPI (blue) and Alexa Fluor -488 Phalloidin (green) actin staining were used. The tubes then were imaged with a fluorescent microscope to image individual stains (DAPI or Phalloidin) and ultimately merge these images. The DAPI images of the three different conditions were compared for cell density difference. All of the collagen tubes had ~1.5-2 million 3T3 cells/tube, a standard amount of cells seeded in the tubes before the treatment test. The collagen tube within the control condition had the least amount of cells when compared to the ascorbic acid treated tubes shown in Table 14 (expressed in the 3T3 cell nuclei). The tube that were cultured in the two different concentration of ascorbic acid were compared with the nuclei images. The 110 μg /ml concentration of ascorbic acid treated tubes showed a significantly higher amount of cells and multiple layers where the cells were seeded compared to the 50 μg /ml concentration. The 110 μg /ml concentration of ascorbic acid also showed a healthier cell attachment to the collagen hydrogel, because it displayed less actin fiber stress on the cytoskeleton images (Phalloidin staining). This proves that the collagen's pore size is big enough to let the ascorbic acid diffuse and that it was able to reach the cells. We can also say that there is a correlation between the higher concentration of ascorbic acid and cell density within the collagen tubes. The team also found that with higher cell density in a 3D cell culture, cells undergo less tension, causing less number of stress fibers, this is due to less contractile force acting on the tube itself. The mechanics behind this are due to actin fibers stretching in order to attach to the collagen, but also to connect with the cells around them, therefore, if there are more cells, less fiber stress will occur.

7.4 Axonal Extension Testing

The axonal extension testing was done to assess the structure of the scar/fibrotic tissue within the collagen tubes. The goal of this test is to set up the collagen tube in a vertical self-supporting manner to test the prevention of the axonal extensions. This test was negative since, due to time limitations and others, it was not performed. Some of the samples of the collagen tubes that were cultured for 21 days in each treatment condition were sectioned as mentioned in the methods section. The tubes that were sectioned had a height of ~1mm. This height was too high to provide self-support to the tube, therefore the tube were section shorter to 0.5mm. This height did not facilitate the self-support to the tube either, and it made the tubes extremely delicate and hard to manipulate. The tubes were not able to self-support to continue with the axonal extension testing procedures. The limitations were mainly the vertical instability of the collagen tube that could have been the toughness chosen for the tube or the cell treatment did not worked effectively to create a tougher, more fibrous tissue.

7.5 Collagen Structure

The collagen cylinders were fixed with 4% paraformaldehyde and sealed in 2% agarose to prepare it for the histology processes. The collagen tubes were cleared and dehydrated to embed them with paraffin. This is to facilitate the sectioning and staining process of tissues, in this case collagen fibers. The collagen cylinders then were sectioned in two different axis: the vertical and horizontal, after this they were placed on slides and stained following the standard protocol for Picro Sirius Red/ Green (non-polarizing). The slides were coverslipped and imaged

with a brightfield microscope with both 10X & 20X magnification. The histology imaged the two different axis for three differently treated samples. The collagen tubes that were cultured in the control treatment displayed minimal collagen fibers within the vertical axis and little to none alignment in the horizontal axis. The collagen tubes that were treated with $50\,\mu g$/ml concentration of ascorbic acid images showed some collagen fiber alignments on the vertical and horizontal axis. The collagen in these samples appears to be more contracted than the control samples. The collagen tubes that were treated with $110\,\mu g$/ml concentration of ascorbic acid display the highest amount of collagen fiber alignment. These images were then compared to the literature and histology manuals and the team found that the pattern in all of the collagen tubes showed much similarity to a dense irregular connective tissue collagen alignment. The fibroblast are embedded in the matrix and the fibers are densely packed and are primary collagenous fibers interwoven without a regular orientation. They are slightly wavy fibers, which allows the tissue to stretch out until the fibers are straightened out. The collagen tubes culture with 3T3 cells and treated with ascorbic acid resulted in a similar structure to that of a connective tissue matrix rather than a fibrotic/scar tissue.

