Project Numbers: TAD-MQP-AAWM
TAD- MQP- AAWL
SJK-MQP-AAW2

Developing a Device to Investigate Migration of Axon Through a Fibrotic Scar

A Major Qualifying Project Report:

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

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Date: December 18th, 2015

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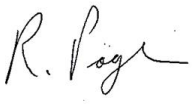

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Authorship

All members contributed equally to the formation and edits of this report.

Acknowledgments

The team would like to acknowledge the following people and organizations for their guidance in the completion of this project:

Tanja Dominko: For advising the project in all aspects.

Raymond Page: For advising the project, significantly in the Biomedical Engineering component.

Stephen Kmiotek: For advising the project, significantly in the Chemical Engineering component.

Elsa Fabbretti: For advising on neuron cultures and other biological processes.

Tanja Bele: For supervising and helping with laboratory techniques.

University of Nova Gorica: For allowing us to use their facilities.

Abstract

Peripheral neural degenerative injuries lead to the formation of fibrotic scars at the site of the lesion which limits axonal regeneration resulting in loss of motor and sensory functionality. This project aims to investigate axonal extension through these fibrotic scars. In order to accomplish this, a device of PDMS and nitrocellulose membrane was designed to simulate the site of injury with a fibrotic scar. Fibroblast NIH 3T3 cells were seeded onto the nitrocellulose membrane to simulate the fibrotic scar. This scar was treated Fibroblast Growth Factor 2 (FGF2) in order to create a less dense extracellular matrix composition. To avoid unwanted fibroblast interaction with the neuron culture, the scar was decellularized before implementation into the device. The device was successful in replicating the environment of a peripheral nerve injury but provided inconclusive results whether axons migrated through the mesh. The validation of the individual parameters of the device suggest future use of treated fibrotic scar in overcoming axonal injuries.

Executive Summary

Injury to the nervous system is one of the most debilitating injuries a person can sustain. The spinal cord injury occurs when a force damages the cells within the spinal cord. It is estimated that 12,000 new cases of spinal cord damage occur annually (National Spinal Cord Injury Statistical Center, 2013). Spinal cord injury (SCI) causes the neurons within the cord to be damaged, resulting in pain, tingling of extremities, loss of feeling, or loss of function. This can result in the patient losing partial or total functionality of their body. After injury, fibroblasts produce collagen to help close up the injury site, while creating a scar formation that makes it hard for axons to grow (Que, 2013). Currently, no alternative treatment has been deemed successful due to the inhibitory factors and physical barrier present via the scars created by the injury in the peripheral nervous system.

The design of our project aims to investigate treatments applied to a fibrotic scar that will show that axonal extension or migration is possible through a scar. To accomplish this goal, we designed a two chamber cellular well plate with a physical barrier in the middle. The barrier was constructed using porous nitrocellulose membrane with 0.45µ pores that was used as a scaffold to seed NIH-3T3 fibroblasts cells to simulate a fibrotic scar. This system allowed neurons to be isolated on one side of the mesh so we could easily image if the axons migrated to the other side of the mesh.

The project was broken into three experimental stages: 1) Testing the viability of using nitrocellulose membrane 2) Testing the implementation of the mesh into the device 3) Testing the modified device (decellularized membrane). The first stage was about getting the independent variables tested and verified. This included making sure that fibroblasts could be seeded and grown on the nitrocellulose membrane. Also it involved the treatment of Mitomycin-C to ensure that fibroblast stop proliferating after reaching confluency. The second stage was

about getting the mesh incorporated into the device. This required that the use of fibrin glue to fix the mesh into the slots created by the PDMS mold and ensuring that neurons were truly isolated on either side. The second experimental stage included the treatment of FGF-2 (Fibroblast Growth Factor- 2) to help reduce the density of the fibrotic scar, changing the percent composition of collagen compared to other extracellular matrix components. Also NGF (neuronal growth factor) was used to help expedite the growth and potential movement of neurons through the modified fibrotic scar. The last stage was taking the results from stage one and two and modifying it as needed. The result of fibroblasts migrating off the mesh onto the neuron culture prompted the team to modify the design by implementing a decellularized mesh in order to migrating cells but keep the extracellular matrix components of the fibrotic scar on the nitrocellulose membrane.

Testing the individual parameters based off of the background research done allowed us to produce a device that in theory should allow axonal migration/extension through a replicated fibrotic scar. The use of nitrocellulose membrane as the barrier was skeptical since there was not much literature on this membrane that is typically used for western blotting. The team was able to successfully seed the fibroblast cells to the nitrocellulose membrane and have the cells be confluent just after 3 days. The fibroblasts were treated with Mitomycin-C at a concentration of 4 ng/ml to stop their proliferation and the results show the same density on the first day but after day 5, the density of the fibroblast cells were significantly reduced in the petri dish treated with Mitomycin-C, showing the successful effect of the treatment. The last individual parameter that had to be checked was treating the fibroblast with FGF-2. FGF2 was introduced into the media upon plating the fibroblasts onto the mesh and it was introduced at a concentration of 4 ng/ ml. The images captured using the immunofluorescence microscope with the Alexa Fluor 488 stain

showed different cell density in the fibrotic scar treated with FGF-2 and the scars treated without FGF-2. The decellularizing of the fibroblast cells from the mesh restricted any cells from migrating onto the neurons and interfering with normal function. The mesh was decellularized using standard protocol which wiped all the cells, except small fragments of DNA left over, and kept the ECM intact on the mesh. Giving the team a replication of a fibrotic scar without having any interference from the migrating fibroblast cells. Due to limited time and resources, the neurons were not concluded to successfully migrate through the fibrotic scar as we had expected in the beginning of the project. However, since the individual parameters were successfully tested to achieve the desired goals, it can be expected that with the proper pore size for the nitrocellulose membrane and higher quality imaging should allow for the axons to migrate through the fibrotic scar treated with FGF-2.

Possible future directions of this project suggested by the team include: larger pore size, 3D culture environment, and possible movement towards a CNS (Central Nervous System) model that included a glial scar. The use of larger pores on the scaffold (nitrocellulose membrane or equivalent) would allow for myelinated and unmyelinated axons to have a higher chance of penetrating through the fibrotic scar that underwent all the previously mentioned treatments. Since this project was restricted in time, the implementation of a 3D culture was never achieved. With more time, a 3D model would allow neurons to display *in vivo* behavior that would allow for better results. The last future direction that the project could go in would be a movement to a CNS model with a glial scar. If it is found that this model, a peripheral nervous system, works with a fibrotic scar, more injuries that stem from the CNS could be studied as regenerative properties are even more limited in that system. Moving to a CNS system would allow scientists to uncover ways to treat some of the most debilitating neuronal injuries.

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Chapter 1 Introduction

The purpose of this chapter is to introduce the audience to the need of a device that would investigate axonal migration through a fibrotic scar. This introductory chapter will be broken down into three major sections: the problem that the project addresses, the goals of the project, and the project approach.

1.1 The Problem Addressed by the Project

Injury to the nervous system is one of the most debilitating injuries a person can sustain. The central (CNS) and the peripheral (PNS) nervous system are comprised of the neurons and glial cells (astrocytes and oligodendrocytes) that are responsible for communication between your brain and all other parts of the body. The nervous system controls thought and movement through physical connections both inside the CNS and outside it into the peripheral nervous system. When either of the nervous system is injured, the neuronal projections - axons are often severed and the connection between neurons and tissues they innervate is lost. The response to injury in the PNS is swelling and the formation of a barrier called a fibrotic scar. The role of the scar is to seclude the damaged area from the healthy tissue surrounding it, reducing the inflammatory response, and facilitates cell degeneration. Long term however, the scar is a physical and chemical barrier that inhibits axonal regrowth.

It is estimated that 1.7 million people sustain a traumatic brain injury (TBI), causing 30.5% of all injury related deaths in the United States, annually (Langlios, 2004), and about 10 million people are annually affected by traumatic brain injuries around the world (Hyder, 2007). TBI is caused by a force that is applied to the head that disrupts the normal functions of the brain. Mild cases could be a brief lapse in mental state or consciousness whereas severe might be an extended period of unconsciousness or even memory loss. Concussions are mild traumatic brain injuries that are well documented and lead to altered brain functions in many cases. The

most common way a TBI occurs is when a patient falls and strikes their head resulting in about 40% of all TBI (CDC, 2011).

The spinal cord injury (SCI) occurs when a force damages the cells within the spinal cord. It is estimated that 12,000 new cases of spinal cord damage occur annually (National Spinal Cord Injury Statistical Center, 2013). SCI causes the neurons within the spinal cord to be damaged, resulting in pain, tingling of extremities, loss of feeling, or loss of function. This can result in the patient losing partial or total functionality of their body.

Immediate treatment for spinal cord injuries most commonly involves immobilizing the movement of the spine. This limits any further damage to the neurons that were harmed by the injury (National Institute of Neurological Disorders and Stroke, 2015). After initial treatment, spinal cord injury victims undergo rehab to try to regain basic functionality lost due to the injury. Physical therapists focus on having the patient maintain and strengthen basic muscle functions however this is all limited due to the effect of the damaged neuronal network. Once the neurons are damaged, fibrotic scars are formed at the site of injury, and represent one of the main factors that prevent axonal regeneration and the reconnection of the neuronal network (National Institute of Neurological Disorders and Stroke, 2015).

The treatments are beneficial in preventing any further damage to the neurons but they do not address the loss of functionality of the neurons. Since neurons are unable to regenerate through a scar, the damaged neurons do not contribute to the neuronal network anymore. Another model for scarring the nervous system is the formation of fibroblasts scarring in the PNS. After injury, fibroblasts produce collagen to help close up the injury site, while creating a scar formation that makes it hard for axons to grow (Que, 2013). The team plans on addressing this issue by working towards developing a fibrotic scar *in vitro* that would allow development

of strategies to facilitate neurons to migrate through a fibrotic scar which could be used as a stepping stone toward regaining full functionality to damaged neurons in an injured site.

1.2 Goals of the Project

Our proposal aims to develop a reliable method to investigate migration of neurons through a fibrotic scar that results from the aforementioned injuries. The team sees this method as being instrumental in the ultimate goal of designing a method to return neuronal function within the body after neuronal degeneration or death has occurred. The team aims towards designing a cost effective, bio-inert device that encourages the growth of neurons using both a synthetic scaffold and growth factors known to encourage such growth.

Since current treatments of neuronal injury only limit any further injury to the neurons, researchers have been looking into alternative methods to help millions of victims regain functionality by facilitating regeneration of neurons through the fibrotic scars. Currently, no alternative treatment has been deemed successful due to the inhibitory factors and physical barrier present by the scars created after the injury in the peripheral nervous system. The component of the fibrotic scar that interests the team is the extracellular matrix (ECM) produced by fibroblast, which creates an impermeable barrier to axonal regrowth. Fibroblast growth factor 2 (FGF2) has been deemed successful in alternating expression of the extracellular matrix (ECM) genes by fibroblasts and creating a more pro-regenerative ECM (Page et al.,2011; Kashpur et al., 2013). The hypothesis for the design of our project is that a scar produced by FGF2-treated fibroblasts should allow the axons to extend through it successfully or migrate through to the other side of the barrier created by the scar; thus showing that axonal extension or migration of neurons is possible if treated with certain growth factors.

1.3 The Project Approach

In order to achieve our goal of axonal extension through the fibrotic scar, the team plans to use a project strategy that takes advantage of the current research being conducted in the field of neuronal regeneration. The first step of the project strategy was to take the initial client statement by our sponsor the University of Nova Gorica, "...fabricate and test prototypes of an artificial cerebral cortex, made of neurons and/or neuronal stem cells and of appropriate 3D supporting scaffolds of novel materials." and to generate a more feasible goal that was mentioned in the previous section. Once we revised the initial client statement, we constructed objectives which included: the device must enable neuronal growth, the device must be cost effective, the device must not induce any toxicity, the device must be durable and transportable; lastly we revised our client statement; "Design a device to enable the testing of neuronal axon migration through a fibrotic scar that can be applied to further research into the treatment of neuronal based injuries". This goal seemed more feasible given the fourteen-week timeframe available to the team.

When designing the device, several constraints were taken into consideration. The cost of the material for building the device must not exceed our budget. Since the device will be used often to test different variables, the number of uses for it must be able to accommodate that. The device should be sized appropriately to promote the cell migration. The device must satisfy all FDA regulations as well as not violate any patents if the device is to be ever commercialized. After the objectives and constraints were established, the team consulted with the clients and advisors to redefine the direction of the project, the agreed upon proposal was to stimulate a fibrotic scar and to migrate axons through it. The device would include two wells plated with neuronal cells and a vertical mesh barrier in the middle seeded with fibroblasts that would produce the fibrotic scar. With only fourteen weeks to successfully complete our project, it was

imperative to have a well-organized timeline in order to meet the deadline and produce deliverables. For the seven weeks in Worcester, MA, the team focused heavily on completing the background research in order to grasp the problem and the need for our project. This would give the team ample time to enter the testing phase of the design process when we arrived at the University of Nova Gorica in Slovenia. After getting the bulk of our design process, the last step was to implement logical metrics to help the team measure the success of the device.

Some metrics for this would be seeing the neurons grow through the fabricated scar. We can test the effects of the growth factors using live image microscopy. If axonal extensions are observed on the other side of the scar during the live imaging, then this metric would be deemed met. Once we deem the structure and the medium to be adequate, we would have to do a cost analysis on the materials used to make the device along with the cost of the materials used for testing purposes. If the cost for this system is less than the current day "golden standard" devices, then this metric has been met. If the team has successfully completed the objectives: 1) growth of neurons through a fibrotic scar 2) cost effectiveness, then the device for reestablishing neuronal communication would be considered ready for application.

Since the needs, the goals and approach of this project has been addressed in this introductory chapter, the following chapter of a literature review will go into finer details about the field of neuronal regeneration and will help contextualize the project even further. The final chapter will be the team's fully detailed approach to our design, which will cover the technical aspects, standard operating procedures in place, as well as the management approach.

Chapter 2 Literature Review

The purpose of this chapter is to provide a background to the field of neuron regeneration. To accomplish this, information from literature was gathered and assessed to connect what has been done before and what still needs to be accomplished in the field of neuron regeneration. This chapter has three sections: anatomy and physiology of neurons, project relations to the field of neuron regeneration, background on neuron regeneration.

2.1 Anatomy and Physiology of Neurons

There are about 10,000 specific types of neurons in the human brain, but the three overarching categories are motor neurons, sensory neurons, and interneurons (Stufflebeam, 2008). Each type of neuron is made up of 4 parts. The first is the cell body, which is the control center and area where neuronal proteins are synthesized. The second is the dendrites. The dendrites are terminals that receive incoming signals from other neurons and transmit them to the cell body. The third portion of a neuron is the axon. An axon is an extension of the cell body that results from a growth cone on the cell body. Axons transmit impulses to the fourth portion of the neuron, the axon terminals. The axon terminals are structures that contain neurotransmitters, which are the chemical medium that transfers impulses from the axon of one neuron to the dendrites of another.

With more than 100 billion neurons in the nervous system (Robnett, 2013), they are the most essential part of our brain makeup, which drives all of our actions. The mixture of different types of neurons in our nervous system make it possible for our bodies to move an arm when required or tell us that the pot we are touching is burning your skin. Different types of neurons have different responsibilities in regards to what is their primary function (Robnett, 2013). For example, sensory neurons are responsible for telling our brain how something feels or smells; whereas the motor neurons are responsible for telling our muscles when to move and lastly, the

interneurons are responsible for communication between the motor, sensory, and other interneurons. The neuron's transfer of electric signal happens in the space between two neurons called synapse, and allows them to communicate with each other. Talking between neurons is made possible by dendrites and axons, which are extension from the cell body. The most important part of the neurons responsible in communication between two neurons are the axon endings at the distal end of the neuron that create synapses with dendritic ending allowing the electrical impulse to jump from neuron to another.

The neurons are supported in place by glial cells (astrocytes and oligodendrocytes), which do not participate in the electrical impulse transfer but do participate in the synapse as a whole. Astrocytes, a type of glial cell, responds to the electrical impulse emitted by the neurons around them by increasing the calcium level in the environment. The increase of calcium induces the release of neuroactive substances-neurotransmitters that control the neuron's excitability and thus the transmission of a synapse. This assistance makes glial cells integral to the regulation of neuronal signals.

2.1.1 Neuron Signaling

Neurons communicate between one another through an action potential. An action potential is an electrical signal created due to a membrane potential. This membrane potential is created by a difference in voltage on either side of the membrane (Kandel, 2014). The resting potential of the membrane is -70 millivolts (mV), which is polarized. The first step in creating an impulse is the dendrites of a neuron receiving a stimulus. The result occurs in milliseconds and begins by the opening of sodium channels allowing sodium to cross the membrane. This charges the membrane to -55mV, which opens up more sodium channels. This charges the membrane to +30 mV. This is when the membrane reaches its threshold, which results in the depolarization of the membrane. This occurs by closing the sodium pumps and the opening of the potassium

channels. This repolarizes the membrane towards its resting potential of -70 mV. The repolarization of the membrane usually hyperpolarizes the membrane, meaning it passes the resting potential and reaches -90 mV. The sodium and potassium pumps then bring the membrane to its resting potential of -70 mV (Kandel, 2014).

Action potentials are conducted through the axon but are assisted by myelin sheaths (Brady, 2005). Myelin sheaths are a type of modified plasma membrane that assists in the transmission of an impulse. Myelin is an insulator, but aids in the conduction in the axon. This is because the myelin sheath is broken into sections by nodes of Ranvier. The nodes are sections of the axon that are not covered by a myelin sheath, thus depolarization of the membrane is possible. This occurs because the sections of the axon covered with myelin cannot generate a flow through the sheath, thus causing the impulse to travel further down the axon to the next node to depolarize. This increases the speed of impulses and also reduces the amount of energy needed to transfer this signal down the axon to the dendrites of another neuron (Brady, 2005).

2.1.2 Central Nervous System & Peripheral Nervous System

There are two sections of the nervous system, determined by their location and function. The central nervous system (CNS) is comprised of the neurons in the brain and spine while the peripheral nervous system (PNS) is made up of all the neurons outside of the brain and spinal cord. The functions they perform vary and thus their anatomy varies as well. The PNS is broken into two subcategories: somatic and autonomic. The autonomous is the portion that receives environmental stimuli and produces an automatic response. Examples of the autonomous region of the PNS are heart, smooth muscle, glands, metabolic tissues, and immune cells (Robnett, 2013). This system controls the body's response to emergency situations (fight or flight), and controls the cardiovascular system, gland function, and digestive, and happens involuntarily.

The somatic nervous system is made up of nerves that connect sensory receptors in the body to the CNS. These nerves are connected to muscles, skin, and sensory organs. This system receives the signals from the sensory neurons in the aforementioned systems and controls the voluntary response to the stimuli. Some voluntary actions controlled by somatic nervous systems include walking as well as facial expressions (Brodal, 2004).

The CNS, composed of the brain and spinal cord, process the information received from the PNS and interpret that information to form a response. The brain is broken into four lobes; the cerebrum, the cerebral cortex, the cerebellum, and the medulla. Cranial neurons and the cells that support them populate all of these lobes. The spinal cord is the pathway between the brain and the rest of the body. The nerves in the spinal cord are organized in a cylindrical pattern that runs down the spine and connects to the PNS by pseudounipolar neurons (Brodal, 2004).

The PNS and CNS vastly differ in function and anatomy, despite both being comprised of neurons. This project is interested in the integral difference between them, which is regeneration. The PNS regenerates naturally through the formation of new membrane, expression of adhesion molecules, and active growth cones. (Seijffers, 2007) Regeneration of neurons can be observed after damage such as a burn to skin has transpired. The first process that occurs is the degradation of the injured material. This allows for a suitable environment for axon regrowth and extension. This allows fibrin to form across the wound in the form of a cable, which allows Schwann cells to migrate and connect the severed nerve ends, promoting the outgrowth of the neurite (Brodal, 2004). An injury to the CNS has drastic difference in the response of neurons to the injury site.

When the CNS is injured glial scars form to create a physical barrier restricting regrowth while an accumulation of inhibitors down regulates the genes needed to remyelinate an axon (Huang, 2014). All of these factors create a “hostile” environment that prevents regeneration,

and thus do not allow the neurons to reconnect to one another once severed. This damage can occur when there is excessive force applied to the skull, a lack of nutrients to the brain, or excessive pressure within the cranial cavity. This project will narrow the focus to injuries sustained from lack of nutrients and excessive pressure that both can occur from a stroke.

2.1.3 Glial Scars

Glial scars are barriers within the brain that form when the brain experiences a trauma. Gliosis is the reaction that transforms glial cells and the cells in the surrounding area in response to the trauma the brain experiences. These transformed cells are what make up glial scars. Glial scars are composed of astrocytes and a multitude of fibrotic cells. These cells form a dense connective matrix that is usually classified in two parts, glial and fibrotic (Yuan, 2013). The part of the scar that is fibrotic is formed from perivascular and meningeal cells that migrate to the injury from the surrounding area, and forms fully fourteen days post injury. These cells secrete mainly extracellular fibronectin, collagen, and laminin (Cregg, 2014). This portion of the scar is found at the center of the lesion that the scar occupies. On the outer edges of the fibrotic part of the scar is the glial limiting membrane that separates the fibrotic and the glial portion of the scar. The glial portion of the scar is composed mainly of reactive astrocytes that form a mesh around the aforementioned portions of the scar. These portions of the scar create a physical barrier between the damaged portion of the CNS and the unaffected portion. The scar also forms a barrier against axonal extension through the scar. The role of the scar is to seclude the damaged area from the healthy tissue surrounding it, inducing a controlled inflammatory response, and facilitates cell degeneration. Long term however, the scar is a physical and chemical barrier that inhibits axonal regrowth.

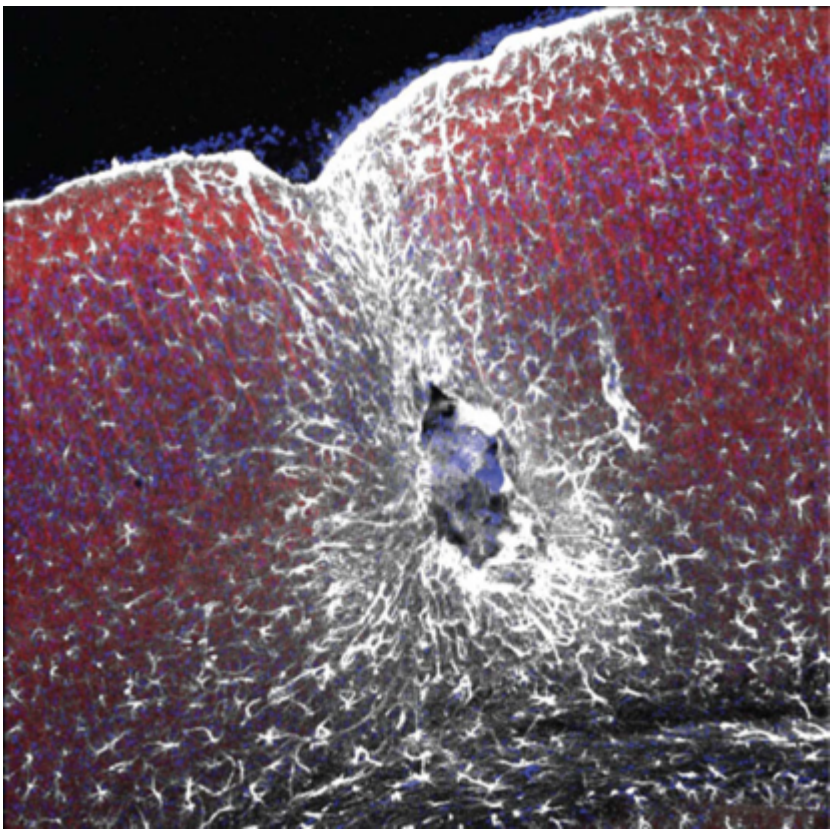


Figure 1: Glial scar with fibroblast in the middle (blue) and reactive astrocytes (white) around the fibroblast.

2.2 Project Relations to the Field of Neuron Regeneration

This project is instrumental in the field of neuron regeneration. The central nervous system does not have the ability to repair and regenerate functional axons that are damaged from injury (Horner, 2000). It has been shown that the environment of the CNS after injury inhibits the regrowth of axons. The physical barriers and chemical inhibitors of glial scars bar the re-extension of axons once injured. This project hopes to further explore methods to manipulate scar composition to allow extension of axons, and reestablish function. This research does not only impact the narrow field of CNS axon regeneration but can also be applied to genetic disorders that affect axonal path finding and degenerative diseases like Alzheimer's (Horner, 2000).

2.3 Background of Neuron Regeneration

Since the beginning of research into how our brain works and how neurons interact with other neurons, conventional cell cultures, has been used by researchers as an in vitro model. Neurons are very sensitive cells that need to be cultured with care to ensure that their functionality is not disrupted since they are terminally differentiated, arrested in G0 phase of the cell cycle and do not replicate and divide. Neuronal apoptosis and necrosis are natural events that occur gradually in our bodies due to neurodegenerative diseases and aging, and have been studied to elucidate death mechanism in vitro (Diemert, 2012). In vitro neuron culture can be studied by microscopy along with live imaging. Cells can be marked for electrophysiological recording and pharmacological manipulation to help researchers understand how neural activity and morphology interact (Potter, 2001). Neuronal cell cultures, however, require special conditions.

2.3.1 Environment of Cell Cultures (2D V. 3D)

Cell culture plates must be prepared properly in order to handle the sensitivity of neuronal cells. The cell plates must be sterilized and coated with Poly-D-Lysine before culturing the cells (Hilgenberg, 2007). All steps in the culturing process must be done under sterile conditions and follow proper protocol to produce successful results when examining the cells for further research. The cell plates are being continuously developed to allow less exertion from the user that allow for more usability in terms of monitoring the activities of neuronal cells. A non-invasive and label-free way to continuously monitor cellular behavior can be achieved using the "xCELLigence System of Real-Time Cell Analyzer (RTCA)" (Diemert, 2012). ACEA Biosciences is developing a real time device, which would take advantage of RTCA to specifically study neuronal activity. The live imaging concept will be beneficial to the growing

research of neurons in order to capture the dynamic nature of endosomes and the increasingly sophisticated glimpses into trafficking processes in live neurons (Lasiecka, 2015).
Without any major success in vivo with reestablishing connection in the CNS, researchers have been trying to understand the behavior of neurons in vitro as a stepping-stone towards full incorporation of neuronal regeneration within the body. Three dimensional culture systems are useful in understanding the true nature and the activity of neurons in a pseudo in vivo environment. Most commonly, researchers culture neurons in hydrogels, agarose, polyglactin scaffolds or Matrigel matrices in order to develop biomaterials that could provide guidance cues to the growing axons. (Gingras, 2003)

In these three-dimensional cell cultures, specific neuronal models are used that would best cooperate to allow the researchers to best understand the activity behind neurons. As explained earlier, specific steps must be taken in order to adequately culture neuronal cells on cell plates. This corresponds to taking the extracted neurons from various sources such as dorsal root ganglia, to be cultured on plastic dishes coated with the Poly-D-lysine (Gingras, 2003). The coating allows the neurons to be studied without having any reaction with the plastic plate. The Poly-D-lysine simulates a similar environment for the neuronal cells allowing them to be studied in depth without interfering with their natural function. Even though poly-D-lysine has been proven to work well with the neuronal cultures, other substrates can be used on the cell plates to assess the potential of neuronal outgrowth and neuronal attachment with different molecules (Gingras, 2003).

Two dimensional cell cultures have been used to study the behavior between cells and the microenvironments for years as it helps provide a base for starting research as 2D plating has been proven to work and is well documented (Tibbitt, 2009). The advantages of using a 2D cell culture is that cells can be plated easily and provide easy imaging of the cells. Also controlling

different external factors are easier with a 2D culture than a 3D one as they have been proven for years and the behavior of the cells is well documented. The reason two-dimensional neuronal cell cultures have been overtaken by 3D cultures because 3D cultures better simulate the in vivo environment of the body. This is not to say that 2D cultures have little scientific value because of this advancement. 2D cultures allow easy visibility of cells to track growth and migration. This method of cell culture can be used as a stepping stone when testing new methods to manipulate cells and their growth patterns (Khoruzhenko, 2011).

2.3.2 Novel Materials of Cell Cultures

In this section, the use of specific materials relevant to the project design will be discussed. It should be noted that materials/methods for regular cell cultures including medium, cell plates, techniques will be discussed later on.

2.3.2.1 PDMS

PDMS or polydimethylsiloxane has been used in cell culturing due to its unique sets of physical attributes. The advantages of using PDMS are: fabrication of the material is fairly easy and cheap, optical transparency, the ability to be molded into different shapes due to its wide elasticity range, gas permeability and biological inertness (Zhang, 2013). The fabrication process includes a cross linker and a curing agent at about 1:10 ratio. The greater the cross linker in the ratio, the higher the rigidity of the PDMS will be (Friend, 2010). The optical transparency helps with imaging, and due to the changing properties of PDMS when it is first mixed and when it is cured it is able to be molded from a viscous liquid into a rigid solid. This has allowed this material to integral in providing molds for cell culturing. PDMS also has been used as the substrate for cell cultures itself because of its gas permeability and biological inertness. With the right coating and preparations HEPG2 cells were able to be seeded onto PDMS and monitored (Leclerc, 2003). With all the current applications of PDMS, it is clear that PDMS will continue

to be used for cell culturing purposes. The only drawbacks of PDMS are discussed in "Biological implications of polydimethylsiloxane-based microfluidic cell culture" by the Regehr et al. (Regehr, 2009), explaining that there is sometimes leaching of uncured oligomers and absorption of hydrophobic molecules.

2.3.2.2 Glass Plates

Typically, glass plates and plastics dishes have been widely used in the industry to culture cells and examine the cell behavior in an in vitro model. Each of these devices have their own advantages and disadvantages. For example, glass plates are better to image cells with but when under the microscope the glass surface is more likely to get scratch compared to a plastic dish. Plastic dishes are much cheaper than glass plates so they are useful in experiments where multiple different variables are being tested and financials are to be considered, but the image clarity is not as high as with glass plates.

When specifically talking about neurons, it is crucial to use glass plates as the neurons are more prone to culturing and surviving on a glass surface than a plastic surface (Cooke, 2008). Even with glass plates being used, neurons are highly unlikely to adhere to the surface unless it is coated with an appropriate extracellular matrix (ECM) proteins that represent the in vivo environment more accurately. Particularly, PC12 cells were used in the study by Cooke to look at the cell's adherence properties to the glass coverslips when coated with different ECM proteins. The PC12 cells attached poorly to glass surfaces but cell adherence was increased when the glass coverslips were coated with collagen I, collagen IV, fibronectin, or laminin (Cooke, 2008). It is to be noted that the rising concentration of the adsorbed ECM proteins promoted better adherence up to a point where no further improvement was witnessed. Out of the four ECM proteins used, collagen IV (8 μg/mL) and laminin (10 μg/mL) were the most effective in promoting cell adherence (Cooke, 2008). Coating the glass coverslips with the appropriate

concentrated ECM proteins will mimic the natural environment that promote cell migration and survival.

2.3.2.3 Transwell & Meshes

Transwell plates are a two-part system that includes a lower cell chamber with an insert chamber that is placed within the lower cell chamber. It allows for co-culturing processes as well the observation of migration and or invasions of cell cultures. The inner chamber insert has a membrane that has a range of pore sizes that allows the migration of cells or molecules through it. This membrane or mesh has allowed to closely mimic an in vivo environments for cells to be cultured in. This microporous mesh has allowed basic cell functions such as transport, absorption and excretion to be viewed in a natural fashion such as traditional cell culturing (Corning, 2013). The applications for transwells are vast, however, most common applications are the invasion or migration of cells. An example of using transwells in neuronal research is discussed in the article "Endothelial Cells Stimulate Self-Renewal and Expand Neurogenesis of Neural Stem Cells" by Shen et al, in which they used a transwell system to co-culture endothelial cells to achieve results which would not be possible in a singular culture system. The specifics are discussed in the article, but the summary of the experiment is that Shen et al. used neural and vascular cells in a transwell system to see what effect of secretions by the endothelial cells had on the neural cells. They found out it had a positive effect as it not only proliferates the neural cells but gives them undifferentiated stem cells (Shen, 2004). This was made possible by microporous membranes in the inner chamber of transwell plates.

These meshes are usually made up of Polyesters(PET), Polycarbonate (PC), Polytetrafluoroethylene (PTFE) or Nylon Micro Mesh (NMM). Applications of the meshes depend on what experiment is being run, as each mesh has its own set of properties. The polyester membrane transwell plate has good cell viability due to the clear optical properties for

the filter. The polycarbonate has poor optical properties so it is usually used for diffusion based research rather than cell imaging. The collagen treated PTFE has a thicker membrane than the PC mesh, and it is used for cell attachment and monitoring of them while the culturing processing is taking place (Corning, 2013).

Nitrocellulose membrane is another type of semipermeable membrane derived from cellulose most commonly used for western blotting to detect specific nucleic acids and proteins. More recently, few studies have utilized this membrane as a scaffold for culturing cells and observed successful proliferation of the cells. Particularly in the study done by Li et al, "Use of nitrocellulose membranes as a scaffold in cell culture", the team grew human cells and examined for adherence, growth, spread, and survival on this nitrocellulose membrane. The high tensile strength of the membrane allowed the team to easily handle it, as well as notice no significant problems after autoclaving it (Li, 2013). The cells were able to adhere well to the membrane and culture without any chemical or physical problem created by the nitrocellulose membrane (Li, 2013). Since the membrane is opaque, it was difficult to measure cell proliferation without special treatment. In order to look at the membrane under an optical microscope, the team treated it with a microscope immersion oil. The immersion oil reacted with the surface of the membrane to instantly make it transparent, which allowed the team to observe and count the number of cells and prove that proliferation on the nitrocellulose membrane was successful (Li, 2013). Lastly, the team also assessed the cytotoxicity of the membrane and noticed no significant effect on apoptosis of the cells. The use of this membrane to grow human cells proves promising in acting as a scaffold to support culturing of human cells, and more particularly fibroblasts for our purposes.

Nylon Micro Mesh is a novel mesh that has been used in 3D cultures as a scaffold for cells. In the article, "Simple and Novel Three Dimensional Neuronal Cell Culture Using a Micro

Mesh Scaffold" by Yoo et al., they used a mesh to further research into the 3D cultures as traditional 2D cultures would not produce the result desired. The specifics are discussed fully in the article, but the summary of the experiment is that Yoo et al., used NMM to create a 3D scaffold that could culture the cells in 3D. Since the Nylon had good biocompatibility and good visibility when imaged, the team was able to observe neuronal cells in a 3D culture (of hydrogels) to further observe neurons in a more natural state of differentiation and behavior (Yoo, 2011).

2.3.2.4 Fibroblasts

Fibroblasts are cells found throughout the body that secrete macromolecules that become the structure of every tissue within the body. Fibroblasts produce collagen, fibronectin, laminin and elastin; molecules that are then modified by the cells in the surrounding area and the chemicals they produce to create tissue specific scaffolds that support cells growth and proliferation (Crapo, 2011). Because fibroblasts migrate freely throughout the body they are an incredibly versatile cells that not only create the structure of tissues but respond when a tissue is damaged. When the tissue is injured, fibroblasts migrate to the injury and secrete macromolecules that become a fibrous scar that isolates the injury and allows for repair of the tissue. The fibroblasts change behavior based on the chemicals released in the environment indicating whether they should secrete a scar or a scaffold (Alberts, 2002). This makes each scar unique based on the cells injured and the chemicals they release as a response. Some scars produced are temporary, acting as a barrier to allow the cells to heal and normal function to be reestablished. Other scars are permanent, blocking normal function from being reestablished.

2.3.2.5 Mitomycin C

Cell cultures of fibroblasts are prone to rapid proliferation if not treated with certain agent. Mitomycin-C has been proven to stop rapid proliferation of dermal fibroblasts if present at

the optimum concentration. Mitomycin-C is an antineoplastic drug which functions as an agent that causes cross-linking in DNA and inhibits RNA transcription into proteins (Chen, 2009). Typically, mitomycin-C is used as an antiproliferative agent to inhibit proliferation of fibroblasts (Chen, 2009). However, it is important to note that fibroblasts exposed to mitomycin-C for a long duration at high concentration leads to cell death. In the study by Chen et al, the group exposed fibroblasts at different concentration ranging from 4 mg/mL to 0.0004 mg/mL. Two experiments were ran with different exposure time. When the fibroblast cells were exposed to mitomycin-C at a concentration of 0.4 mg/mL, cell death was witnessed within hours of being exposed to the agent. In a separate experiment, the fibroblasts cells were exposed to mitomycin-C for 4 minutes at different concentrations available in the first experiment. Mitomycin-C concentration of 4 mg/mL resulted in cell death even if the fibroblast cells were exposed for only 4 minutes. The optimal concentration of mitomycin-C of 0.4 mg/mL made the population doubling time significantly longer for the fibroblasts yet did not cause cell death. In another recent study cited by Chen, a concentration of 0.1 mg/mL of mitomycin-C suppressed the proliferation of fibroblasts for almost 3 weeks when exposed for 5 minutes. This study paralleled the work done by Chen and verified the use of mitomycin-C to suppress the proliferation of fibroblasts.

2.3.2.5 Fibrin Glue

Fibrin glue is a two component material consisting of fibrinogen and thrombin which turns into an insoluble fibrin when it comes in contact with calcium and factor XIII (Spotnitz, 2014). Fibrin glue has been used in research for the past century in sealing structures to prevent leakage of liquid or gas. Fibrin sealants are inert to the cell behavior of neuronal cells as well as nonneuronal cells such as glial cells and fibroblasts (Cox, 2013). The sealant prevents the neuronal cells from migrating through the fibrin glue which proves its effectiveness for our

project. To maximize the longevity and integrity of the fibrin clot, manufacturers incorporate fibrinolysis inhibitor into the product. The common inhibitors are aprotinin and tranexamic acid which have been proven by Cox to be inert to both the neuronal and non-neuronal cells. However excess tranexamic acid concentration, 300-450 mM, in some fibrin sealants detached cells from matrix coated cell plates (Cox, 2013). The data from Cox's experiment supports the model in which cell detachment from a coated culture dish in the presence of tranexamic acid is due to loss of cell adhesion and not to cell toxicity. Fibrin glue is beneficial to seal the small space between two surfaces that are in contact but not impermeable. The opening between the two surfaces, for example PDMS and a glass coverslip, would be sufficient for cells to squeeze through and migrate under the PDMS and on top of the coverslips.

2.3.2.6 Decellularizing Mesh

The reason to decellularized cultures and tissues is to remove the cellular material from the tissue, leaving the extracellular matrix (ECM) behind. The ECM is made up of macromolecules secreted by the cells around it to form a mesh that is shaped by the cells around it. This means that different tissues and organs in the body have ECM's that are unique to their cell composition. The ECM is an important portion of the body because it creates a scaffold within the tissue that supports and structures the cells within the tissue. ECM is also crucial during injury to the body because it contributes to the formation of a scar to protect the healthy portion of the body from the injured portion.

The scar created during injury is the main focus of this project, thus cells that create that scar will be focused on. These cells would be fibroblasts, one of the main contributors to ECM throughout the body. The fibroblasts secrete collagen and fibronectin that the fibroblasts then manipulate with mechanical movement in addition to the chemicals in the environment secreted by the cells in the area. Once this ECM is created, the fibroblasts are no longer needed for the

scar to function as a barrier so they are taken off of the mesh. This process is called decellularization.

Decellularization removes all cellular matter to leave the ECM behind. This process is used for the transplantation of an organ or tissue. The donors' cellular material is removed leaving behind a scaffold of the organ or tissue which can then be repopulated with cells compatible with the patient receiving the organ or tissue.

Decellularization is going to be used to remove the fibroblasts from our mesh, leaving just the ECM, but the ECM is not going to be repopulated. The decellularized mesh is going to be placed into the mold to recreate a scar without the interference live cells would have on the results.