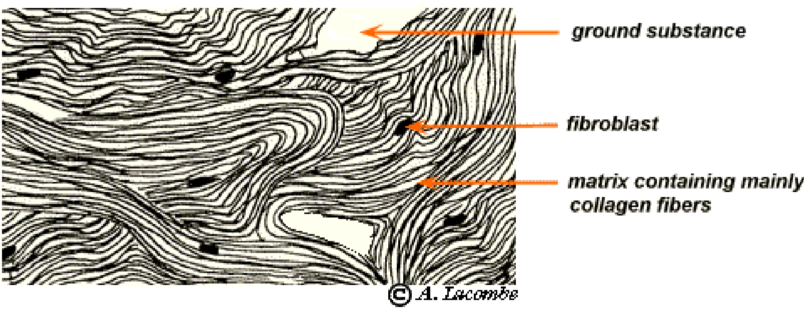


Fig. 17 Proper dense irregular connective tissue structure.

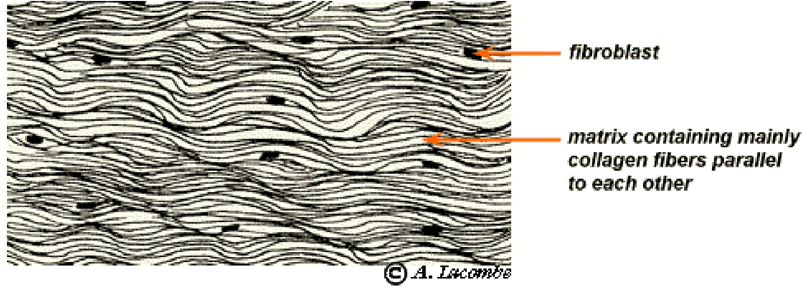


Fig. 18 Proper dense regular connective tissue structure.

Chapter 8 Conclusion and Recommendations

This chapter provides analysis of our results, further recommendations for the project, and any future work needed.

8.1 Conclusions

The project was concluded with designing a device process/methodology that would enable us to investigate the histology of a treated fibrotic scar. Although the project did not produce results to establish whether or not axons could extend through the collagen cylinder tissue, it did make strides towards using this device for further experiments that would actually be able to test for axonal extension. This project established that it is possible to grow cells on a 3D collagen cylindrical tube and treat them to form collagenous fiber alignment/production.

The final design of the collagen mold with NIH3T3 cells seeded within the cylinders proved to survive three weeks, which made handling the device easier. The collagen molds were reproducible and assuming there's standard materials (for cell culture) in the lab the collagen cylindrical tissue was very inexpensive (~$7), which makes them disposable. Using the collagen hydrogel molds as the structure for the scar was beneficial for the team as it allowed us to successfully seed fibroblasts cells, size the membrane to proper dimensions, and implement various treatments.

Throughout the year the team encountered some limitations which interfered with positive results in that allowed the completion of this project. Time and laboratory skills were quite possibly the major limitations which interfered with the successful completion of the

project. If we were able to produce a stronger structure collagen tubes, we would have had a better answer to whether or not axons could migrate through a tissue/scar treated with ascorbic acid. The limitations did restrict the device from achieving the goal of testing for axonal extension through the tissue/scar but future recommendations for the device provide further use for the device by making some modifications and continue the experimental process of this design.

8.2 Recommendations

If further development on this project is to be conducted, there are multiple recommendations the team would like to present. The collagen molds can be switched to a smaller silicone tubing to reach a maximum of 200 μm of thickness due to non-vascularization limitation. The collagen structure strength can be changed by using a slightly tougher type I collagen hydrogel around 0.7% or 1 % toughness. The structure could be fixed with the cell density as well, especially if the cells are more responsive to the treatments. Therefore, another suggestion that can benefit the structure of the cylinder is using primary cells. Human neonatal dermal fibroblasts (HNF) can be a cultured in the collagen cylinders to form a device that has a better structure and responds similar to an *in-vivo* structure. HNF cells will be more responsive to the ascorbic acid treatment and can be also be treated with other supplements such as TGF-β. If the HNF cells respond to the treatments then the structure of the cylinder becomes stronger due to the collagen fiber production of HNF cells. Another benefit from using the HNF cells would be the immunocytochemistry to stain the two different species (human fibroblast and rat

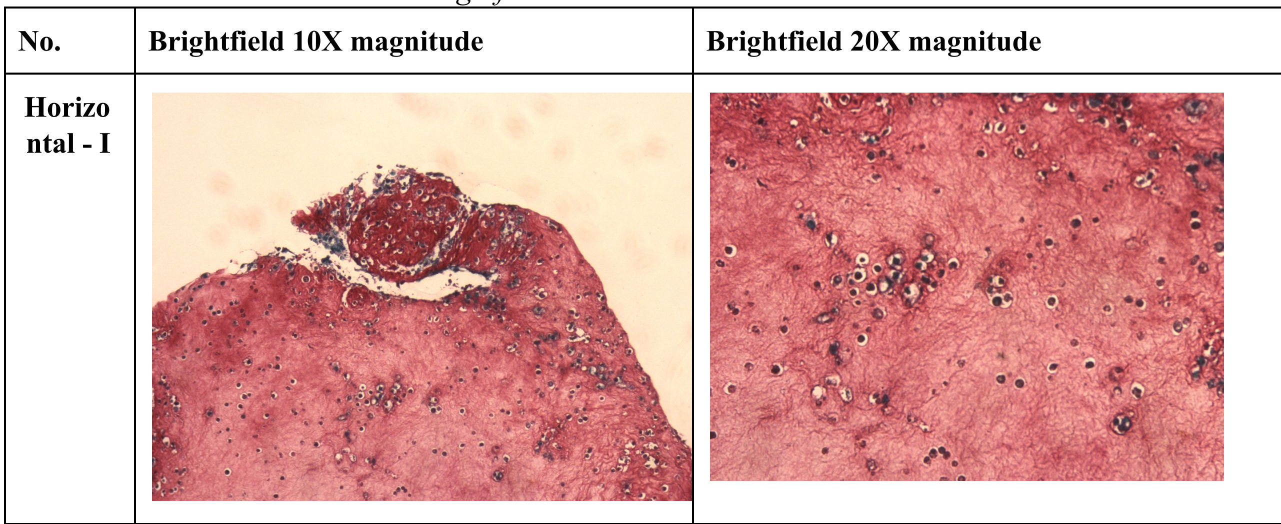
neuroscreen-1) to test the axonal extension or cell migration under a fluorescent microscope in an accurate manner.