2.3.3 Growth of Neurons

Neuroregeneration, is the growth or repair of tissues, cells and by products in the nervous systems. Most importantly, neuroregeneration is the regrowth and reestablishing of neuronal connection that are harmed after various kinds of injuries. Neuron regeneration is particularly different between the Peripheral Nervous System (PNS) and the Central Nervous System (CNS). Since the CNS is the main target site for our project, PNS neuroregeneration was excluded in the in-depth literature review done for our project. It should be noted that damaged neurons in the PNS have the ability to regrow length and connection naturally whereas the neurons in the CNS lack this ability. In order to further understand the regeneration process for the CNS, many articles were reviewed to see what methods were being used in the attempt to regrow connection.

One method attempting to regenerate neuronal cells is the use of autologous Schwann cells in the assistance of axonal regeneration (Subramanian, 2009). Schwann cells have been investigated because of their function in the PNS as myelinating cells. These myelinating cells are a glial cell that promotes regeneration of the axon and the remyelination of that axon by

secreting adhesion molecules, extracellular molecules, and trophic factors. Schwann cells, while having regenerative properties, have been studied and exhibit undesirable effects. The cells do not migrate into the CNS effectively, exhibit delayed functional recovery, and in some cases form white matter instead of the intended gray matter pathway (Subramanian, 2009). This was the most promising route for regeneration until neuronal stem cells could be differentiated from pluripotent cells. When oligodendrocytes are derived from embryonic stem cells, they have been shown to myelinate axons in culture and replace lost myelin in an injured CNS (Subramanian, 2009).

Astrocytes have also been investigated to assist in the outgrowth and differentiation of neuronal stem cells (Song, 2002). Astrocytes release cytotropic effects that assist in neural repair, which prompted the neuronal regrowth studies. The astrocytes were grown on polymer substrates to promote the differentiation and outgrowth of the neuronal stem cells, but when applied in the studies, the results were not what were desired. The astrocytes did contribute to the neural repair, but inhibited the neurite outgrowth by releasing inhibitory factors (Nisbet, 2009). Astrocytes are still being studied to rectify this.

Additionally, olfactory ensheathing cells (OEC) have been shown to promote recovery and functionality to injured nerves in the spinal cord. The OEC's are present in both the PNS and the CNS, which promotes axonal growth from the PNS into the CNS, giving the OEC's the properties of both Schwann cells and astrocytes. These properties facilitate the OEC's in encouraging functional recovery at an accelerated rate compared to Schwann cells, in addition to secreting trophic factors and extracellular molecules (Subramanian, 2009).

Stem cell research has shown that neuronal stem cells are a successful method to repair and extend neurons, but the issue with stem cells is they are hard to control. Stem cells tend to differentiate unchecked, creating tumors, negating their applications in vivo. The goal is to learn

to control the differentiation of these stem cells, thus inhibiting the creation of tumors (Subramanian, 2009).

Growth factors are also instrumental in the repair and extension of neurons. Countless growth factors have been used to target myelination, axonal extension, migration, and up regulation of repressed genes. Nerve growth factor (NGF) are crucial in the development of neuronal cells and it promotes the survival maturation of several populations of sympathetic and sensory neurons in the PNS (Sofroniew, 2001). Hence the use of NGF in culturing the neurons is beneficial in proper development of the neurons and their axons. NGF allows the neurons to develop at a more rapid pace and allows the axons the extend quicker than cultures without the presence of NGF (Sofroniew, 2001).

2.3.4 State of the Art in Neural Regeneration Research

Since neuron regeneration is a growing field of research, many new developments are being uncovered in the use of new substrates that allows for effective growth of damaged neurons resulting in regained functionality. These developments move past the types of neurons used but rather what substances should be used to promote the growth of these neurons. Since nanotechnology is a fast developing field for all sorts of applications, researchers have looked into nanotubes in order to promote neuronal growth. Particularly, carbon nanotubes have been proposed as growth substrates promoting neuronal development (Fabbro, 2012). The use of newer technology suggests that the field of neuronal regeneration is still an unexplored field. The carbon nanotubes (CNTs) have been discovered to have multiple purposes in being used as scaffold for nerve tissue engineering, electrode coating and even neuronal interfaces for long-term implants. These carbon nanotubes have emerged as effective tool for manipulating certain neuronal activity whether it is in single cells, in a network of synapses or even multilayered tissue explants. (Fabbro, 2012) The use of carbon nanotubes is still an early discovery so the

success rate for using such tools will be based on experimental results that show successful growth of neuronal connections. The main characteristic of these carbon nanotubes for use in neuronal regeneration is the organization of these nanotubes that mimic the natural structure of axonal pathways within the brain and spinal cord. (Fabbro, 2012) This specific characteristic seems promising for neuronal regeneration since the neurons in the brain are in such a complex network that without a proper guidance directional growth could not be controlled.

In the case of the CNTs, scanning electron microscopy was used to measure the growth and contact between the neuronal membrane with a 500 nm scale underneath the CNT coated plates (Fabbro, 2012). In order to assess the effectiveness of regrowth of neurons, standardized testing had been put in place to assess how effectiveness of neuronal regrowth by Chu and his team who were able to assess the regrowth by various different methods. The diverse methods allowed the regrowth to be tracked during the initial stages, middle and after two days of transfection. Chu and his team first assessed how many tracks were visible in the dish which defined the initiation of regrowth, then the number of branches were counted per regrowing neurite and lastly the average elongation per hour of each branch was tracked using the scanning electron microscope (Chu, 2001).

Although further developments are being made in discovering the state of the art technology to promote neuronal regrowth, the field of neuroregeneration still has unknowns. For example, the brain-immune signaling after a brain injury is still largely untested. After acute brain injury, the peripheral immune response is altered compromising the opposing phenomena of early immune activation and subsequent immunosuppression (Liesz, 2015). This leaves patients prone to infections since the immune system is occupied by handling the acute brain lesions, leaving the victims vulnerable and this brain signaling has been unknown for many years. Another unknown signaling involves the activity that goes on in the peripheral nervous

system in axon regeneration. “The exact signal for initiating regrowth after axonal transection is not known” (Chu, 2001). According to Chu, some of the possibilities for this signal include the membrane depolarizing, loss of action potentials and the manipulation of the calcium ion could induce the signal but no solid evidence has been provided to explain this phenomenon. Understanding the complexities of the brain requires current day practices as well as previous body of works in order to move forward in neuroregeneration research.

2.3.5 Methods to Treat the Fibrotic Scar

The immediate inhibitory factor to axonal growth are the factors secreted by the astrocytes that create the outer portion of the scar. Because the fibrotic portion of the scar takes two weeks after injury to fully form, researchers have used this time frame to extend axons through the scar by using different molecules to block the inhibitory factors secreted. The astrocytes found in the scar produce extracellular matrix molecules (ECM’s) such as chondroitin sulfate proteoglycans (CSPG’s), phosphacan, neurocan, and brevican that have been found to inhibit axonal growth (Kawano, 2012). These factors have been studied and produced successful results in prohibiting axonal growth. Studies have been done concerning the inhibitory property within CSPG by targeting the chondroitin sulfate (CS) side chains of the molecules. This was conducted by introducing chondroitinase ABC (ChABC) into the injury site. ChABC is an enzyme that degrades CS, and when introduced into the environment, it promoted the regeneration of severed axons (Moon, et al. 2001) (Kawano, 2012). Suppression of fibrotic scar formation has also been investigated by targeting Type IV collagen synthesis. The use of antibodies against Type IV collagen as well as DPY, an inhibitor of Type IV collagen synthesis, has been successful in the suppression of fibrotic scar formation and it has been reported that they allow the regeneration of axons and the recovery of motor function (Kawano, 2012).

Another method is the use of fibroblast growth factors to extend through a glial scar. FGF2 the fibroblast growth factor that is responsible for modifying the macromolecules secreted by fibroblasts and for the modification of the organization of those secreted macromolecules. These modifications reduced the density of the scar produced and have been shown to promote the formation of glial bridges across the scar. This gene was first tested in vivo when the zebrafish was investigated for its ability to repair its injured spinal cord. It was found that FGF2 signaling induced zebrafish glia to form a bridge between the two sides of the injury, allowing axons to migrate across (Goldshmit, 2012). When FGF2 signaling was interrupted, the glia cells did not bridge the gap and axonal regeneration was not achieved. This lead to the application of FGF2 signaling with mammalian cells to test whether the results would be similar. FGF2 was first tested with primate cells then when it was found to be successful, FGF2 was tested in mice models (Goldshmit, 2012).

This test was conducted by creating a lesion in mice spinal cords to simulate an SCI. This lesion was then treated with FGF2 to induce increased fibroblast growth factor production at the site of injury. The treatment was administered subcutaneously started 30 minutes after injury and lasted for two weeks. The study found that this treatment promoted functional recovery, decreased inflammation and astrocyte reactivity, and supported neurite elongation and axonal regeneration (Goldshmit, 2014).

Chapter 3 Project Strategy

The purpose of this chapter is to guide the reader through the project approach. This will include intensive sections that cover from the conception of the client statement through the design process to the finalized client statement. This chapter has five main sections: Project Statements, Technical Design Requirements, Standard Design Procedure Requirements, Financial Approach, and Experimental Approach.

3.1 Project Statements

Here the initial and then revised client statements are discussed.

3.1.1 Initial Project Statement:

Our initial client statement came from the University of Nova Gorica, Tanja Dominko, and Elsa Fabbretti. The interest stems from the limited methods currently available to reestablish neuronal connections within the brain after injury. From the initial client statement shown below, the team developed objectives and constraints for the project. Initial Client Statement: Design a device or method to extend an axon through a hostile space.

As our team met with our advisors and our contact at the University of Nova Gorica, it was noted that the client statement must be revised in order to fulfill the client's need. The first thing needing revision was the part of a “hostile space”. Since the definition of a hostile space is very broad, this was defined further as a fibrotic scar preventing the extension of neurites and axons. Once the team arrived in Vipava, the team met with Tanja and Elsa and further narrowed the scope of the project to develop the final project statement. This revision to the project statement is seen below.

3.1.2 Revised Project Statement:

Based on the needs of the client, the project statement was revised to read:

Design a device to enable the testing of neuronal axon extension through a fibrotic scar.

3.2 Technical Design Requirements

The next step in the design process was to compile a list of objectives, constraints, metrics, functions and specifications for the device in order to satisfy the technical design requirements.

3.2.1 Objectives

The team formulated multiple objectives in order to achieve the project goals. These objectives include: functionality, cost effectiveness, biocompatibility, reproducibility, customizability, and quantifiability. These objectives were placed into an objective tree that branched into sub-objectives.

3.2.1.1 Primary Objectives

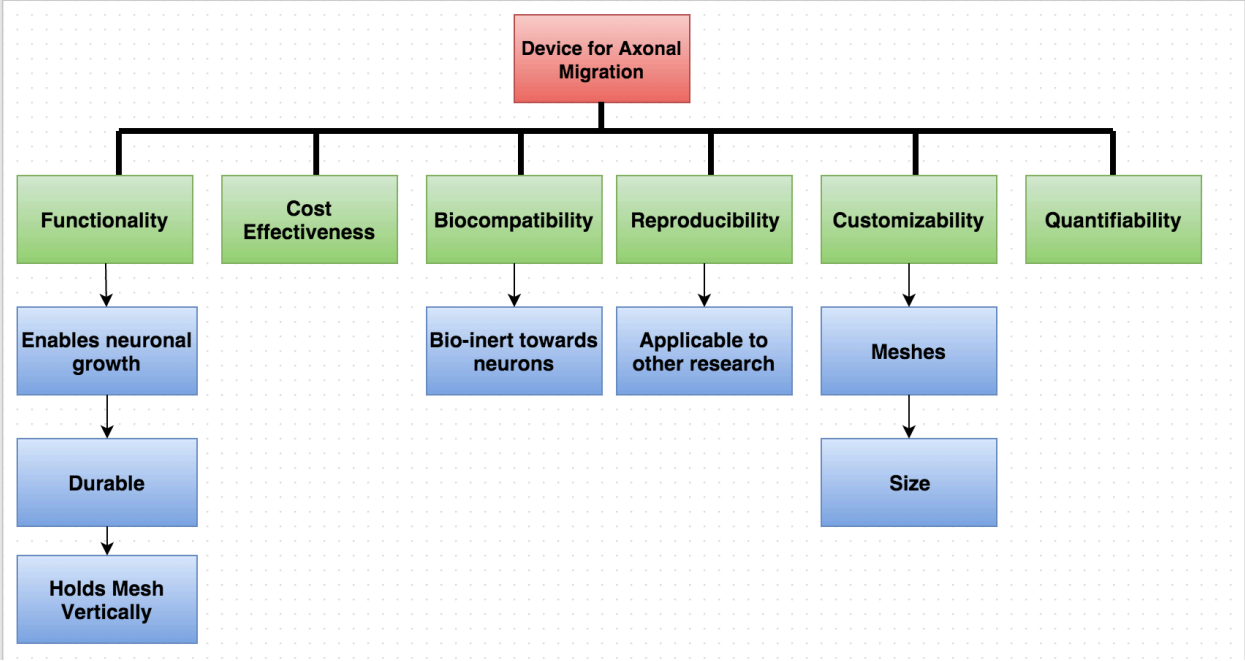


Figure 2: Objective Tree

Functionality: Many users will handle the device regularly; this makes the functionality of the device very important. Included in the functionality, the ability to grow neurons within the device will be paramount as success of the device hinges on neuronal survival. Also the durability of the device will increase the ease of function and handling of this device greatly. Lastly the mesh needs to be able to hold the mesh vertically to act as the barrier between the two wells of the device.

Cost effectiveness: A device to study axon extension is extremely useful in preclinical research for development of treatments towards neuronal regeneration after traumatic brain injury, stroke, and peripheral nerve injury. The type of research that uses devices such as ours would not be commercially feasible if the price is too steep as the cost of machinery and materials for testing is already high enough. The final price for this device will be discussed later on in the cost analysis section.

Biocompatibility: Since the device will be using neuronal cells, the device and all its components must not elicit any cytotoxicity.

Reproducibility: Since the mold for the device will be implemented into other research, the process of constructing the PDMS mold and any testing procedures should be able to be reproduced easily by others looking for a device such as ours as this will standardize results. The PDMS mold must be identical for every run of the experiment so it is necessary for the negative impression of our device (3D printed CAD drawing) to be able to be used multiple times without losing its shape while curing the PDMS or extracting the PDMS for use. Also mesh that emulates a fibrotic scar must be able to be reproduced easily in order for others to use it to further their research.

Customizability: Since the device will be applied to other research, there will be many variables that will change between researches, therefore it the device must be able to be customized for the test that is being performed in it. The customizability of the device will be important to help other research be furthered by being able to be tailored to their needs. Some of the customizability lies within the dimensions of the slit where the mesh will be placed, the shape of the PDMS well required by future researching teams, and the height of the mold in order to construct a deeper PDMS device for use with 3D migration of axons in the future. Lastly the composition and density of mesh must be customizable.

Quantifiability: Since this device is designed to test whether neurons can bridge a fibrotic scar, the device should allow monitoring and quantifying axonal extensions. In order to take these measurements, the device must be made of materials that can be used in fluorescence microscopy.

3.2.1.2 Secondary Objectives

Enable neuronal growth: Since the focus of the design is to extend axons through a fibrotic scar, the device must support the culture of neurons. In order to achieve the primary objective of functionality, this secondary objective of enabling neuron growth serves as a functional block. This means that without this sub-objective being met, the primary objective would not be complete.

Durability: Handling of this device will be frequent as many test will be performed in the hopes of culturing neurons. In order to achieve the primary objective of functionality, the secondary objective of durability must be met in the device. This means that the device must be able to be handled, sterilized, and stored without failures.

Holds Mesh Vertically: Since the device will study a fibrotic scar and migration of axons through the scar, the device must be able to hold the mesh vertically as to provide a stimulated scar and pseudo 3-D hostile environment in which the axons must migrate through.

Bio-inertness: Since this device will not be placed inside the body, the compatibility with body tissues will not be a concern, however, since the cells (neurons) will be alive, so at the very least bio-inertness must be taken into account. If the material of the device causes unwanted infection to the neurons, then the secondary objective of bio-inertness will not be met. If the neurons get infected by anything in the device, or any tools used in the process of setting up the experiment then any results following will be deemed inconclusive. If the bio-inertness is not met, then the primary objective of biocompatibility will be incomplete. The importance of this secondary objective is that ensures that the device is at least bio-inert towards the cell lines.

Applicable: This device must fulfill the needs of the current research techniques/methods. This means that other research teams must find this device relevant enough to implement it into their research. If this secondary objective cannot be met, then the primary objective of reproducibility will not be complete. The importance of this secondary objective is that ensures that the primary objective of reproducibility is met, as if other people find this device useful, they will be use it and reproduce it.

Mesh to culture fibroblast scar: The simulated fibrotic scar is to be grown on a biocompatible mesh. The pore size and biocompatibility of this mesh is of great importance because it needs to be bio-inert, enable the attachment of fibroblasts, as well as having a pore size that allows axons to migrate through.

Size: Incorporating an adaptable size for the overall experiment can allow for further research to be conducted on a large scale.

3.2.2 Evaluations of Objectives

A pairwise comparison chart was used to rank objectives to give them significance. In order to do this, the objectives were matched up against one another. If the objective was deemed to be more important to the project goals, then it received a score of one. If that objective was deemed less important to the project goals, then it received a score of zero. If both objectives were deemed to be equally as important then the score received a half (.5). In the tables below, the primary objectives were first ranked then the secondary objectives within the applicable primary objectives were ranked using the pairwise comparison chart.

Table 1: Primary Objectives Pairwise Comparison

Objectives	Functionality	Cost-Effectiveness	Biocompatibility	Reproducibility	Customizability	Quantifiability	Score	Rank
Functionality	x	1	0.5	1	1	1	4.5	1
Cost-Effectiveness	0	x	0	0.5	1	0	1.5	3
Biocompatibility	0.5	1	x	1	1	1	4.5	1
Reproducibility	0	0.5	0	x	1	0.5	2	2
Customizability	0	0	0	0	x	0.5	0.5	4
Quantifiability	0	1	0	0.5	0.5	x	2	2

Table 2: Secondary Objective Pairwise Comparison

	Enables Neuronal Growth	Holds Mesh Vertically	Durable	Score	Rank
Enable Neuronal Growth	x	1	1	2	1
Holds Mesh Vertically	0	x	1	1	2
Durable	0	0	x	0	3

Table 3: Secondary Objective Pairwise Comparison

	Meshes	Size	Score	Rank
Meshes	x	1	1	1
Size	0	x	0	2

Note: A score of 0 does not mean that the objective is not relevant, it was just ranked lower than the other objectives present.

Based on the pairwise comparison chart the ranked primary objectives are as follows:
Functionality/ Biocompatibility
Reproducibility/ Quantifiability
Cost-Effectiveness
Customizability

Based on the pairwise comparison chart the secondary objectives ranked were as follows:
Enabling neuron growth
Holds mesh vertically
Durable

Based on the pairwise comparison charts the secondary objectives ranked were as follows:
Mesh to culture fibroblast scar
Size

3.24 Constraints

From the initial client statement, we have identified several constraints to our design of the device that helped us revise the client statement to something more suitable for the amount of time available to us in Slovenia. The constraints are described below.

Cost: Since the device requires bio-inert materials and changeable attributes, the cost must not exceed the material budget. This will ensure that it can compete with the current day devices.

Number of uses: In order maximize the cost effectiveness of this device; the number of uses must exceed at least one. This means that the device must be able to be sterilized for multiple uses.

Sizing: The device must not be smaller than standard neuron cell culture plates, also it can not exceed standard cell plate size too as this will become cumbersome to transport and handle. Also the pore sizes of the membrane/ mesh must allow for some molecules to pass through while limiting others.

Viability of Cells: Since the device will be interacting with neuron culture, components that prevent toxins from the air must be in place (such as covers for the cell plate). Also proper handling and storing procedure of the device must be followed in order to ensure that the neuronal cells survive the testing process.

FDA Regulations and Patent Copyrights: Since this device deals with live cells in hopes for clinical treatments, all FDA regulations must be adhered in order to ensure that this device can be placed on the market. Also since nerve regeneration has been a topic of interest for many

years, it will be important to look at patents and products currently in the market to avoid any copyright infringements by our device.