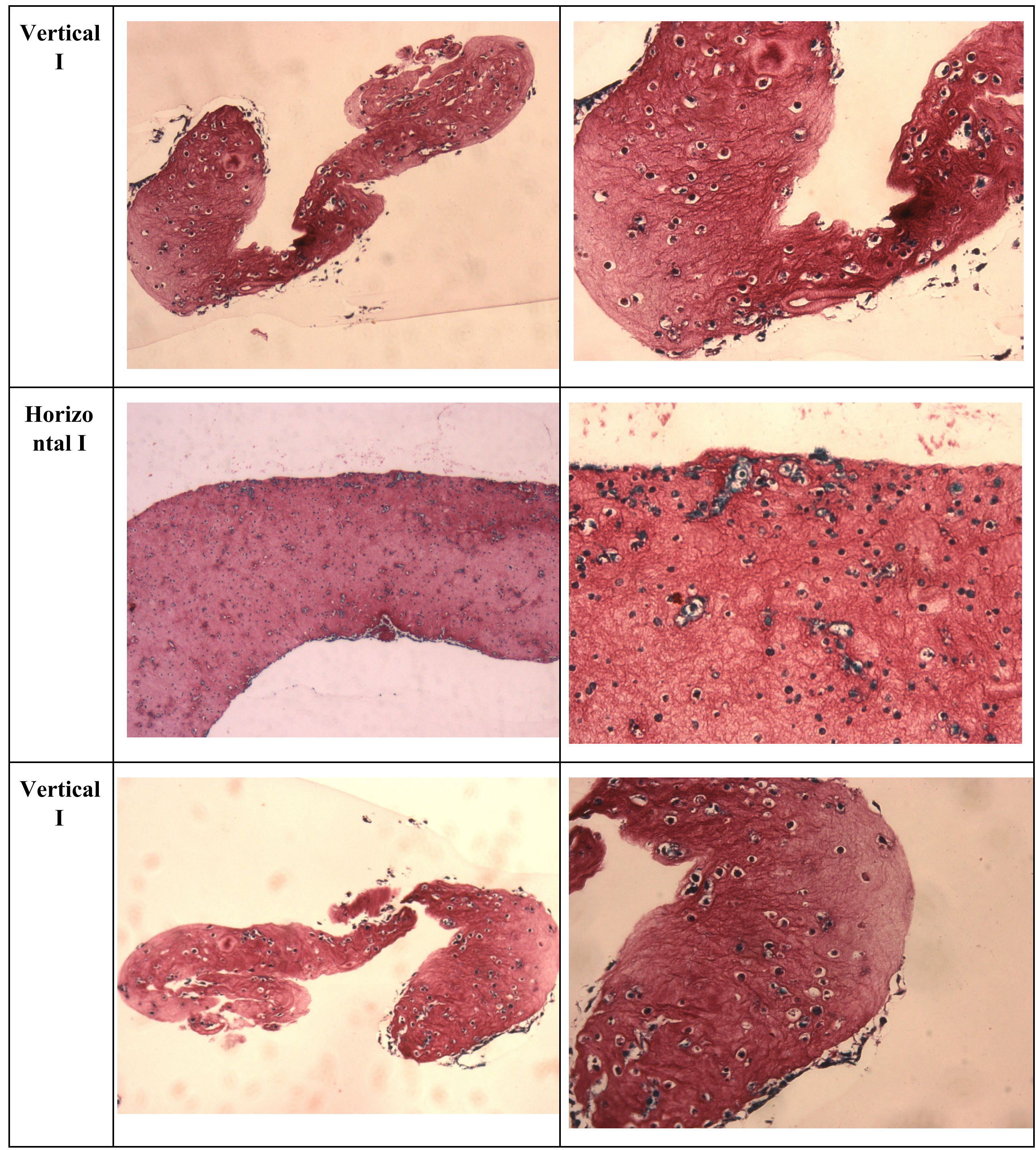
Another limitation the team encountered was not being able to leave the collagen cylinder on the silicone mandrel. The benefits of leaving the collagen on the silicone mandrel would be to have an anchored tissue to it, making it a sturdier structure, facilitating for the axonal extension testing since we would surely have a hole in the middle of the tube. For the histology testing a better method of fixing the tissue could be done by using the silicone mandrel to prevent the tubes to collapse into a ribbon shape. Keeping them on the silicone mandrel has several benefits for the experiments and the tissue itself. For more testing on the collagenous fiber structure a great test would be immunohistochemistry. This can test different types of collagen within the cylindrical tissue.

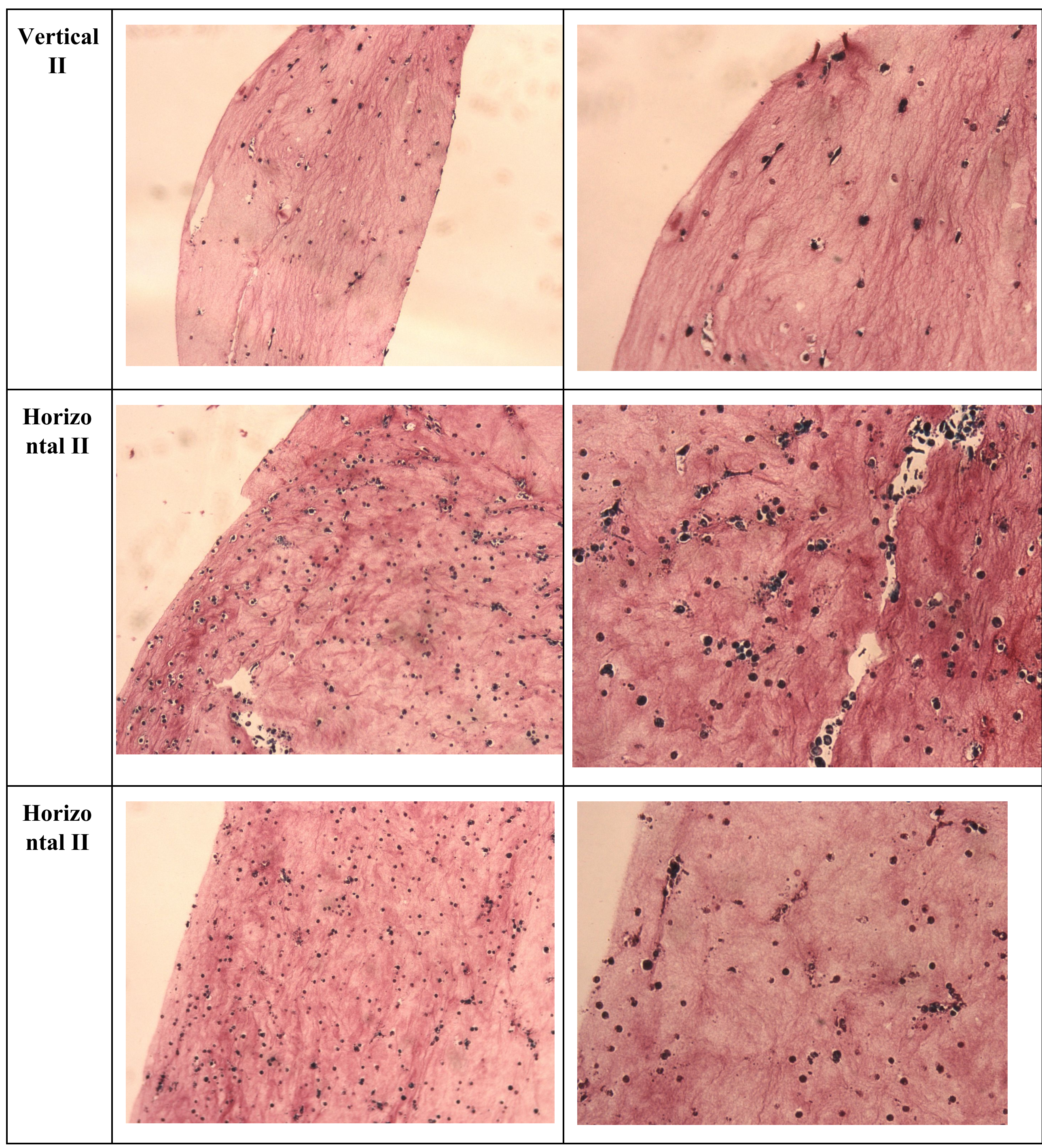
Appendix A

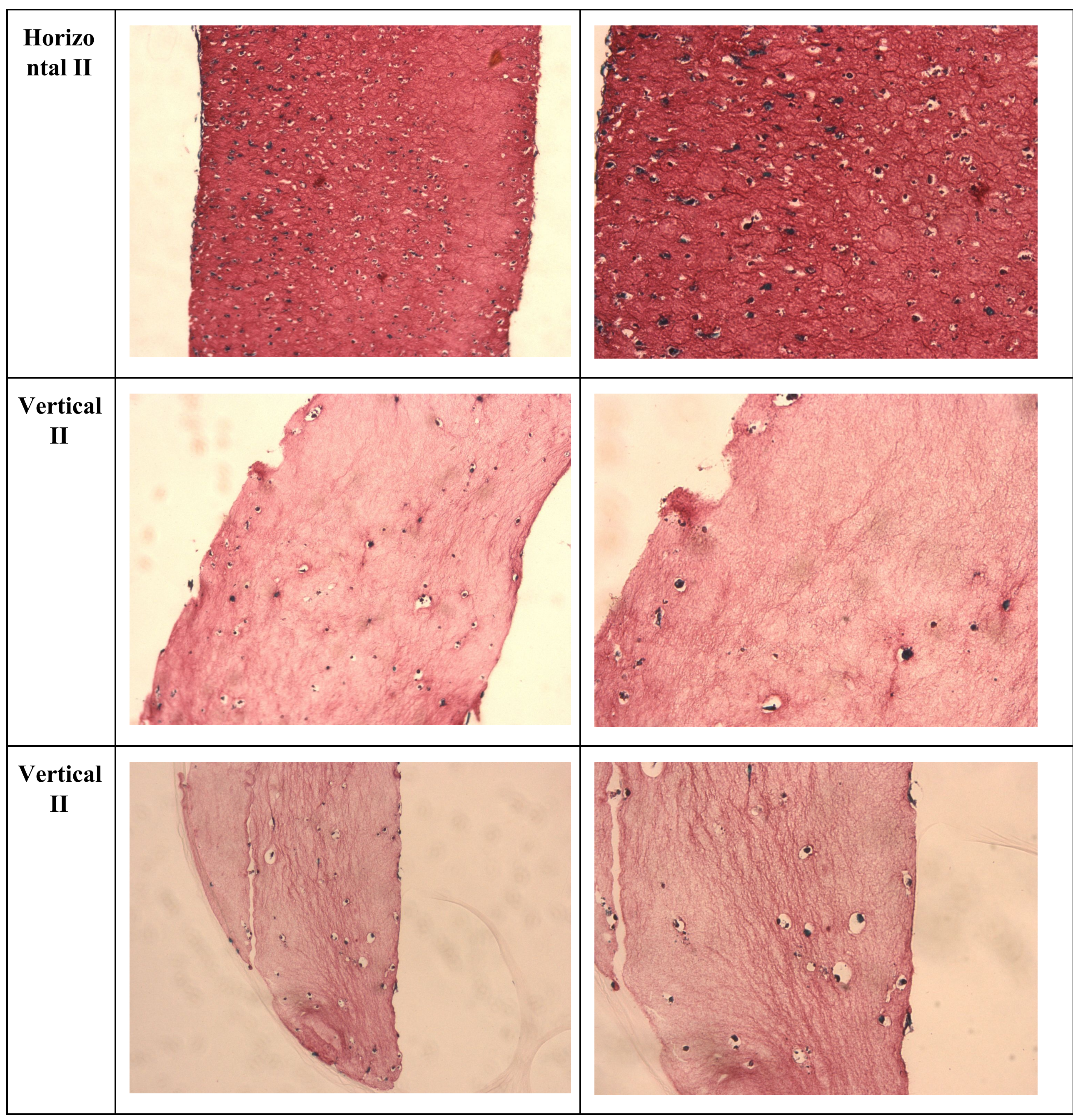
Histology of the collagen tubes that were treated with ascorbic acid and the tube that were controls. The table below has all the images taken in to different angles: vertical and horizontal axis. There are three different samples that are labeled I, II, and III. Sample Is are the collagen tubes that were treated with 50 μg /ml of ascorbic acid in the culture media. Sample IIs are the collagen tubes culture with complete media only. Sample IIIs are the collagen tubes treated with 110 μg /ml of ascorbic acid in the culture media. All the results are shown in 10X & 20X magnitude in brightfield microscope.