3.3 Standard Design Procedure Requirements

In order to ensure the success of our project and device, industry regulations must be adhered to. Some primary regulations that must be considered are sterilization and biocompatibility. From the International Organization for Standardization (ISO), the major standard procedure for sterilization is ISO 11737-2:2009 which outlines strict rules for how medical devices must be sterilized. For biocompatibility, the standard procedure is ISO 10993-1, which helps determine if a device is biocompatible. Another standard that need to be met was ISO 10993-5. This dealt with cytotoxicity, which is important since the device is dealing with live cells. Other regulations are governed by United States Pharmacopeia (USP), which oversees reagents that are used in devices. Some reagents that are used in our device are the medium and other supplements that are needed to grow neuron cells; these must be within the USP guidelines. Also lab safety standard operating procedures need to be met at all times, within this the right biosafety level must be met and the right equipment to handle that must be met as well. Lastly since the team is testing materials that could be implemented into a medical device, the American Society for Testing and Materials (ASTM), an international standardization organization oversees the testing of materials. In particular, ASTM F813 is used to evaluate "Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices". (ASTM, 2012).

3.4 Financial Approach

For the purpose of budgeting the project, current prices of materials were used to estimate the total cost in order to complete the project. Many of the expenses for the project were supported by the University of Nova Gorica but it is important to note the price for the testing material as they were well above the budget allotted by WPI for reimbursement. Without the right testing material, this project would not be deemed possible. The main expense for the study will be from the neurons that we obtain to study and use in the device. The neurons will be supplied by the University of Nova Gorica however it is important to note that typically these cells cost about $128 for 1 x 106 cells. Other material used for testing include FGF-2, Mitomycin-c, Fibrin glue and PDMS. The prices for these testing material also covered by University of Nova Gorica are shown in the table below.

Table 4: Testing Material Cost

Testing Material	Price for stock material
Nitrocellulose membrane	~320 USD for one roll
Neurobasal media with NGF (neuronal growth factor)	~60 USD per bottle
3T3 fibroblasts for production of scar tissue	~120 USD for 100ug/ml
Fibroblast growth media:	~690 USD for 1 mg
Fetal bovine serum (FBS)	~212 USD for 100 mL
Trypsin/EDTA	~13 USD for 100 mL
Phalloidin 488 for visualization of axonal extensions	~175 USD for 100 µg
DAPI for DNA staining	~86 USD for 10 mL
Decellularization solutions with Triton X-100	~60 USD for 100 mL
6-well tissue culture plates (glass)	~25 USD for 5 plates
Other tissue culture plastic (pipettes, Petri dishes, media bottles, filters, pipette tips, centrifuge tubes, tissue culture flasks)	Inconclusive

3.5 Experimental Approach

The experimental approach involves the procedure and description of the cell culture methods, the quantification process and description of experiments used to validate the individual system parameters.

3.5.1 Cell Culture Methods

The cells used in the project were mouse NIH 3T3 fibroblast cells, rat dorsal root ganglion cells (DRG), and Trigeminal ganglion neuron (TG) cells. This immortalized 3T3 cell line was originally isolated and initiated in 1963 from mouse embryonic tissue (Leibiger, 2013). DRG are live primary neuronal cells that are retrieved from the 18th day of embryonic Sprague/Dawley rats from regions of their brain (DRGs, 2013). TG are sensory neurons taken from the trigeminal nerve or fifth cranial nerve (CN V) of mice.

3.5.1.1 Fibroblast cell culturing procedure

In order culture fibroblast (NIH 3T3) for use, the following protocol was used:

Cultures were originally kept in DMEM with 10% FBS.

For sub-culturing the fibroblast:

1. Aspirate out medium and wash with .25 % Trypsin and .53 mM EDTA.
2. Add 1-2 ml of Trypsin
3. Let it incubate for 5 minutes
4. Check that the cells have detached under the microscope
5. Add 3-4 ml of DMEM to neutralize the Trypsin
6. Take the 5 ml of medium, cells and Trypsin and place in a centrifuge tube
7. Centrifuge for 5 minutes at 1,500 RPM
8. Aspirate out the medium leaving just the pellet of cells
9. Re-suspend the cells with new medium
10. Take 200 microliters of medium and place on hemocytometer for counting
11. Once number of cells has been calculated, place desired concentration of cells into culture flask and add desired amount of medium and incubate

3.5.1.2 Neurons cell culturing procedure

The Trigeminal ganglia sensory neurons and dorsal root ganglion used in the project were supplied by The International School for Advanced Studies (SISSA) research facility in Trieste, Italy. The neurons were cultured following this protocol:

1. TG/DRG neurons came with enzymes that included collagenase, DNAse, and trypsin in a 500 microliter tube.
2. The cells were shaken at 37 degrees Celsius
3. Cells were re-suspended using a pipette.
4. 25 microliters of trypsin inhibitor and 25 microliters of FCS was added to the tube.
5. It was spun down at 1000 RPM for 5 minutes
6. Medium was aspirated out and new medium was added
7. The cells were re-suspended with new medium and plated

3.5.2 Experiments to Validate Individual System Parameters

In order to test the device, experiments were run on the individual system parameters.

3.5.2.1 Mesh

Nitrocellulose membrane (Thermo Scientific) was cut into rectangles 28 mm long and 7 mm wide, the size customized for the PDMS device. Membranes were sterilized by autoclaving and placed into 6-well plates. Fibroblasts were seeded onto membranes at a density of 50,000 cells/ml and 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% CO2 in air in a humidified atmosphere. Cell density was monitored daily and evaluated by DAPI fluorescence (200 ng/ml). At the time of 90% of confluency, fibroblast populated membranes were treated with xx mg/ml Mitomycin-C for 3 hours to permanently inhibit cell proliferation.

Hypothesis:

The team expects the fibroblasts to produce an effective extracellular matrix-derived scar after 3 days of incubation.

Experimental design:

The team used a 6 well plate to run the experiment. Each plate was seeded with the same number of cells. The mesh was tested for growth on days 1,2,3,6,7, and 8. On each day, the mesh being tested that day was treated with for 15 minutes to fix and permeabilize the membrane. The mesh was then treated with Hoechst 33342 (Trihydrochloride, Trihydrate - 10 mg/mL; ThermoFisher Scientific) to stain the nuclei of the cells. The Hoechst stain was applied at a concentration of 1:1000 for 5 minutes then the mesh was washed with PBS 2-3 times before imaging. Each sample was imaged using fluorescence (excitation 350, emission 461) on Olympus X81, images acquired using SlideBook software (Olympus) and the cell count was determined using ImageJ (NIH).

3.5.2.2 FGF-2

FGF-2, as discussed in 2.3.5, is a fibroblast growth factors that has been described to alter expression of several extracellular matrix coding genes (Kashpur et al., 2013). The primary expected effect of FGF-2 is to induce fibroblasts to produce extracellular matrix scar with less collagen I, more collagenase I (MMP1), more fibronectin and more laminin.

Hypothesis:

The team expects the medium with FGF-2 will cause fibroblasts to produce a less fibrotic (collagen I containing) scar and consequently allow for axonal penetration.

Experimental design:

The team cultured the fibroblast cells on the nitrocellulose mesh with a medium that already includes the FGF-2 (4ng/ml) and culture fibroblast in a medium without the FGF-2 as a control. Depending on the first experiment of culturing fibroblasts on the mesh, the team determined how long to incubate the cells in the medium of FGF-2. The team used a concentrations of FGF-2 at 4 ng/mL of medium as published previously (Page et al., 2009, 2011,

Kashpur et al., 2013, Grella et al., 2015). In order to test the results, the cells were fixed and stained in a similar fashion to the experiment in 3.5.3.2 with fixing the cells with Paraformaldehyde (4% in PBS; Chem Cruz) for 15 minutes and staining with DAPI and Alexa Fluor 488® in order to observe the different extracellular material around each nucleus and more importantly see the difference between the fibrotic scar created in presence of FGF-2 and without the presence of FGF-2 in the culturing media. These results will be discussed in Chapter 5.

3.5.2.3 Decellularization of Nitrocellulose Membrane

The decellularization of nitrocellulose membrane will allow us to prepare a fibrotic scar to use as part of our device, without having any interference on neuronal activity from the fibroblast cells. Since the fibroblast cells have a tendency to migrate quickly, the team did not want those cells to migrate on top of the neurons and disrupt normal behavior. Decellularizing the mesh will leave behind the extracellular matrix without any reactive cells which the axons can hopefully pass through.

Hypothesis:

Decellularizing the fibrotic scar will leave behind only the extracellular matrix created by the fibroblast and get rid of any reactive fibroblast cells that might interfere with the neurons.

Experimental design:

Once the fibroblasts on the mesh are confluent, decellularization of the mesh is performed. The decellularization method that was used was by using Triton-X-100 as presented in the article by Xu et al. (2014). First the confluent mesh is taken out of the media they were incubated in and washed with PBS twice. Then the mesh is placed in a bath of Tris-HCl buffer with 3% Triton X-100 (Sigma), 0.1% EDTA and complete ULTRA Tablets (Roche), and washed for 24 hours under continuous shaking. After 24 hours, the mesh is removed from the bath and placed in a PBS bath and washed for 24 hours. This removes any cellular material form the mesh leaving behind the ECM.

3.5.2.4 Axonal Extension or Migration

The most important parameter that needed to be validated was the axonal extension or migration of the neurons through the mesh alone. This was an important parameter for the design because if the team was able to validate that axonal migration is possible through the 0.4 µm pores in the mesh, then we can move forward with the current design of the device in using the 0.4 µm porous mesh.

Hypothesis:

The team expects the axons to migrate/ extend through the 0.4 µm porous mesh and the neuron cell body to be held back because the neuron is too big to pass through the mesh.

Experimental design:

In order to test whether the axons will extend through the mesh without any fibroblast, the team cultured neurons in one of the wells in the device with a nitrocellulose mesh acting as the barrier. The neurons were tagged with Alexa Fluor 488® to track the axonal extension/ migration. It was important to verify that no cell bodies were present on the other side of the mesh. If the cell bodies were present on both sides than the axonal extension would not verify correctly that the axons migrated through the mesh and not just extended on their sides. Once this was verified, the immunofluorescence imaged the outer membrane of the axons to show the extension. The culture was also tagged with Hoechst 33342 diluted at 1:1000 in PBS to track the nuclei of the neurons.

Chapter 4 Alternative Designs

The purpose of this chapter is to guide the reader through the different designs that were considered before the final design was conceived. Having all the designs compared against each other provides the reader with a clear understanding of why the final design was chosen over the alternative designs and how it best fit the client's needs.

4.1 Needs Analysis

A needs analysis is vital to providing the functions and specifications that the device must meet in order to be considered viable. After functions and specifications were determined as a guideline for the device, means for accomplishing the functions were established and implemented into the design.

4.1.1 Design Functions

First step in a need analysis was to determine the principal functions that would be needed to ensure the success of the final design. The functions were ranked in importance to the completion of the design below:

1. Enable neuronal extension
2. Enable seeding on mesh
3. Provide access for measurements
4. Retain integrity through sterilization process for reuses

Enable neuronal extension: The design must meet the expectations of the advisors and client that the device will be fully functional in neuron culture, therefore the enabling of neuronal extensions is paramount. This function is necessary in showing that neuronal extension is possible even in the presence of scarring.

Enable seeding on mesh: It is known that axons can extend normally, however, the scarring process after an injury often limits this. The necessity of being able to complete this function is

that providing a scarred region for the neurons to attempt to pass through will serve as a model for modifying a scar-containing hostile space in an in vivo injury.

Provide access for measurements: Without measurements a design and or device will be deemed null. Therefore, the materials and methods used will allow for measurements to be taken in the form of imaging different aspects of the process of neuronal extension through scarring.

Retain integrity through sterilization process for reuses: The cost of projects usually depends on how many times an experiment must be run; therefore, it is important that the device be able to be sterilized for multiple uses without losing any integrity. This function will allow the team to keep cost low while giving reproducibility in not only the results but the uses as well.

4.1.2 Design Specifications

Once the functions had been determined, specifications for the device were conceived to ensure success of the device. After close examination of the client statement and functions for the design, the specifications are as follows:

1. Pore sizes that allow axon extension but not fibroblast
2. Ensure that the fibroblast is seeded directly onto the mesh and allowed to proliferate until the required confluency is met so treatment can be implemented.

Pore sizes that allow axon extension but not fibroblast: Making a scarred region is critical in the success of the project, therefore the pore sizes of the mesh must serve dual purposes. First it must allow axonal extension through it as this will show that migration through a scarred region is possible. Secondly it must be able to support fibroblast growth without allowing it to penetrate the pores as this will ensure that fibroblast does not interfere with the axons attempting to pass through the pores. The optimal pore size for both purposes was found to be .45 microns.

Ensure that the fibroblasts are properly seeded to implement treatment: It is important that the fibroblast are properly seeded as treatment of FGF-2 and Mitomycin-C will be applied to

them. FGF2 has been shown to help rearrange the extracellular matrix to allow for openings in the fibroblast that the axons can extend through, and the Mitomycin-C will stop replication of the fibroblast after the mesh has been covered with them to ensure that the scarred region is contained. Mitomycin-C still allows for regular cellular behavior however; this means that extracellular matrix will still be created by the fibroblast. These treatments are pending on the seeding of fibroblast on the mesh and the culture of them onto the materials chosen for the mesh. Without this proper seeding of fibroblast, the treatments cannot be applied.

4.1.3 Functions-Means Analysis

Once functions and specifications have been established, means or how these functions would be completed were established. A chart below shows the relationship between functions and means with functions on the left and the corresponding means on the right hand side.

Table 5: Function-Means Table

Functions	**Means**		
Enable neuronal extension	FGF2	Proper Seeding	Labeling
Enable seeding on mesh	Treatments	Transwell	Nitrocellulose
Provide access for measurements	Proper imaging	Proper labeling	Standardized
Retain integrity through sterilization process for reuses	Autoclaving	Oven	Ethanol

Enable neuronal extension: In order to ensure full functionality of the device, enabling neuronal extension must be met. To complete this function, FGF-2 was used to change the composition of the extracellular matrix (ECM). It has been published by Kashpur et al. (2013) that FGF-2 down regulates most types of collagen and up regulates some laminin. This means that while regular fibrotic scars are mostly collagen based, which makes them dense, treated

fibrotic scars with FGF-2 can make the scar less dense due to the down regulation of collagen giving axons more a chance to extend through the scar. The second means to accomplish this would be proper seeding techniques, if the neurons cells are seeded properly either on top of the fibroblast or on the plate on either side of the scarred region, then with the right culturing they will begin to extend towards the scarred region, and with the less dense scar treated with FGF-2, migration through the scar could be possible. The last mean in determining extension of neurons is the labeling of neurons on one side of the mesh differently than the ones on the other side. If labeled neurons or parts of them are seen to be on the other side of the mesh, then this function of enabling neuronal extension is met.

Enable seeding on mesh: To satisfy the objective of a fabricated scarred region, enabling seeding of fibroblast on mesh must be met. To complete this function, the first mean is to use treatments of the fibroblast such as FGF-2 and Mitomycin-C. FGF-2 is a gene that helps up regulate certain parts of extracellular matrix such as laminin and down regulates collagen therefore making the scar less dense, which allows for a more optimal scar to be seeded onto the mesh. Mitomycin-C stops rapid proliferation of fibroblasts while keeping other normal functionalities such as formation of extracellular matrix (ECM). These two treatments will allow for fibroblast to be seeded on the mesh. The second mean is transwell plates, these special cell culture plates are used for invasion and migration studies where there is an insert with a mesh that allows for secretion of molecules it. Using this system would allow the team to forgo creating meshes and seeding, it would reduce the number of steps to just seeding fibroblast onto the mesh in the transwell. The last mean is the use of nitrocellulose membrane to seed the fibroblast on. This material is usually used for Western Blotting however; the properties of adhesion has made it a viable option to seed fibroblast onto it.

Provide access for measurements: In order to verify that axons successfully extended through the mesh, the neurons were imaged by tagging them with immunofluorescence stains to get a picture that specifically distinguishes how the cell bodies remained on one side of the mesh while the axons were able to extend to the other side. The immunofluorescence stains used for measuring the success of the device were Hoechst 33342 (for DNA staining) and Alexa Fluor 488® (for staining the actin). The combination of these stains provides a picture of the nucleus and actin present in cells in order to visualize extension of axons throughout the cell culture. Retain integrity through sterilization process for reuses: In order to reproduce results and uses, the sterilization of the device must be met. To accomplish this function, the first mean is autoclaving. This is a method where contents are subjected to high pressure via steam to sterilize them. Since the device will be made of mostly polystyrene and PDMS, these materials have been noted to withstand autoclaving without changes to their structural integrity. The second mean is to use an oven to sterilize the device. This method uses high heat to sterilize the device, where polystyrene and PDMS if kept in an oven safe container will be able to sterilized via this method. Ethanol is used to sterilize between uses as it kills cells and provides on the spot sterilization. Both polystyrene and PDMS are noted to be able to within ethanol treatment of sterilization.

4.2 Conceptual Designs

Transwell Plate:

The team first investigated the use of a transwell plate to conduct the experiment. The team theorized that the fibroblasts would be able to grow in the transwell insert on the mesh in various conditions then treated with Mitomycin-C. This would create a confluent, non multiplying scar that would simulate the fibrotic portion of a glial scar. Neurons could then be placed on top of the scar and encouraged with a neuronal growth factor to travel through the scar and membrane into the multiple well plate well.

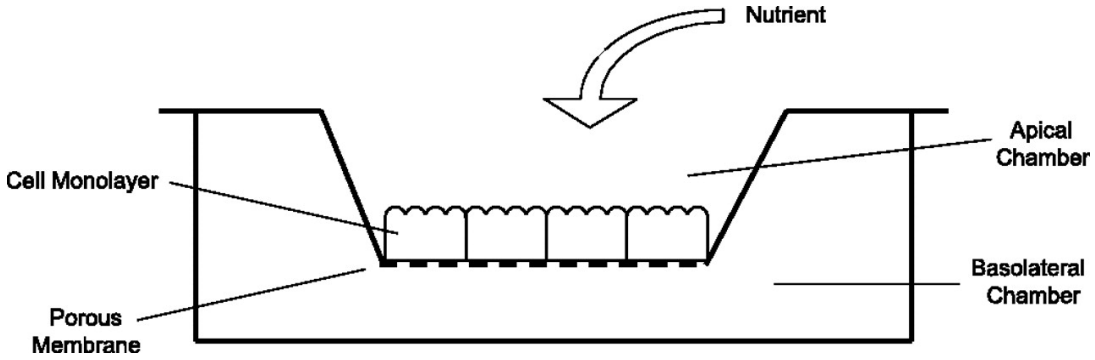


Figure 3: Transwell Schematic (Schnabl, 2009)

Figure 4: Transwell Dishes (Krackeler, 2014)

Design 1:

To create a scar, the fibroblasts must be seeded onto a mesh and then the mesh placed perpendicular to the plate. This design uses glass slides to mold PDMS into two rectangles. The glass slides would be held by a stand, and PDMS would be poured around the slides to create the mold. The smaller rectangles created would hold the simulated scar perpendicular to the plate bottom to create two chambers to hold neurons and their growth factors.