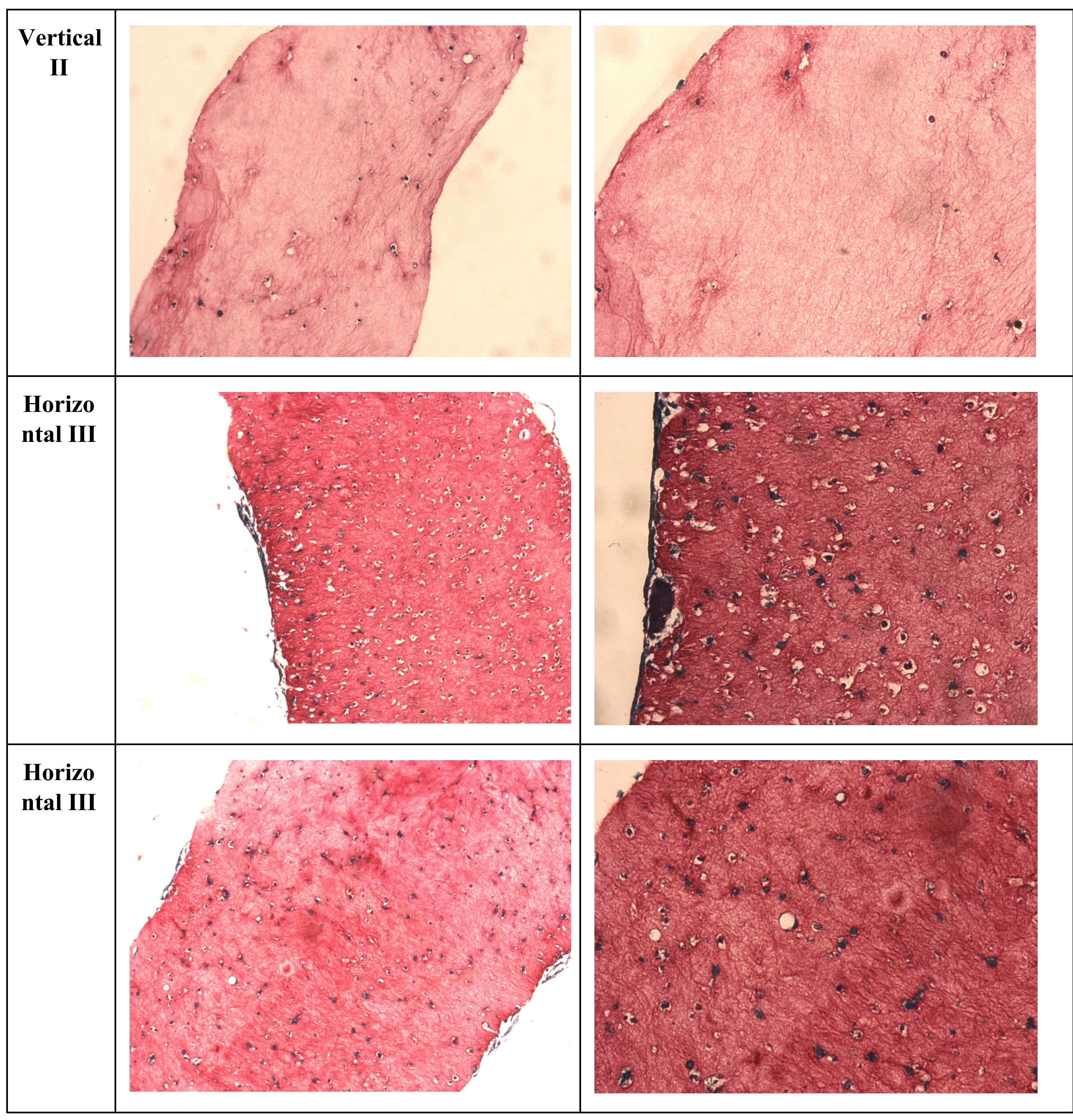
Table 16. All the histology images for all the three different sample on the vertical and horizontal axis with 10X & 20X magnification.