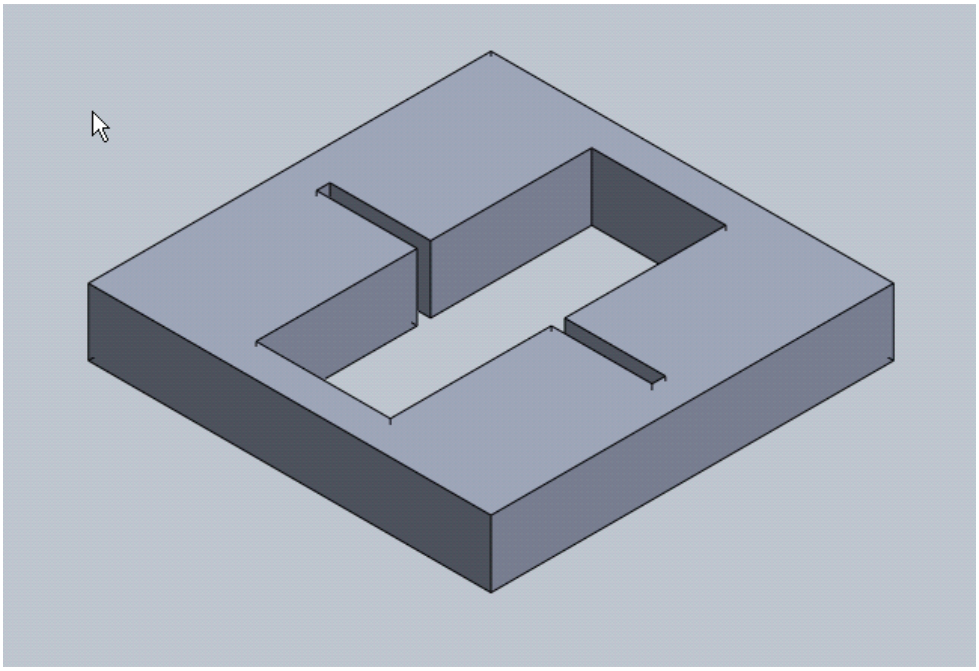


Figure 5: CAD Schematic of Device Design 1

Design 2:

The CAD drawing shown would be used to mold PDMS to create channels to hold the scar and create two chambers to hold neurons and their growth factors. The PDMS would be poured into the mold, allowed to set, and then removed.

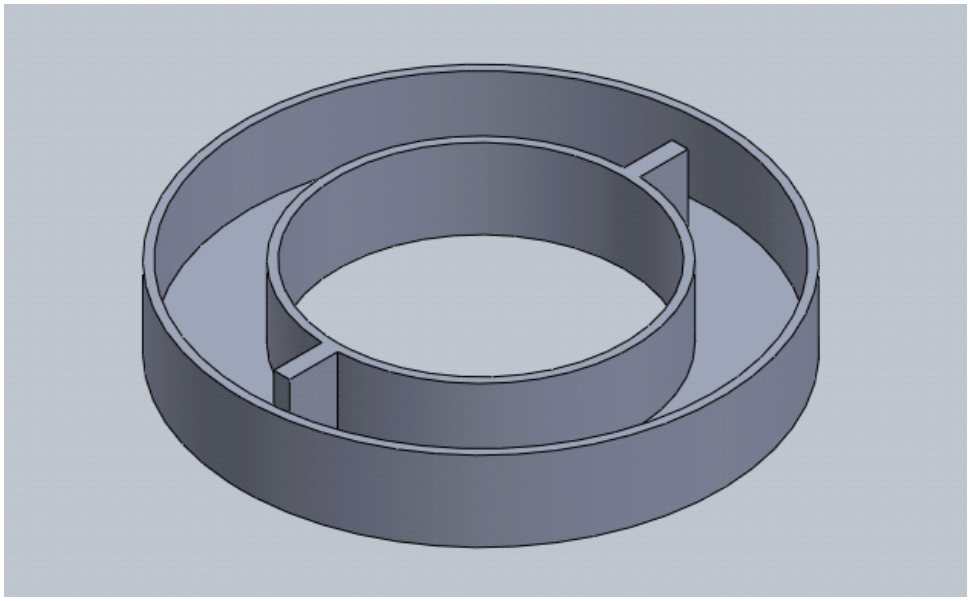


Figure 6: CAD Schematic of Device Design 2

Design 3:

The CAD drawing shown would be used to mold PDMS around the outside of the model. It would be placed in the well plate and PDMS would be poured around it and allowed to set. The drafted edges would make removal of the mold easier, reducing the chance of damaging the PDMS. The result would be a ring of PDMS on the outer edge of the plate with two grooves to hold the scar.

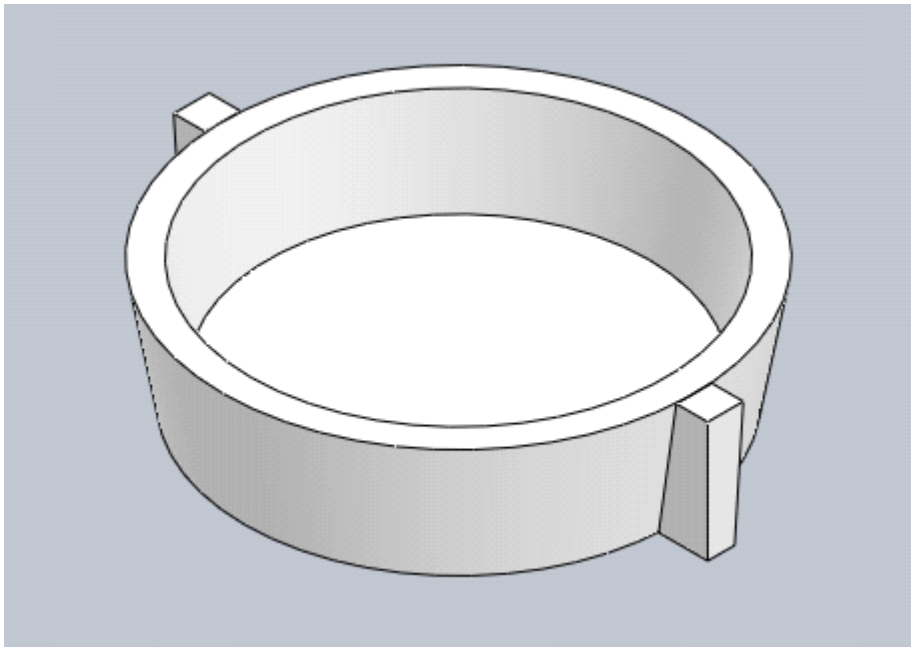


Figure 7: CAD Schematic of Device Design 3

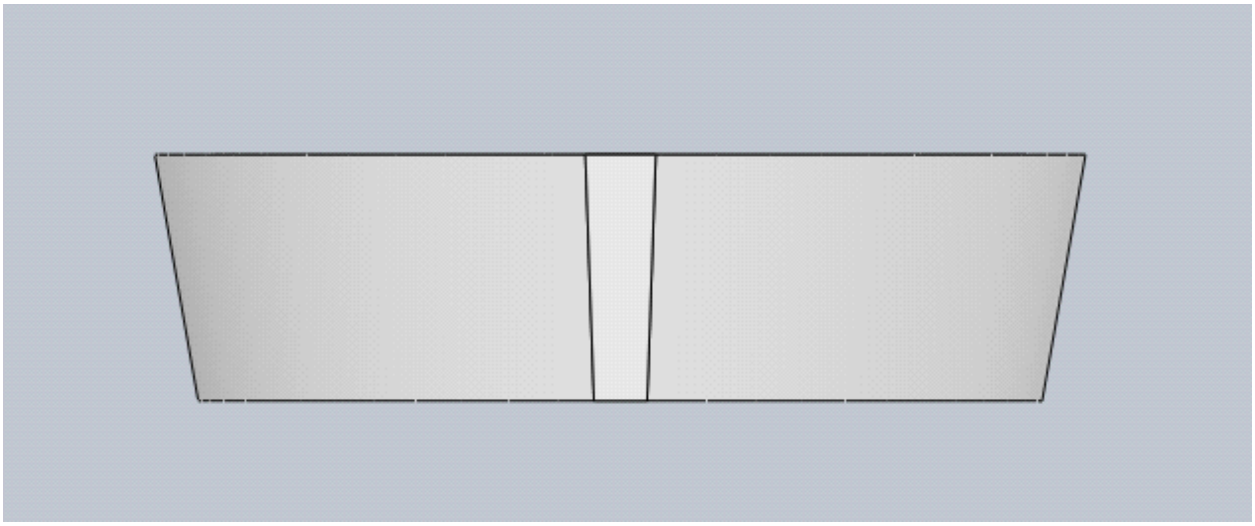


Figure 8: CAD Side View of Device Design 3

4.3 Final Design Selection

After analyzing different meshes and conceptual designs, a final design was selected that best met the client's needs and wants. Also a cost-benefit analysis and design matrix with the objectives and constraints of all the designs was conducted to further finalize the design.

4.3.1 Cost-Benefit Analysis of Designs

A cost-benefit analysis is used to help select a final design, it takes the designs and see which one is the most beneficial at the lowest cost. Since all the designs require PDMS, the deciding factors were the mesh material and the plating material. The transwell alternative was beneficial because it included the mesh and the plate in one system. This cut down on the cost of the device. The nitrocellulose model cost a little more for a roll of the membrane however, the multiple uses that can be obtained from a roll is worth the cost of the material, also needed was a well plate which is inexpensive. The designs would all use a nitrocellulose membrane or an equivalent polymer. The biggest factor was the ability to be imaged, and nitrocellulose was vastly easier to image than the transwell, this made the cost of the nitrocellulose worth it as having an easy and effective way to image the processes of the neurons was paramount to the success of the project.

Table 6: Product Cost of Designs

Product	**Cost**
Transwell Plate	~ 230 $ (Sigma-Aldrich)
Nitrocellulose Membrane	~ 320 $ a roll (Sigma-Aldrich)
Glass Well Plates	~ 5 $ per plate
PDMS	60-200 $ (Sigma-Aldrich)

As seen, per uses, the nitrocellulose was the most cost effective and the easiest to image. This made it the clear choice for mesh. The different designs all cost about the same in manufacturing cost, however, design 3 was easier to handle with the PDMS. Therefore, design 3 was the most beneficial with the nitrocellulose membrane.

4.3.2 Alternative Design Evaluations Matrix

An Alternative Design Evaluations Matrix is shown below. This chart takes the objectives and constraints previously established for the device and compares them to each other. The scoring is based on a 0-3 scale with 3 being the highest.

Table 7: Alternative Design Evaluations Matrix

	Functionality	Cost	Biocompatible	Reproducible	Customizable	Quantifiable	Viability of Cells	Sizing	Sterilization	Total
Transwell	3	2	3	3	2	2	Y	Y	Y	15
Nitrocellulose	3	2	3	3	3	3	Y	Y	Y	17
Design 1	2	3	2	3	2	3	Y	Y	Y	15
Design 2	3	3	3	2	2	3	Y	Y	Y	16
Design 3	3	3	3	3	3	3	Y	Y	Y	18

Following the analysis of the matrix, it was deemed that design 3 was most effective in meeting all the client's needs as well as being the most feasible alternative designs of all the designs. It should also be noted that the first two designs were a comparison of the type of membrane/mesh to use for the design. It was noted that the nitrocellulose was more feasible than the transwell plate.

Chapter 5 Design Verification

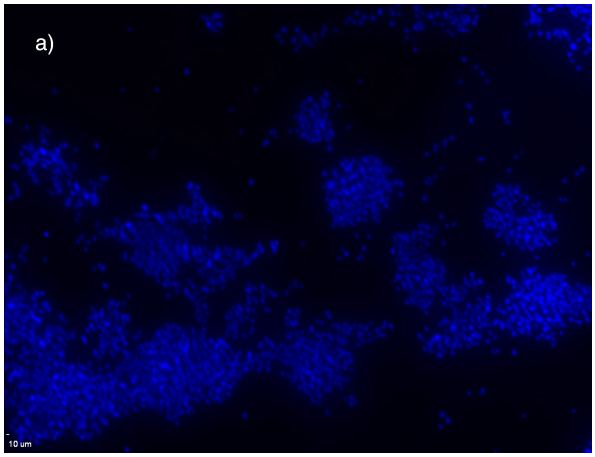
This chapter presents the team results from the experiments that were previously mentioned in the prior chapter. These results help to narrow down the design choice as well as making final conclusions about the project. The results are presented in order of their completion, starting with nitrocellulose membrane testing, to various treatments of membranes, to axonal migration testing and ending with PDMS mold and mesh testing.

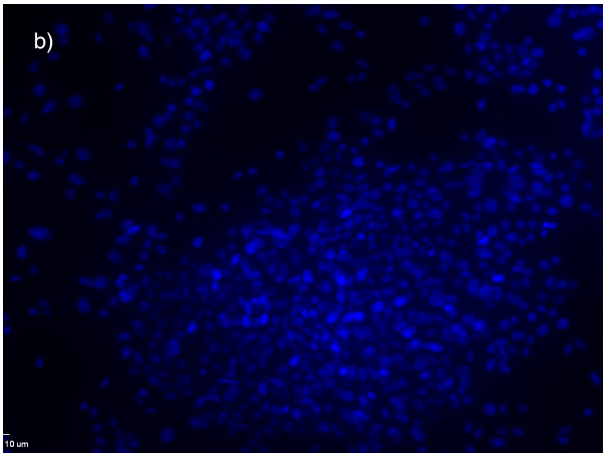
5.1 Nitrocellulose Membrane Experiments

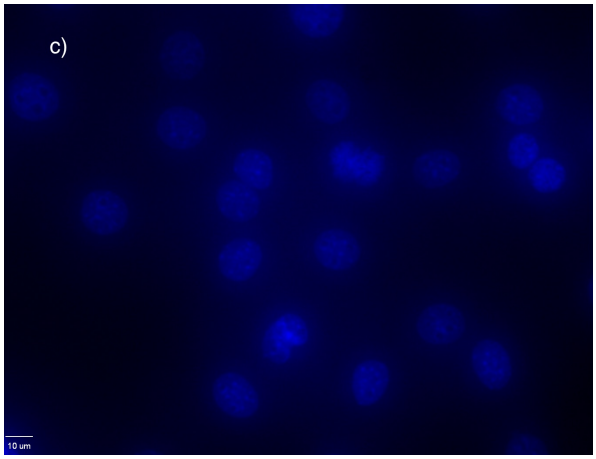
Nitrocellulose membrane has been used a scaffold for cell culture according to Li et al. article, "Use of nitrocellulose membrane as a scaffold in cell culture", therefore the team hypothesized that fibroblast would be able to grow on this membrane. The pore size that the nitrocellulose membrane was .45 microns to ensure that fibroblast would not grow through the pores (Li, 2013).

5.1.1 Initial Testing

To test that nitrocellulose membrane would enable fibroblast growth, the team cut out rectangular shaped membranes and placed them into petri dishes. Then the cells were taken from a separate culture and plated onto the mesh. After a day, the mesh was fixed with 4% paraformaldehyde and stained with Hoechst 33342 at a 1:1000 concentration. Also fixed and stained were a control of just mesh. Below are pictures of the initial cut mesh stained with DAPI. The blue shows nuclei from each cell of the fibroblasts.


a)
10 um


b)
10 um


c)
10 um


d)
10 um

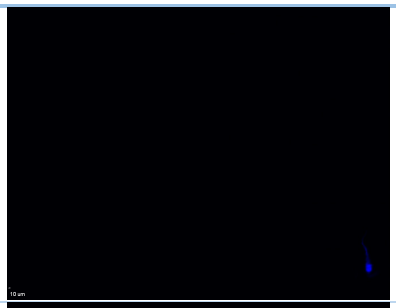
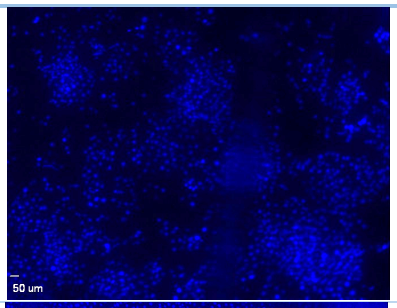
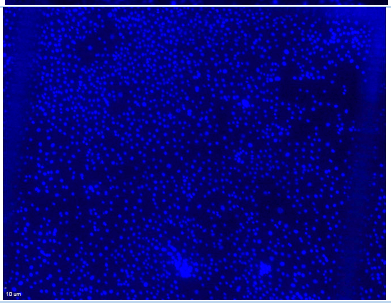
Figure 9: Pictures are of the nitrocellulose membrane plated with fibroblast and stained with Hoechst 33342 (blue) at a) 4x b) 10x c) 40x and d) control

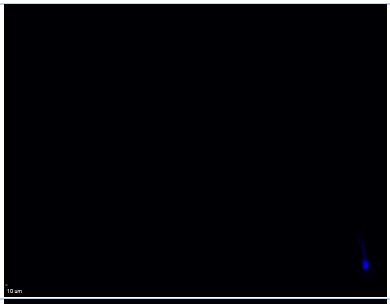
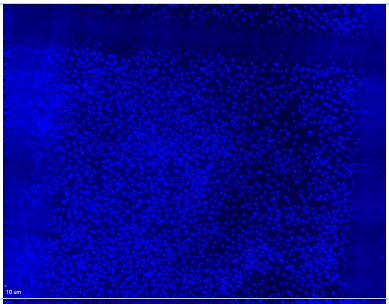
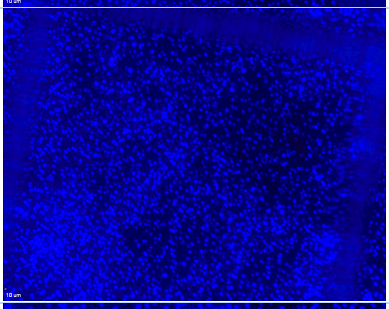
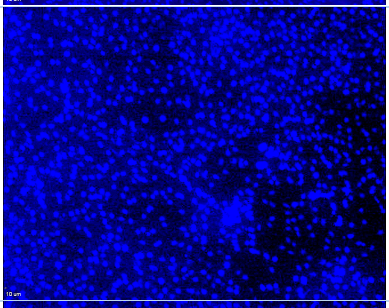
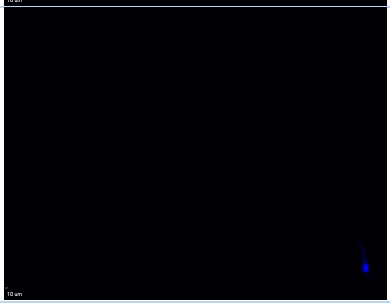
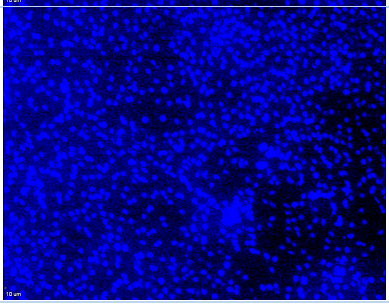
As the pictures show, the fibroblast was successfully seeded onto the nitrocellulose membrane. This is due to the blue staining of DAPI that allows the user to see the nuclei of the cells, since the nuclei are present, the cells successfully adhered to the mesh and proved that nitrocellulose membrane could be a scaffold for fibroblasts cells.

5.1.2 Confluency Testing

Once it was confirmed that fibroblasts were able to attach and proliferate on the membrane, the correct incubation period had to be determined. The team set up experiments to test the fibroblasts proliferation on days 1,2,3,6,7 and 8. Cells were plated by taking 500 microliters of cells and medium from a 5 ml mixture of cells and medium, this gave the starting cultures approximately 500,000 cells. After each day, the meshes were fixed and stained using the same method in the initial testing section. Also a control of just mesh was fixed and stained.

Table 8: Proliferation of Fibroblasts Confluency Experiment

Day	Control (Just Mesh)	4x Magnification of Fibroblasts on Mesh
Day 1		
Day 2		

Day 3		
Day 6		
Day 7		
Day 8		

As the pictures in the table show, at day 3 the fibroblast is mostly confluent on the mesh, any other day post day 3 is unnecessary if the desire results is a confluent mesh. Since fibroblasts proliferate at high rate, it does not take eight days to have them totally confluent on the mesh. Day 3 is an appropriate incubation period for getting full confluency on the mesh.

5.2 Treatment of Meshes

After the proper number of days for incubation for confluency was determined, different treatments of the mesh were performed. These treatments included fibroblast growth factor 2 (FGF2) and Mitomycin-C.

5.2.1 Fibroblast Growth Factor 2

FGF2 was introduced into the media upon plating the fibroblasts onto the mesh. The FGF2 was introduced at 4 ng/ ml. Two plates in a 6 well plate were treated with this concentration of FGF2 to give multiple results of the same treatment. Figure 10 shows the slight difference visible under the microscope between the two different types of scars, fibrotic scar cultured with and without FGF-2.

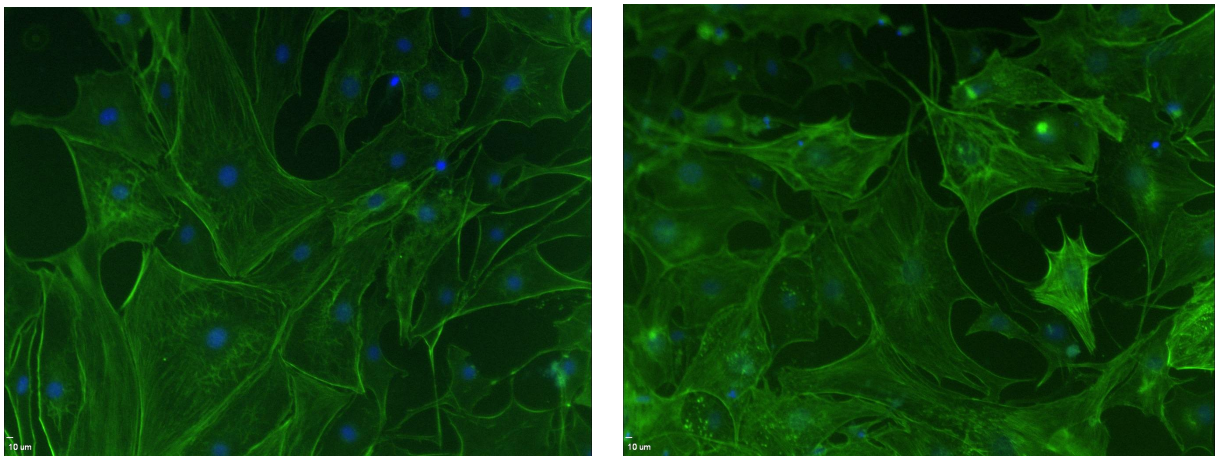


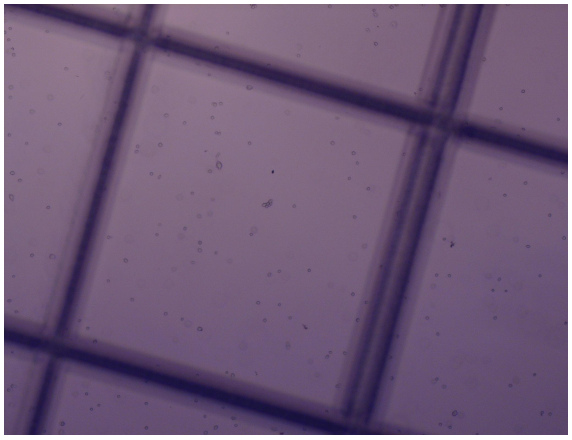
Figure 10: Fibrotic scar cultured without the presence of FGF-2 (Left) and fibrotic scar culture in medium containing FGF-2 (Right). Actin (green) and DNA (blue).

5.2.2 Mitomycin C

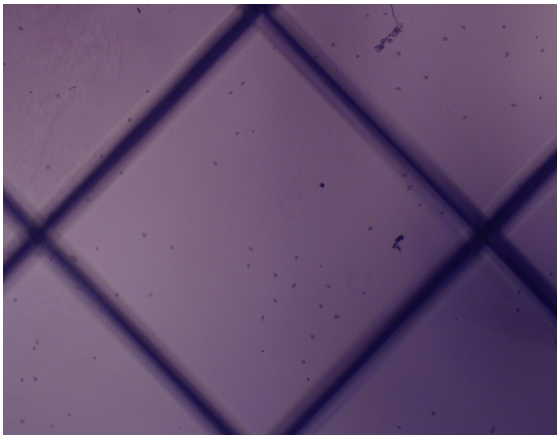
The fibroblasts were treated with Mitomycin-C at a concentration of 4 ng/ml to stop their proliferation. This was to keep the cells on the mesh and not allow them to proliferate onto the bottom of the wells where the neurons were being cultured. To test the effect of Mitomycin-C,

two plates were cultured with the same cell density of fibroblasts. One was treated with Mitomycin-C and the other was treated with regular medium. Pictures was taken from Day 1, Day 2, and Day 5. The densities of the two cultures were measured at the end of the fifth day to determine the effect of Mitomycin-C.

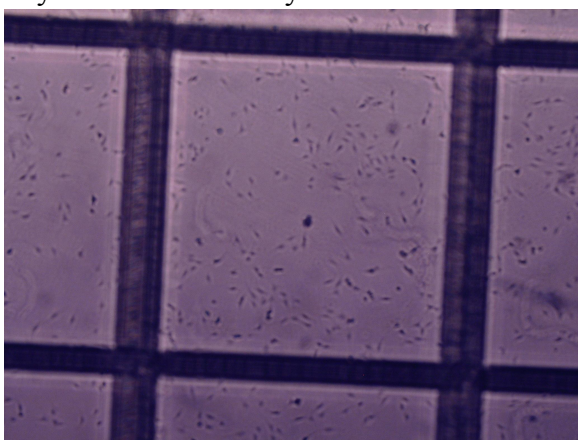
Table 9: Effect of Mitomycin-C



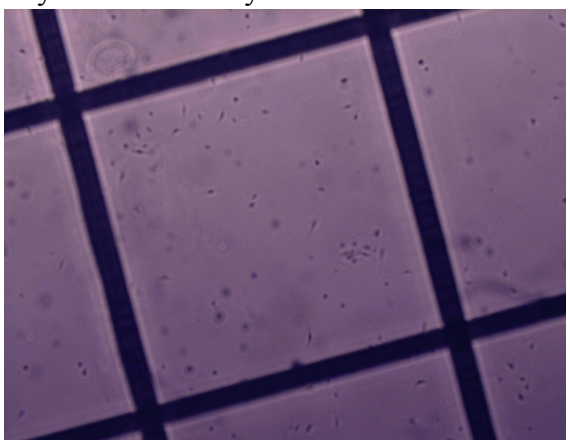
Day 1 Without Mitomycin-C



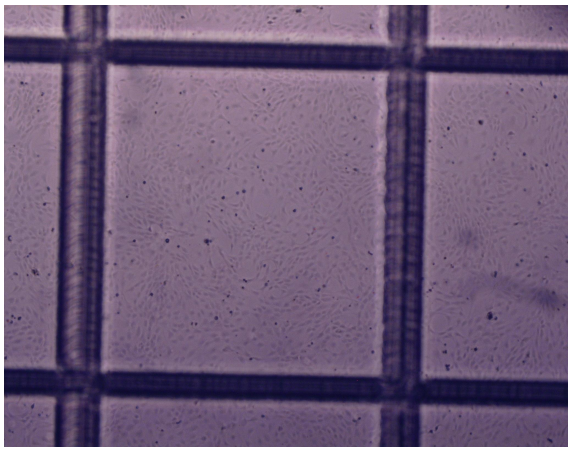
Day 1 With Mitomycin-C



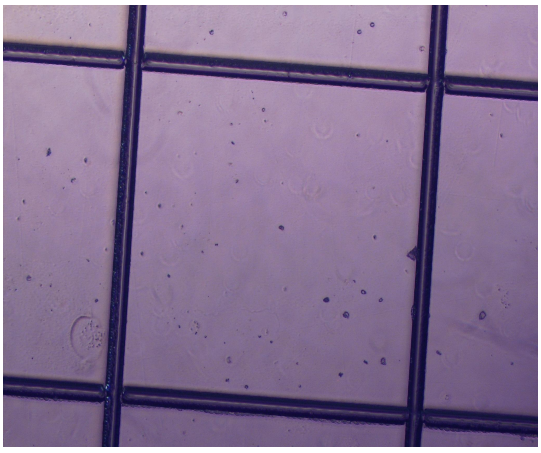
Day 2 Without Mitomycin-C



Day 2 With Mitomycin-C



Day 5 Without Mitomycin-C



Day 5 With Mitomycin-C

As the pictures indicate, the mitomycin-C treated culture did not proliferate and stayed about the same density. The untreated culture, proliferated normally and is shown to have a lot more confluency just after one day of culture.

5.2.3 Decellularization

Decellularization of the mesh was conducted once the fibroblasts on the mesh became confluent. Decellularization removed the cellular components leaving the extracellular matrix behind. The meshes were stained with DAPI DNA stain and Phalloidin-conjugated Alexafluor- 488 (green) to stain F-actin to visualize cellular cytoskeletal components that remained on the mesh. The images below show the results of the staining. The mesh was then stained with ponceau S dye to stain any protein on the mesh pink. The images below are the results the team got after staining the decellularized mesh.

Figure 11: Decellularized mesh Stained with DAPI

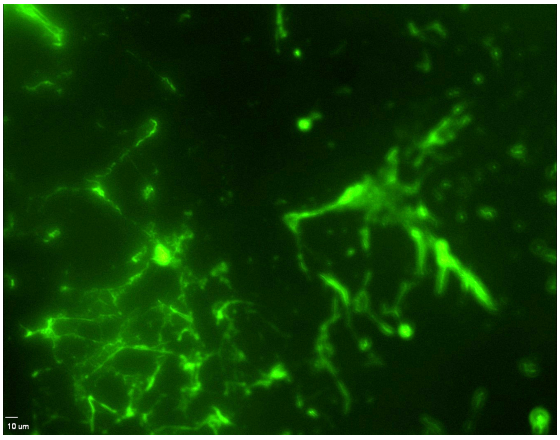


Figure 12: Decellularized mesh stained with Phalloidin

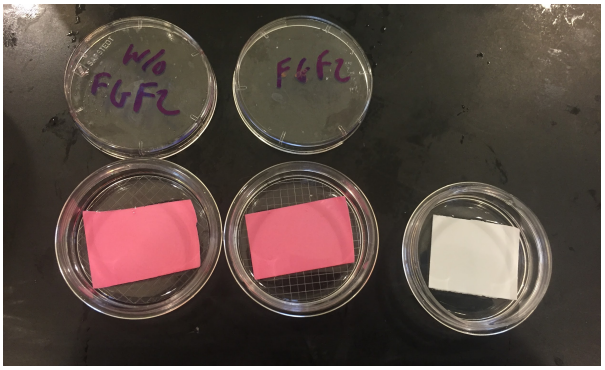


Figure 13: Decellularized mesh and control stained with Ponceau S stain.

5.3 Axonal Migration Through Mesh Experiments

After treated membrane tests were concluded, the axonal migration test through the mesh was conducted. This was done with three separate tests: just the mesh with no external treatment, fibroblasts with Mitomycin-C treatment, and FGF2 treated fibroblast with Mitomycin-C treatment as well. In all of the following experiments, fibrin glue was prepared and used to fix the meshes vertically in the well plates.

5.3.1 Fibrin Glue

In order to make the fibrin glue used in fixing the meshes vertically in the well plates, fibrinogen and thrombin were mixed and applied immediately to fix the mesh. The fibrinogen was available at a concentration of 0.0699 g/mL in Hepes/NaCl solution and thrombin was available at a concentration of 8 units/200 uL. The thrombin was diluted to a concentration of 10 units/ mL in a solution of 40 mM $CaCl_2$. The fibrinogen and the thrombin solution were kept on ice when mixing to prevent the fibrinogen from activating. For each fixation, 100 uL of fibrinogen and 100 uL of thrombin solution was mixed in a vial and placed at the bottom of the vertical mesh using a micropipette to obtain accuracy of placement. The fibrin glue was left alone for 10 minutes at room temperature to allow it to solidify.

5.3.2 Only Mesh (Not Mitomycin-C Treated)

To prove that axons could migrate through the pore size of the nitrocellulose membrane, the team seeded neurons onto one side of the plate with the mesh acting as the barrier. One well was treated with NGF to encourage a more rapid growth so that axons may extend through the membrane. One well was not treated with NGF to determine the effect of NGF on migration of the axons. The images below show results from staining after five days of incubation. The image on the left shows: the blurry regions caused by the fibrin glue, the dark region is the nitrocellulose membrane, the blue staining is the DNA of the neurons and lastly the red staining represents the actin present in the axons. The image on the right are the axons stained with Phalloidin 588 along with the DNA of the neurons in blue.

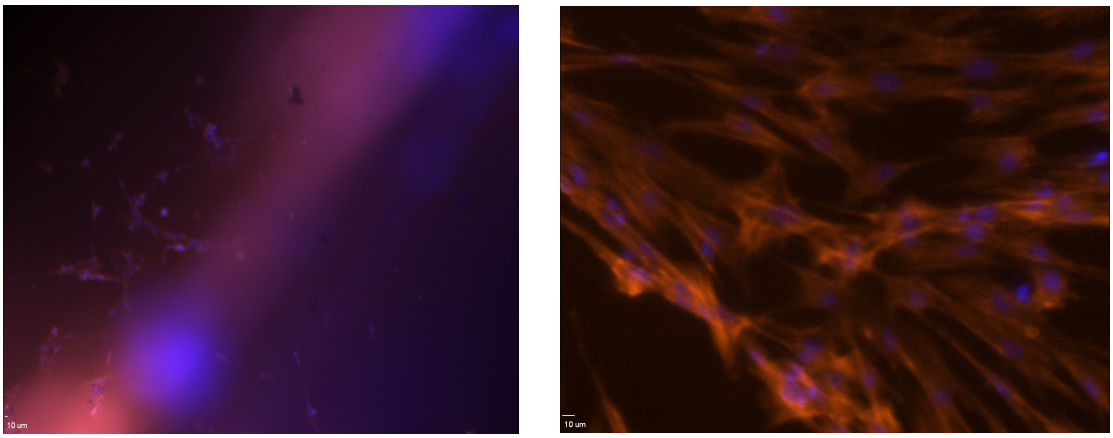


Figure 14: (Left) Image of the well showing both compartments separated by the mesh. (Right) Axonal extension stained with Phalloidin 588 (red) and the nucleus stained with DAPI (blue).

5.3.3 Only Fibroblast Mesh (Mitomycin-C Treated)

Following the experiment testing only the mesh, a fibroblast populated mesh was implemented to provide a scar like barrier for the axons to migrate through. One well was again treated with NGF and one without NGF. This mesh was treated with Mitomycin-C to ensure that the fibroblast would not proliferate anymore on the mesh as well as not into the neuronal culture. The images below show the axonal extensions that was viewed under the microscope in the

experiment where the fibrotic scar mesh was not treated with FGF-2. The blurry regions in the image depict the area where the fibrin glue was used to fix the mesh vertically in the device. The red stain in the image shows the presence of actin while the blue stain in the images represents the DNA, however, it was observed that fibroblasts had migrated off the mesh and mixed in with the neuronal culturing, making it inconclusive whether it was neurons, fibroblasts or both being stained.

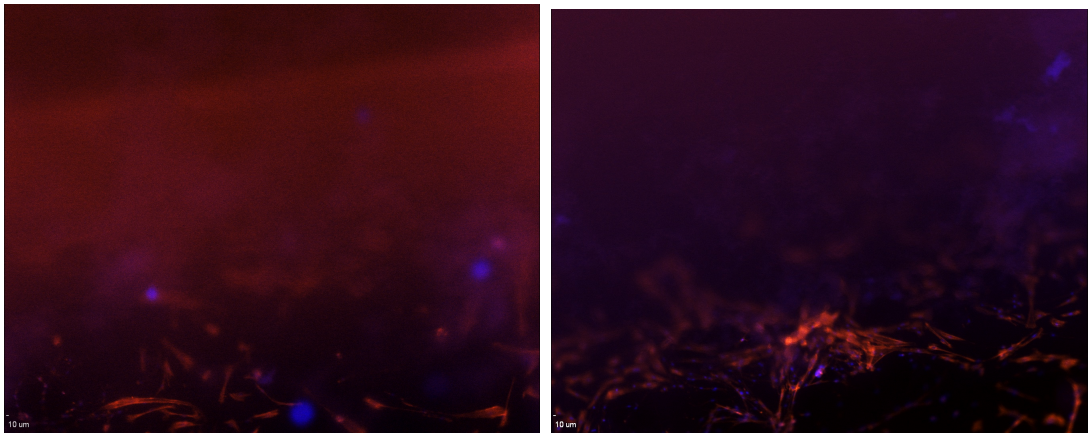


Figure 15: Axonal extension in the well with the fibrotic scar not treated with FGF-2.

5.3.4 FGF2 Mesh (Mitomycin-C Treated)

After testing the experiment with only the fibroblast mesh without any treatments, the final test was to treat the fibroblast with FGF2 to see if this would help with creating a less dense fibrotic scar for the axons to migrate through. One well was treated with NGF and another was without NGF. Both the meshes were treated with Mitomycin-C as well. The images below show two different regions along the mesh where the black region in the middle of the image is the mesh, the red stain shows the presence of actin and lastly the blue stain is the representation of the DNA present in the culture. It should be noted that fibroblasts migrated off the mesh and mixed in with the neuronal culture, making it inconclusive whether it was neurons, fibroblast or both being stained.

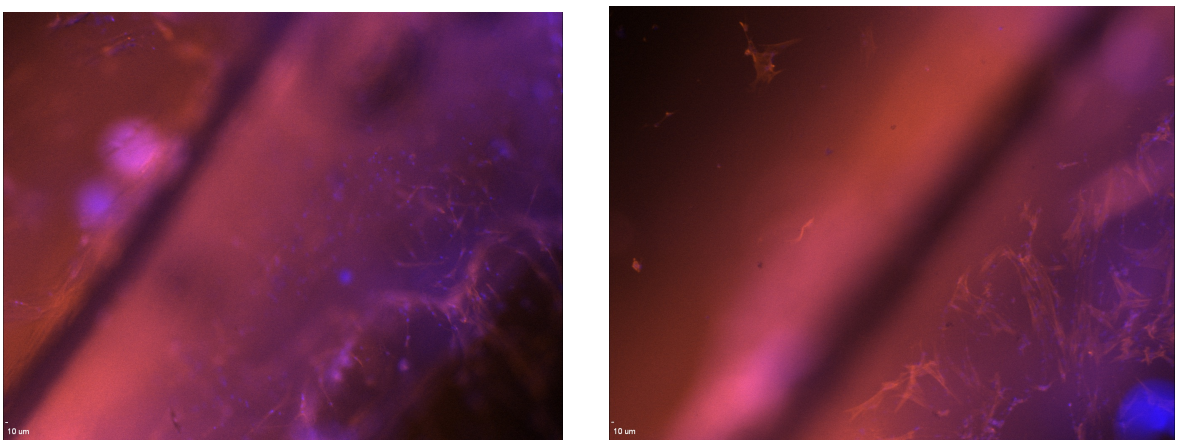


Figure 16: A mixture of fibroblast and neuronal cells near a fibrotic scar treated with FGF-2. Left picture culture was treated with NGF and the right picture culture was not treated.

Chapter 6 Final Design and Validation

In this chapter the final design will be discussed and verified that it met all the requirements set out by the team in previous chapters. Also details on the creation and implementation of the device are shown for those looking to reproduce the results and or the device.

6.1 Objectives Verification

Functionality

The requirements set by functionality are that the device enables neuronal growth, is durable, and holds the mesh vertically in the mold. The device successfully fulfilled all the subsections of functionality thereby verify that the objective of functionality was met.

Cost Effectiveness

The cost effectiveness of the device itself is met as the manufacturing cost is relatively low. The only materials needed are PDMS, the rapid prototype mold, nitrocellulose membrane, one glass well and fibrin glue. The estimated cost of one fully constructed device would be: $32

Table 10: Pricing for One Device

Product	Cost
PDMS (5 ml max)	$ 5.80 from a 50 ml bottle
Rapid Prototype Mold 1x	$ .70 each
Nitrocellulose Membrane	~ $ 9 from a pack of 10 (8.5 x 13.5 cm)
Glass well (1x)	$1.75
Fibrin Glue (200 microliters)	$14.64 from 2 ml bottle
Total (5 components)	~ $ 32 for one fully constructed device

Biocompatibility

The components of the device, the PDMS mold and the mesh, are not cytotoxic and combined did not produce a cytotoxic response. This indicates that the device meets the biocompatibility objective of the project.

Reproducibility

The device can be easily reproduced if the SolidWorks file as well as this report are available. By following the steps and procedures found in this report, anyone trying to reproduce the production of the device would be able to. Because the results from the axon migration were inconclusive, it cannot be said whether others would be able to reproduce these results.