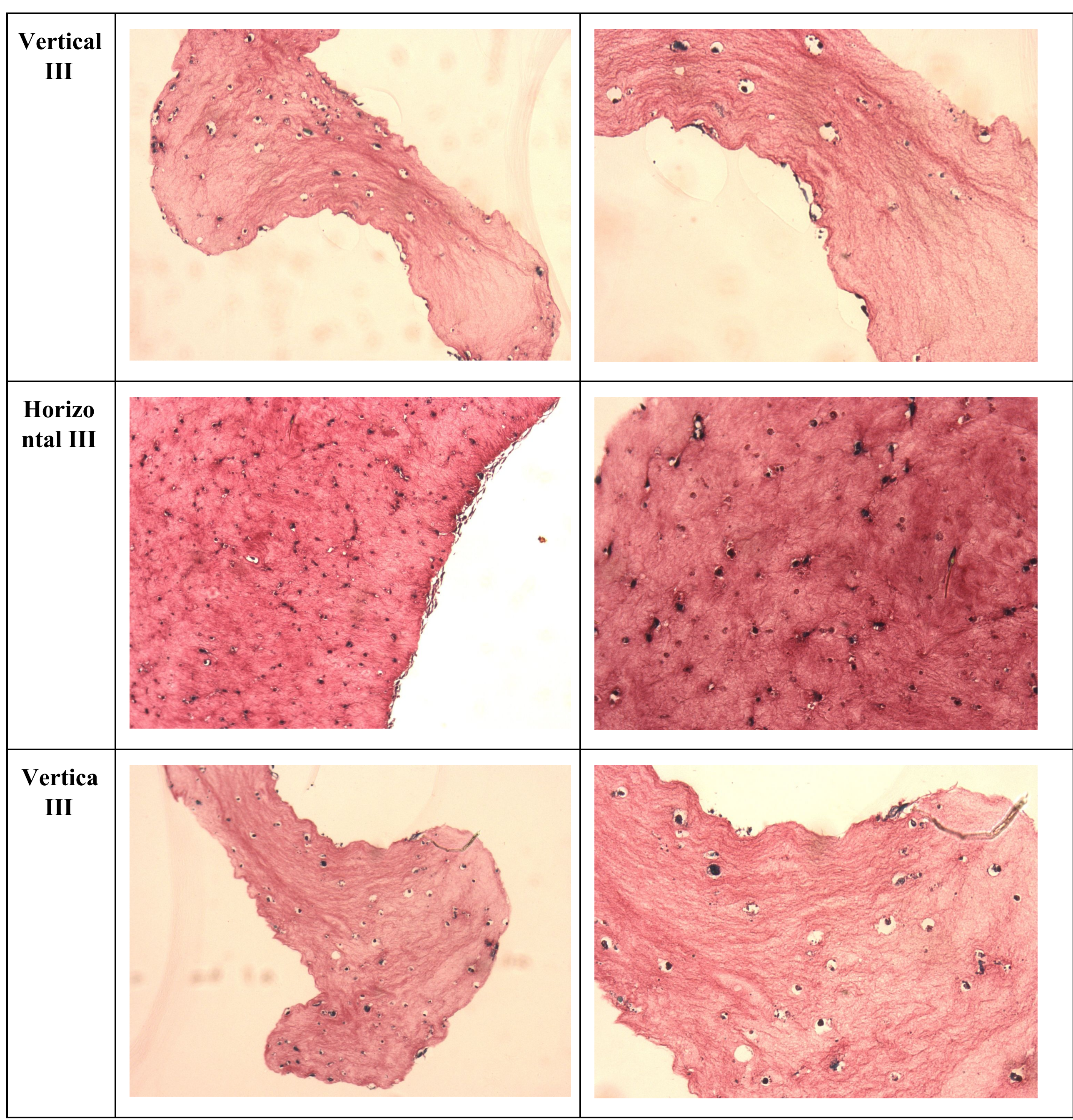
No.	Brightfield 10X magnitude	Brightfield 20X magnitude
Horizontal - I		


Vertical I
Horizontal I
Vertical I


Vertical II
Horizo ntal II
Horizo ntal II


Horizontal II
Vertical II
Vertical II


Vertical II
Horizo ntal III
Horizo ntal III


Vertical III
Horizo ntal III
Vertica III

Appendix B

Stages/Milestones		A Term	A Term	A Term	A Term	B Term	B Term	B Term	B Term	C Term	C Term	C Term	C Term	D Term	D Term	D Term	D Term
Research																	
Determine User, Stakeholders, Client	Finalize stakeholder needs and determine focus of project																
Research	Research PNS physiology, neurons																
Research	Research connective tissue scarring																
Research	Research 3D cell culture																
Research	Research wound healing process																
Competitor Research	Conduct market research; determine state-of-the-art and caveats of current solutions																
Financial Approach	Delineate potential resource expenses																
Brainstorm																	
Design Ideas	Generate conceptual designs																
Design Ideas	Generate alternative designs																
Design Criteria	Generate objectives, constraints, functions																

Materials Sourcing	Work with BME department to acquire appropriate materials needed within budget															
Sketching	Create detailed mock-ups of full design ideas, including labeled diagrams															
Prototyping																
Design Selection	Review all designs and ensure feature incorporation															
Evaluate	Analyze alternative designs; experimentation															
Manufacturing	Incorporate manufacturing industry standards															
Testing/Bench marking	Determine a series of tasks and evaluations that evaluate the prototype and prove the prototype's efficacy															
Feasibility	Conduct a review of the product evaluating its cost, efficiency, and all other design criteria to determine if the design should be actually made															
Final design interaction and completion																
Presentation/Paper																

Write, edit	Chapters 1, 3 and 4																
Write, edit	Chapter 2																
Write, edit	Chapters 5 and 7																
Write, edit	Chapter 6 and 8																
Comple	Compile and format paper																
Financial Report	Create comprehensive list of finances related to the project																
Presentation	Create and edit presentation																
Practice	Practice presenting																

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