Customizability

The PDMS mold can be modified for size and depth if the SolidWorks file is available. The only constraint to the customizability objective would be the size of the plate being used, because the ISO standard for plate sizes is set and cannot be adjusted.

Quantifiability

Though the device allowed for cells to be seeded directly onto the plate bottom, the visibility of the cells was still difficult to determine. The fibrin glue used to secure the mesh into place did not provide clear visibility of the neurons, and as the fibrin glue surrounded both sides of the mesh, it was impossible to determine whether axons were able to migrate through the mesh. The objective for quantifiability was not met.

6.2 Industry Standard Verification

Our final design of a PDMS mold with a neuronal culture must meet several industry standards if it were ever to be manufactured. The first being the International Organization for Standardization (ISO) 11737-2:2009 which is the major standard procedure for sterilization of a device. Our device adhered to this standard as the device was sterilized using various methods. The mesh was autoclaved which is an accepted method of sterilizing materials that are to be used in cell culture. The PDMS mold was sterilized using a combination of UltraViolet (UV) light and ethanol (70%) washing to ensure that no bacterial cells could contaminate our cell culture. Other standards that were taken into account were ISO 10993-1 (biocompatible), ISO 10993-5 (cytotoxicity), reagents standards governed by the United States Pharmacopeia (USP), and the

American Society for Testing and Materials (ASTM) F813. Our device met ISO 10993-1 and 10993-5 as PDMS, nitrocellulose, and fibrin glue are known to be bio-inert and not cytotoxic to the cells. Reagents such as fibroblast growth factor 2, mitomycin-C, and all media and other cellular reagents were purchased from accredited companies that follow the USP regulations for reagents. ASTM F813, is a standard that covers the evaluation of materials for cytotoxicity when in direct contact with cell cultures geared towards medical devices. This standard would need to be met if the device where to be manufactured into a medical device.

6.3 Final Device: PDMS Mold with Decellularized Mesh

A ring of PDMS was molded from a 3D printed mold designed to hold the mesh horizontal on the plate. The mold was placed in the center of each well in a plastic 6 well plate and held in place while the PDMS was poured around it and allowed to solidify by placing in the oven at 60 ℃for 1 hour. This was the maximum temperature allowed by the small oven available to us at the University. After the PDMS mold was solidified, the negative plastic 3D mold was detached from the PDMS. This was considered to be the final device used for the final experiment. The PDMS mold was sterilized prior to use with the neurons by being placed in a 70% ethanol bath for 30 minutes under the UV light in the Biosafety Cabinet (BSC). After 30 minutes, the PDMS mold was washed with PBS multiple times before being placed in the glass 6 well plate (coated with collagen to allow the neurons to fix to the well plate) The meshes seeded with fibroblasts (2 meshes with FGF-2 and 2 meshes without FGF-2) and the control nitrocellulose membranes (2x) were sterilized prior to being installed in the device. The fibrotic scar mesh was decellularized following protocol and allowed to sit in a PBS bath for 24 hours while being shaken. After the decellularization process, the mesh was sterilized under the BSC by being placed in an ethanol bath for 10 minutes. After being washed with PBS multiple times,

the mesh was ready to be fixed in the device. 6 different meshes were used in the experiment with the following characteristics:

Table 11: Mesh Treatment Experiments

Mesh Type	Treatment
Fibrotic Scar	With FGF-2, Neurons with NGF
Fibrotic Scar	With FGF-2, Neurons without NGF
Fibrotic Scar	Without FGF-2, Neurons with NGF
Fibrotic Scar	Without FGF-2, Neurons without NGF
Nitrocellulose Membrane	Neurons with NGF
Nitrocellulose Membrane	Neurons without NGF

The 6 different meshes were placed perpendicular to the bottom of the plate with the edges placed in the grooves created by the mold and fixed in that position with fibrin glue as can be seen below.

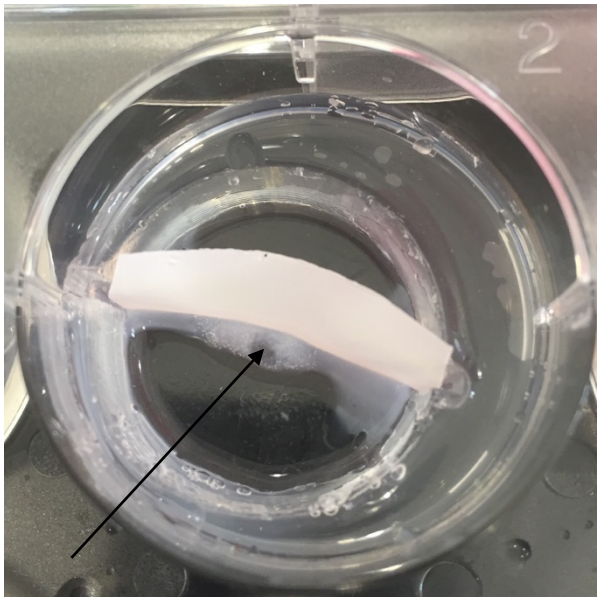


Figure 17: The Opaque White Region Represents the Fibrin Glue

In the six well plate shown below in Figure x., as aforementioned two of the plates had meshes that were treated with FGF2 (A1, B1), two had meshes that were not treated with FGF2 (A2, B2), and the last two of the plates had meshes without fibroblasts or FGF2 treated "fibrotic scar" (A3, B3).

Figure 18: Final Test Plate

Neurons were plated on one side of each of the meshes and incubated for 4 days with media changes every day to avoid infection. The top row of well plates had NGF present in the media with the neurons, while the bottom row of the well plates did not have any NGF present in the neuronal media. The side where the neurons were not plated had only the neuronal media. After 4 days, the neurons were fixed and stained with DAPI and phalloidin stain to image the nucleus and the actin in the cells.

6.4 Impact Analysis

The research conducted by the team to produce and overcome the migration of axons through the fibrotic scar has implications outside of the biomedical environment where the research was conducted. In this portion of the document, we will discuss the economic, environmental, societal, and ethical impacts this project could have. Also taken into account are the health and safety issues, manufacturability, and sustainability of the system created and tested.

6.4.1 Economics

There is little evidence suggesting successful integration of our device into medical research would change the economics of everyday living. The device, if integrated, would have an impact on scar research, but as this branch of research is relatively small the economic impact would not significantly alter the everyday living of society.

6.4.2 Environmental impact

The environmental impact our device would have is not concrete but the team does not predict that the device would cause a large negative or positive impact. The only foreseeable way our device would cause an impact on the environment would be if the cells used as well as the device itself were not disposed according to Environmental Protection Agency's protocol for lab waste disposal. The harmful waste must be disposed of in the specified manner because it could cause pollution of the environment the lab is located in as well as having an effect further away by potentially polluting the water supply

6.4.3 Societal Influence

Our device as is would not have any immediate societal influence, but if improved on and implemented into further research, our device concept could contribute to a successful treatment of fibrotic scars. A successful treatment of fibrotic scars would help to improve nerve reconnection in a number of patients and injuries. This would have a great impact on the lives of people with injuries and their families.

6.4.4 Political Ramifications

Our device would have very little influence on the global market even if it were to be successfully implemented into the research of scars within the body. There is very little possibility that our device would be heard of on a global scale, let alone affect it.

6.4.5 Ethical Concern

The only ethical concern that was associated with this device is the source from which the cells were derived from. There were no human subjects involved in the making and testing of this device so no human ethical concerns. The cell culture of neurons and fibroblasts are the only ethical concern presented in the creation and testing of the device. To resolve this concern, the neurons and fibroblast that were purchased were approved for use by Scuola Internazionale Superiore di Studi Avanzati (SISSA) in Trieste, Italy. Also under the HIPPA law, the patient from with the fibroblast and neuronal lines were taken was unknown to the researcher, therefore protecting them against any ethical concern in that regards.

6.4.6 Health and Safety Issue

This project has the possibility, if expanded on, to drastically improve the health of individuals affected by nerve injury. At its current stage, the project's only safety concerns would be the materials used in lab, and if all safety protocols and disposal protocols are followed by each member of the team. Since live cells are being dealt with, user safety is crucial in order to restrict any unwanted proliferation of cells on any of the team members. Proper use of the level 2 biosafety cabinet was practiced in order to keep the cells from getting infected. If the cells were to be infected, proper disposal using ethanol and aspiration of the cells was performed to keep the team members away from getting the infection in their system.

6.4.7 Manufacturability

This device could be easily reproduced if the report is read and followed and those trying to reproduce it has access to the SolidWorks files used to design the mold. Also the formation of the device would easily be reproduced as the only components are the PDMS and the nitrocellulose membrane. A working knowledge with making PDMS and being able to make

fibrin glue to hold the mesh into place will provide our device to those trying to replicate it for their own uses.

6.4.8 Sustainability

Because our device is based around cell culture, the only portion of the design that is reusable is the rapid prototyped mold. This mold can be used multiple times with no detrimental effects of the resulting PDMS structure. Single use disposable materials such as pipette tips and well plates were disposed of in accordance to Environmental Protection Agency (EPA) guidelines for removal of laboratory waste. This included grouping sharps such as metal and glass in appropriate containers, as well as removing plastic waste in separate containers. All chemical waste was placed in appropriate containers that matched their toxicity requirements. Finally, once the waste was ready for removal, approved companies would remove the waste and recycled the appropriate material in order to help with the sustainability of laboratory work.

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 Nitrocellulose Membrane Experiments

This experiment was performed to test the first hypothesis of the project of using nitrocellulose membrane as the scaffold for producing a fibrotic scar to be used as a platform to test the ability of treatments that could enable axonal migration. This was the first step of completing our design since a vertical barrier (nitrocellulose membrane fixed vertically) was required to separate the well into two sections. One section of the well cultured the neurons and the other side carried only the medium. This was a crucial step for our design because if the nitrocellulose membrane with 0.45 micron pores was not able to act as a scaffolding for fibrotic scar formation, then we would have to search for another scaffold that we could fix vertically while being inert to the health of the neurons. As seen in the results, the fibroblasts were seeded on a rectangular piece of nitrocellulose membrane and imaged to see if the fibroblast would actually culture on it. Following the verification of culturing the fibroblast, another test was executed to test the confluency of the scar on days 1,2,3,6,7 and 8. Originally we had hoped to look at the nitrocellulose membrane under a light microscope, however the opaque properties of the membrane did not allow the light to pass through hence not allowing us to prove that the fibroblasts were cultured successfully on the membrane. To overcome the opaque nature of the membrane, DAPI was used to stain the nuclei of the fibroblast on the membrane. After imaging under an immunofluorescence microscope, the nitrocellulose membrane was deemed successful to act as a scaffold for culturing the fibrotic scar.

Looking at the results of the first experiment using the nitrocellulose membrane, it is clear that fibroblast successfully cultured on the membrane. This was promising in proceeding to the next step of our design to see how long the team should culture the fibrotic scar on the membrane before applying treatment, as discussed in the next section. The confluency of the "fibrotic scar" was important for testing in order to give the best representation of a fibrotic scar that injured peripheral neurons would normally encounter in the body. After looking at the density of the fibroblasts on the membrane, it was decided by the team that 3 days of culturing the fibroblast cells on the membrane gave an appropriate confluency for a scar. Any further culturing did not have an effect on further proliferation of the fibroblast which can be seen in the results on days 6,7 and 8. This first experiment gave us a clear indication that fibroblast does proliferate on the nitrocellulose membrane and how long the fibroblast cells need to be cultured to give an appropriate "fibrotic scar" which was then further treated to allow axonal migration through the mesh.

7.2 Treatment of Membrane Experiments

The treatment of certain membranes with FGF-2 was hypothesized to change the biochemical makeup of the fibrotic scar formed, inducing the formation of channels that would allow axonal migration. The presence of FGF-2 has been shown to increase the production of laminin, a regenerative molecule and the down regulation of collagen and fibronectin, two scar forming molecules (Kashpur et al., 2013). These concentrations were found using qRT-PCR, a technique this lab is unable to perform. Additionally, the team found published work that states the presence of FGF-2 in fibroblast cultures encourages the formation of channels through the scar formed. Regarding the effects of FGF-2, a report by Kashpur et al. states, "FGF-2 induced effects on transcriptome associated with regeneration competence in adult human fibroblasts", the report explores deeply into the effects of FGF-2 on adult human fibroblasts at the genomic

level, looking at the upregulation and downregulation of common extracellular matrix components like collagen, laminin, fibronectin etc.

This treatment was applied to a portion of the meshes seeded with fibroblasts to create a physical and chemical environment that supports and allows the extension of axons through. FGF-2 was present in the medium when the fibroblasts were seeded onto the mesh and remained in the medium for at least 3 days of incubation to allow the fibroblasts to become confluent on the mesh. The use of FGF-2 has been proven successful by multiple research teams in creating channels between the extracellular matrix as the levels of collagen are decreased which in turn reduces the scarring. These “channels” are beneficial for our project as it gives way for the axons to migrate from one side of the well to the other. Even after imaging with Alexa Fluor® 488 phalloidin to look at the extracellular matrix, it was difficult to notice any major differences in between the “fibrotic scar” treated with and without FGF-2. The in depth use of FGF-2 and its effect on the fibroblast can be further explained in the scholarly article, "FGF2-induced effects on transcriptome associated with regeneration competence in adult human fibroblasts" (Kashpur et al., 2013). Due to time constraints, an in depth analysis such as Western Blotting for the effects of FGF-2 on fibroblasts was not conducted. It was believed that previous work done in this regard such as the Kashpur et al. article above provided adequate enough knowledge of the effects of FGF-2 on fibroblasts.

7.3 Mitomycin-C Experiment

Once all the meshes were confluent, Mitomycin-C was added to the cultures to stop the proliferation of the cells. To test that our aliquot of Mitomycin-C would have the desired effect, a test was conducted with two plates seeded with 100K cells. Once the cells attached to the bottom of the plate, one plate was treated with Mitomycin-C and incubated for 3 hours. Once the plate was washed with PBS and provided with new media, images were taken of the Mitomycin-C treated plate as well as the untreated plate and labeled day 1. Images were taken at days 2 and 3 of both the plates.

The results showed that the Mitomycin-C stopped the division of the fibroblasts, leaving the treated plate at the concentration it was seeded at. This treatment was used on the meshes to reduce the chance that the fibroblast cells' growth will interfere with the neurons on the bottom of the plate when the meshes were placed vertically in the culture. The interference from the fibroblast by proliferating on top of the neurons could have restricted the neurons' normal function and growth. Hence, it was important to use the Mitomycin-C to allow the neurons to extend their axons and help us move forward with our project.

7.4 Decellularization of the mesh

Even though proliferation of the fibroblast was halted, migration of the fibroblast into the neuronal culture could not be stopped. In order to address this issue, decellularizing the mesh was proposed when it became clear that the fibroblast cells could interfere with the final imaging of the device. The fibroblasts migrated off of the mesh and onto the bottom of the plate, invading the space the axons are extending into. The team consulted with Professor Dominko and came to the conclusion that the fibroblasts were not needed on the mesh as long as the ECM had time to be secreted and deposited. This lead to the decision to decellularize the meshes following the protocol explained in chapter 3. This protocol was modified from a protocol found from

Comparison of decellularization protocols for preparing a decellularized porcine annulus fibrosus scaffold (Xu, 2014). The original protocol read:

> "Pig AF was placed in hypotonic Tris-HCl buffer (10 mM, pH 8.0) with 0.1% ethylenediaminetetraacetic acid (EDTA; Sigma) and 10 KIU/ml aprotinin (Sigma) at 4°C for 48 h. Then AF samples were agitated in Tris-HCl buffer with 3% Triton X-100 (Sigma), 0.1% EDTA and 10 KIU/ml aprotinin at 4°C for 72 h. The solution was changed every 24 h. Then AF samples were incubated with 0.2 μg/mL ribonuclease A (RNase A; Sigma) and 0.2 mg/mL deoxyribonuclease I (DNase I; Sigma) at 37°C for 24 h. Finally, decellularized AF was washed with PBS for 24 h to remove residual reagents." (Xu, 2014)

This protocol was modified under the guidance of professor Dominko to reduce the time needed to decellularize the meshes and to accommodate for the thinness of the meshes. The team only had 48 hours to complete the decellularization process but due to the thickness of the mesh, the amount of cells that needed to be broken down and washed away was significantly less than the amount of cells in the paper above.

Once the team decellularized the meshes, one of the meshes was stained with DAPI to stain the DNA as well as Phalloidin to stain the actin. The mesh was imaged using the immunofluorescence microscope and the images showed fragments of stain throughout the mesh. There was no significant DAPI staining present in the images that would normally show a healthy cell. This indicated that the DNA and actin were simply residual cellular components that did not get washed away completely. This could be attributed to the components attaching to the small pores in the nitrocellulose membrane making them difficult to wash off. This did not concern the team greatly because the main goal of decellularizing the mesh was to destroy the cells and keep the ECM intact.

To determine whether the ECM did in fact remain on the mesh, the team stained with Ponceau S to visualize the protein. The team stained a control mesh and two decellularized meshes; one mesh treated with FGF-2 and one mesh without treatment. The control mesh, when

stained and washed with deionized water, remained white. The two decellularized meshes when stained and washed with deionized water remained pink indicating that the ECM remained intact. This proved that the ECM was still attached to the nitrocellulose membrane since the DAPI stain was scattered throughout the mesh however the Ponceau S pink stain was observed all over the mesh. If the Ponceau S was to bind to the intracellular proteins of the remaining fragmented cells, we would have observed scattered pink staining of the mesh. Since the DAPI was scattered and the Ponceau S stained the entire surface of the mesh, it can be concluded that the ECM did in fact remain intact and the mesh was successfully decellularized.

7.5 Axonal Migration Through Nitrocellulose Membrane

Once the meshes were placed in the PDMS mold and secured with fibrin glue, the sensory neurons were placed in the plate. The goal was to allow the neurons to attach and extend over a three-day period, preferably extending through the mesh. Our first experiment showed to be infected after incubation overnight, so the team reevaluated its sterilization techniques and prepared for a second batch of neurons. The second batch of neurons again became infected after one day of incubation leading the team to believe that it was the media being used that caused the infection. For the third batch of neurons, new media was procured and used. This batch of neurons incubated for 4 days. During that time the neurons attached and extended axons and dendrites without infection occurring. This culture had mesh that was not treated with Mitomycin-C or decellularized because of the tight timeframe the team had available to plate the neurons. This allowed the untreated fibroblasts to migrate off of the mesh and onto the bottom of the plate, making it difficult to verify our results. Another possible limitation to achieving viable results for this experiment included improper plating of neurons. The team believes the neurons plated on the side designated for neuronal culture leaked onto the other side because of the fibrin glue was not fully cured, which lead to medium and cells be able to pass to the other side. The

lack of control of keeping the neuronal cell bodies on one side of the mesh made our results inconclusive as there was no way to tell whether the neurons, fibroblasts or both were being stained. The last batch of neurons was plated with decellularized mesh to exclude fibroblasts from the culture, but this culture showed to be infected after incubation overnight, negating any results that could have been obtained. Overall, it is important to note that lack of proper sterilization, when working with neurons, caused the results to be inconclusive.

An issue with the handling of the device was the method of securing the mesh into place. It was found that the fibrin glue was interfering with the imaging capabilities of the microscope. This limitation was due to the semi-opaque transparency of the cured glue as well as to the height of the glue at the base of the mesh.

The experiments shown in the images in Chapter 5 do suggest some positive results. The pictures show the axons extending towards the fibrin glue and the mesh. However the opaque nature of the fibrin glue restricted the team to get a close look at axons that may have been present right next to the mesh or even migrating through the fibrotic scar mesh.

Since a limited number of neurons were available for testing, it was important to create the best scenarios for the neurons to survive and have the axons extend through the mesh. One major limitation for the entire project and especially the final design experiment was time. With limited amount of time available and the time required for the neurons to culture properly and allow enough time for the axons to extend before having to image the results. Since the life of the cultured neurons was relatively small, every experiment had to be run at a faster pace which could have potentially been the reason behind the infections that we encountered.

Chapter 8 Conclusions and Recommendations

This chapter provides analysis of our results and further recommendations for the device moving forward.

8.1 Conclusions

The project was concluded with designing a device that would enable us to investigate axonal extension through a treated fibrotic scar. Although the project did not produce results to establish whether or not axons could extend through nitrocellulose membrane, it did make strides towards using this device for further experiments that would actually enable axonal extension. This project established that it is possible to grow cells on a nitrocellulose membrane to simulate the formation of a scar within a lesion. In order to remove potential interference with the imaging of a neuronal culture, the team decellularized the mesh in order to eliminates the possibility of the fibroblasts migrating onto the neurons.

The final design of the PDMS mold created from the rapid prototype proved to be durable, which made handling the device easy; also the rapid prototyped portion of the device was reusable, which cut down on cost as well as materials. Using the nitrocellulose membrane as the scaffold for the scar was beneficial for the team as it allowed us to successfully seed the fibroblasts cells, size the membrane to proper dimensions and implement various treatments.

During the project, we did encounter some limitations which interfered with positive results in completing the project. Time and sterilization of the materials were quite possibly the major limitations which interfered with the successful completion of the project. If we were able to keep the neurons healthy and infection-free, we would have had a better answer whether axons would migrate through a fibrotic scar that has been treated with FGF-2. The limitations did restrict the device from achieving the goal of extending axons through the fibrotic scar but future

recommendations for the device provide further use for the device by making some modifications and even in different application.

8.2 Recommendations

If further development on this project is to be conducted, there are multiple recommendations the team would like to present. The mesh used in the device should be secured using an agent that will not interfere with imaging the culture. This may require a redesign of the PDMS mold because the mesh must be secured to the bottom of the plate so neurons don't migrate under the mesh rather than through it. If an alternate agent cannot be found, using a three dimensional cell culture rather than a two dimensional cell culture would allow the neurons to grow in a more natural environment and exhibit normal behavior towards a fibrotic scar. If this recommendation is to be followed, a confocal microscope should be used to image the neurons in the 3D culture.

The mesh should be decellularized in order to remove the possibility of the fibroblast interfering with the neuron culture. This also provides the necessary components of a fibrotic scar that can easily be stored for future uses and experiments. The device with the decellularized mesh can be used for various applications not necessarily tied to neuronal research. The components left on the decellularized mesh can be stored for future uses without having to worry about keeping the cells viable. The modification of the fibrotic scar can be used for future applications such as using the idea to test certain treatments that pharmaceutical companies produce for fibrotic scars that people typically gain from an external injury to the epidermis. Although the decelullarized fibrotic scar is not a perfect replication of an in vivo model, it does provide an accessible way to test certain treatments that can manipulate the ECM. These altered ECM composition will allow further research into providing a way to treat neurodegenerative injuries.

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Fibroblasts and their transformations: The connective-tissue cell family.

ASTM F813 - 07(2012) Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices. (n.d.). Retrieved October 15, 2015, from http://www.astm.org/Standards/F813.htm

ATLANTIS, T. (2004). Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. The Lancet, 363(9411), 768-774.

Bareyre, F. M., Garzorz, N., Lang, C., Misgeld, T., Büning, H., & Kerschensteiner, M. (2011). In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. Proceedings of the National Academy of Sciences, 108(15), 6282-6287.

Brady, S., Siegel, G., Albers, R. W., & Price, D. (Eds.). (2005). Basic neurochemistry: molecular, cellular and medical aspects. Academic Press.

Bret A. Morrow, Robert H. Roth, D. Eugene Redmond Jr., John R. Sladek Jr., John D. Elsworth, Apoptotic natural cell death in developing primate dopamine midbrain neurons occurs during a restricted period in the second trimester of gestation, Experimental Neurology, Volume 204, Issue 2, April 2007, Pages 802-807, ISSN 0014-4886, http://dx.doi.org/10.1016/j.expneurol.2007.01.009.

Brodal, P. (2004). The central nervous system: structure and function. Oxford University Press.

Burroughs, S. L., Duncan, R. S., Rayudu, P., Kandula, P., Payne, A. J., Clark, J. L., … Kaja, S. (2012). Plate reader-based assays for measuring cell viability, neuroprotection and calcium in primary neuronal cultures. Journal of Neuroscience Methods, 203(1), 141–145.

CDC, NCHS. Underlying Cause of Death 1999-2013 on CDC WONDER Online Database, released 2015. Data are from the Multiple Cause of Death Files, 1999-2013, as compiled from data provided by the 57 vital statistics jurisdictions through the Vital Statistics Cooperative Program. Accessed Feb. 3, 2015.

Centers for Disease Control and Prevention. Nonfatal Traumatic Brain Injuries Related to Sports and Recreation Activities Among Persons Aged ≤19 Years — United States, 2001–2009. MMWR 2011; 60(39):1337–1342.

Chen, Theodore, Shaun S. Kunnavatana, and R. James Koch. "Effects of Mitomycin-C on Normal Dermal Fibroblasts." The Laryngoscope 116.4 (2006): 514-7. Web.

Cooke, M. J. et al. “Enhanced Cell Attachment Using a Novel Cell Culture Surface Presenting Functional Domains from Extracellular Matrix Proteins.”Cytotechnology 56.2 (2008): 71–79. PMC. Web. 4 Nov. 2015.

Corning, Transwell Permeable Supports Selection and Use Guide. (2013, June 13). Retrieved November 4, 2015, from http://csmedia2.corning.com/LifeSciences/media/pdf/transwell_guide.pdf

Cox, S., et al. "Effect of Tranexamic Acid Incorporated in Fibrin Sealant Clots on the Cell Behavior of Neuronal and Nonneuronal Cells."Journal of Neuroscience Research 72.6 (2003): 734-46. Web.

Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials*, *32*(12), 3233-3243.

Cregg, J. M., DePaul, M. A., Filous, A. R., Lang, B. T., Tran, A., & Silver, J. (2014). Functional regeneration beyond the glial scar. *Experimental neurology*,*253*, 197-207.
Dawodu, S. T. (2015). Traumatic Brain Injury (TBI) - Definition, Epidemiology, Pathophysiology: Overview, Epidemiology, Pathophysiology: Primary Injury.

de Lima, S., Koriyama, Y., Kurimoto, T., Oliveira, J. T., Yin, Y., Li, Y., ... & Benowitz, L. (2012). Full-length axon regeneration in the adult mouse optic nerve and partial recovery of simple visual behaviors. Proceedings of the National Academy of Sciences, 109(23), 9149-9154.

Deng, L. X., Deng, P., Ruan, Y., Xu, Z. C., Liu, N. K., Wen, X., ... & Xu, X. M. (2013). A novel growth-promoting pathway formed by GDNF-overexpressing Schwann cells promotes propriospinal axonal regeneration, synapse formation, and partial recovery of function after spinal cord injury. The Journal of Neuroscience, 33(13), 5655-5667.

Diemert, S., A. M. Dolga, S. Tobaben, J. Grohm, S. Pfeifer, E. Oexler, and C. Culmsee. "Impedance measurement for real time detection of neuronal cell death." Journal of neuroscience methods 203, no. 1 (2012): 69-77.

DRGs. (2013). Retrieved December 13, 2015, from http://www.neuromics.com/drgs

Fabbro, Alessandra et al. “Carbon Nanotubes: Artificial Nanomaterials to Engineer Single Neurons and Neuronal Networks.” ACS Chemical Neuroscience 3.8 (2012): 611–618. PMC. Web. 28 Sept. 2015.

Fagoe ND, van Heest J, Verhaagen J (2014) Spinal cord injury and the neuron-intrinsic regeneration-associated gene program. Neuromolecular Med 16(4):799–813.

Friend J, Yeo L. Fabrication of microfluidic devices using polydimethylsiloxane. *Biomicrofluidics*. 2010;4(2):026502. doi:10.1063/1.3259624.

G.K.T. Chu, C.H. Tator, Calcium influx is necessary for optimal regrowth of transected neurites of rat sympathetic ganglion neurons in vitro, Neuroscience, Volume 102, Issue 4, 14 February 2001, Pages 945-957.

Gingras, Marie, et al. "In Vitro Development of a Tissue-Engineered Model of Peripheral Nerve Regeneration to Study Neurite Growth." The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17.14 (2003): 2124-6. Web.

Goldshmit, Y., Frisca, F., Pinto, A. R., Pébay, A., Tang, J. K. K., Siegel, A. L., ... & Currie, P. D. (2014). Fgf2 improves functional recovery—decreasing gliosis and increasing radial glia and neural progenitor cells after spinal cord injury.*Brain and behavior*, *4*(2), 187-200.

Goldshmit, Y., Sztal, T. E., Jusuf, P. R., Hall, T. E., Nguyen-Chi, M., & Currie, P. D. (2012). Fgf-dependent glial cell bridges facilitate spinal cord regeneration in zebrafish. *The Journal of Neuroscience*, *32*(22), 7477-7492.

Haile, Y., Haastert, K., Cesnulevicius, K., Stummeyer, K., Timmer, M., Berski, S., ... & Grothe, C. (2007). Culturing of glial and neuronal cells on polysialic acid. Biomaterials, 28(6), 1163-1173.

Hilgenberg, L. G. W., & Smith, M. A. (2007). Preparation of Dissociated Mouse Cortical Neuron Cultures. Journal of Visualized Experiments : JoVE, (10), 562. http://doi.org/10.3791/562

Horner, P. J., & Gage, F. H. (2000). Regenerating the damaged central nervous system. Nature, 407(6807), 963-970.

How Is a Stroke Treated? (2014, March 26). Retrieved September 27, 2015, from https://www.nhlbi.nih.gov/health/health-topics/topics/stroke/treatment

Huang, Z. H., Wang, Y., Cao, L., Zhu, Y. L., Chen, Y. Z., Yuan, X. B., & He, C. (2008). Migratory properties of cultured olfactory ensheathing cells by single-cell migration assay. *Cell research*, *18*(4), 479-490.

Huang, L., Wu, Z. B., ZhuGe, Q., Zheng, W., Shao, B., Wang, B., ... & Jin, K. (2014). Glial scar formation occurs in the human brain after ischemic stroke.International journal of medical sciences, 11(4), 344.

Hyder AA, Wunderlich CA, Puvanachandra P, Gururaj G, Kobusingye OC. The impact of traumatic brain injuries: a global perspective. Neuro Rehabilitation. 2007;22(5):341–353.

Jed A. Hartings, Steven Vidgeon, Anthony J. Strong, Chris Zacko, Achala Vagal, Norberto Andaluz, . . . M. Ross Bullock. (2014). Surgical management of traumatic brain injury: a comparative-effectiveness study of 2 centers. http://dx.doi.org/10.3171/2013.9.JNS13581. doi:2013.9.JNS13581

Kaech, S., Huang, C. F., & Banker, G. (2012). General considerations for live imaging of developing hippocampal neurons in culture. Cold Spring Harbor Protocols, 2012(3), pdb-ip068221.

Kandel, E. R., Schwartz, J. H., Jessell, T. M., Siegelbaum, S. A., & Hudspeth, A. J. (2014). Principles of neural science.

Khoruzhenko A. I. 2D- and 3D-cell culture // Biopolym. and Cell.–2011.–27, N 1.D. 17–24.

Kawano, H., Kimura-Kuroda, J., Komuta, Y., Yoshioka, N., Li, H. P., Kawamura, K., ... & Raisman, G. (2012). Role of the lesion scar in the response to damage and repair of the central nervous system. *Cell and tissue research*, *349*(1), 169-180.

Langlois, J. A., Rutland-Brown, W., & Thomas, K. E. (2004). *Traumatic brain injury in the United States: emergency department visits, hospitalizations, and deaths*. Department of Health and Human Services, Centers for Disease Control and Prevention, Division of Acute Care, Rehabilitation Research and Disability Prevention, National Center for Injury Prevention and Control.

Lasiecka, Z. M., & Winckler, B. (2015). Studying endosomes in cultured neurons by live-cell imaging. Methods in Cell Biology.

Leclerc, E., Sakai, Y., & Fujii, T. (2003). Cell culture in 3-dimensional microfluidic structure of PDMS (polydimethylsiloxane). *Biomedical microdevices*, *5*(2), 109-114.

Leibiger, C., Kosyakova, N., Mkrtchyan, H., Glei, M., Trifonov, V., & Liehr, T. (2013). First Molecular Cytogenetic High Resolution Characterization of the NIH 3T3 Cell Line by Murine Multicolor Banding. *Journal of Histochemistry and Cytochemistry*, *61*(4), 306–312. http://doi.org/10.1369/0022155413476868

Li, Aimin et al. "Use of Nitrocellulose Membranes as a Scaffold in Cell Culture."*Cytotechnology* 65.1 (2013): 71–81. *PMC*. Web. 4 Nov. 2015.

Li, A., Wang, Y., Deng, L., Zhao, X., Yan, Q., Cai, Y., ... & Zhang, Y. (2013). Use of nitrocellulose membranes as a scaffold in cell culture. *Cytotechnology*,*65*(1), 71-81.

Liebeskind, David. "Hemorrhagic Stroke." Medscape. Ed. Robert E O'Connor. 8 Jan. 2015. Web. 13 Oct. 2015.
Liesz, A., Dalpke, A., Mracsko, E., Antoine, D. J., Roth, S., Zhou, W., ... & Veltkamp, R. (2015). DAMP signaling is a key pathway inducing immune modulation after brain injury. The Journal of Neuroscience, 35(2), 583-598.

Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 21.1, Overview of Neuron Structure and Function.

Matthew R.J. Mason, A.R. Lieberman, G. Grenningloh, P.N. Anderson, Transcriptional Upregulation of SCG10 and CAP-23 Is Correlated with Regeneration of the Axons of Peripheral and Central Neurons in Vivo, Molecular and Cellular Neuroscience, Volume 20, Issue 4, August 2002, Pages 595-615, ISSN 1044-7431, http://dx.doi.org/10.1006/mcne.2002.1140.

Mozaffarian D, Benjamin EJ, Go AS, et al. Heart disease and stroke statistics—2015 update: a report from the American Heart Association. Circulation. 2015 ;e29-322.

National Spinal Cord Injury Statistical Center. (2013). Spinal cord injury facts and figures at a glance. *The journal of spinal cord medicine*, *36*(1), 1.

Nisbet, D. R., Rodda, A. E., Horne, M. K., Forsythe, J. S., & Finkelstein, D. I. (2009). Neurite infiltration and cellular response to electrospun polycaprolactone scaffolds implanted into the brain. *Biomaterials*, *30*(27), 4573-4580.

Ninds.nih.gov,. 'Spinal Cord Injury: Hope Through Research'. N.p., 2015. Web. 5 Nov. 2015.
Pandya, R. S., Mao, L., Zhou, H., Zhou, S., Zeng, J., Popp, A. J., & Wang, X. (2011). Central Nervous System Agents for Ischemic Stroke: Neuroprotection Mechanisms. Central Nervous System Agents in Medicinal Chemistry, 11(2), 81–97.

Parenteau-Bareil, R., Gauvin, R., & Berthod, F. (2010). Collagen-based biomaterials for tissue engineering applications. *Materials*, *3*(3), 1863-1887.

Pawar, K., Prang, P., Müller, R., Caioni, M., Bogdahn, U., Kunz, W., & Weidner, N. (2015). Intrinsic and extrinsic determinants of central nervous system axon outgrowth into alginate-based anisotropic hydrogels. Acta biomaterialia.

Pekna, M., & Pekny, M. (2012). The Neurobiology of Brain Injury. Cerebrum: The Dana Forum on Brain Science, 2012, 9.
Potter, S. M., & DeMarse, T. B. (2001). A new approach to neural cell culture for long-term studies. Journal of neuroscience methods, 110(1), 17-24.

Que, J., Cao, Q., Sui, T., Du, S., Kong, D., & Cao, X. (2013). Effect of FK506 in reducing scar formation by inducing fibroblast apoptosis after sciatic nerve injury in rats. *Cell death & disease*, *4*(3), e526.

Raji CA, Tarzwell R, Pavel D, Schneider H, Uszler M, Thornton J, et al. (2014) Clinical Utility of SPECT Neuroimaging in the Diagnosis and Treatment of Traumatic Brain Injury: A Systematic Review. PLoS ONE 9(3): e91088. doi:10.1371/journal.pone.0091088

Regehr, K. J., Domenech, M., Koepsel, J. T., Carver, K. C., Ellison-Zelski, S. J., Murphy, W. L., ... & Beebe, D. J. (2009). Biological implications of polydimethylsiloxane-based microfluidic cell culture. *Lab on a Chip*, *9*(15), 2132-2139.

Robnett, R. H., Shanahan, P., Mullahy, C., Chop, W. C., Hui, Y. H., & Cross, N. (2013). Gerontology for the health care professional. Jones & Bartlett Publishers.

Roos, Y. B. W. E. M., Dijkgraaf, M. G. W., Albrecht, K. W., Beenen, L. F. M., Groen, R. J. M., De Haan, R. J., & Vermeulen, M. (2002). Direct costs of modern treatment of aneurysmal subarachnoid hemorrhage in the first year after diagnosis. Stroke, 33(6), 1595-1599.

Saini, Deepa. "The American Association of Neurological Surgeons." AANS. Ed. Jeffery E. Thomas. 1 Aug. 2009. Web. 13 Oct. 2015.

Schnabl, K. L., Larcelet, M., Thomson, A. B., & Clandinin, M. T. (2009). Uptake and fate of ganglioside GD3 in human intestinal Caco-2 cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *297*(1), G52-G59.

Seijffers, R., Mills, C. D., & Woolf, C. J. (2007). ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. Journal of Neuroscience, 27(30), 7911-7920. doi:10.1523/JNEUROSCI.5313-06.2007

Shen, Q., Goderie, S. K., Jin, L., Karanth, N., Sun, Y., Abramova, N., ... & Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. *Science*, *304*(5675), 1338-1340.

Sigma-Aldrich. Labware Products (n.d.). Retrieved October 15, 2015, from http://www.sigmaaldrich.com/labware/labware-products.html?TablePage=9579136

Sofroniew MV, Howe CL, Mobley WC. Nerve growth factor signaling, neuroprotection, and neural repair.*Annual review of neuroscience*. 2001;24:1217.

Song, H., Stevens, C. F., & Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature*, *417*(6884), 39-44.

Stroke: MedlinePlus. (2015, August 1). Retrieved October 15, 2015.

Stroke Treatments. American Stroke Association. 23 May 2013. Web. 13 Oct. 2015.

Stufflebeam, R. (2008). Neurons, synapses, action potentials, and neurotransmission. Consortium on Cognitive Science Instruction.

Subramanian, A., Krishnan, U. M., & Sethuraman, S. (2009). Development of biomaterial scaffold for nerve tissue engineering: Biomaterial mediated neural regeneration. J Biomed Sci, 16(1), 108.

ThermoFisher, Tools for Neural Cell Culture of Primary Cells. (n.d.). Retrieved October 15, 2015, from https://www.thermofisher.com/us/en/home/life-science/cell-culture/primary-cell-culture/neuronal-cell-culture/primary-cells.html

Tibbitt, M. W., & Anseth, K. S. (2009). Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnology and bioengineering*, *103*(4), 655-663.

U.S. Food and Drug Administration. (n.d.). Retrieved October 15, 2015, from http://www.fda.gov/RegulatoryInformation/Guidances/ucm080735.htm

Wang, G., Zhang, Z., Ayala, C., Dunet, D. O., Fang, J., & George, M. G. (2014). Costs of hospitalization for stroke patients aged 18-64 years in the United States. Journal of Stroke and Cerebrovascular Diseases, 23(5), 861-868.

Xu, H., Xu, B., Yang, Q., Li, X., Ma, X., Xia, Q., ... & Zhang, Y. (2014). Comparison of decellularization protocols for preparing a decellularized porcine annulus fibrosus scaffold. *PloS one*, *9*(1), 86-723.

Yoo, S. J., Kim, J., Lee, C.-S., & Nam, Y. (2011). Simple and Novel Three Dimensional Neuronal Cell Culture Using a Micro Mesh Scaffold. *Experimental Neurobiology*, *20*(2), 110–115. http://doi.org/10.5607/en.2011.20.2.110

Zhang, W., Choi, D. S., Nguyen, Y. H., Chang, J., & Qin, L. (2013). Studying cancer stem cell dynamics on PDMS surfaces for microfluidics device design. *Scientific reports*, *3*.