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Repair severed nerve connections through a multi-branch microchannel scaffold to control the direction of the regenerated nerve

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REPAIR SEVERED NERVE CONNECTIONS THROUGH A MULTI-BRANCH MICROCHANNEL SCAFFOLD TO CONTROL THE DIRECTION OF THE REGENERATED NERVE

A Thesis

by

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ABSTRACT

Castañuela Ramon**,** Luis Enrique**,** Repair severed nerve connections through a multi-branch microchannel scaffold to control the direction of the regenerated nerve. Master of Science (MS), August, 2016, 24 pp, 14 figures, 50 references, 13 titles.

Damage to the peripheral nervous system results in functional abnormalities due to disrupted nerve connections. Existing methods of repairing severed nerve connections lack control of the direction of nerve regeneration within nerve conduits. A handcrafted multi-branch microchannel scaffold incorporates microchannels, which guide and accommodate the nerve regeneration to distal ends, allowing for the treatment of nerve injuries involving multiple branches with fewer surgeries. The scaffold used in the study was designed specifically for the sciatic nerve, which branches out to the tibial, sural, and common peroneal nerves, and was implanted in Lewis rats with a severed sciatic nerve and three distal nerve branches to demonstrate the effectiveness of the nerve scaffold. The devices proved successful after four weeks inside the Lewis rats. A total of eight devices were harvested and all show the presence of growth cones inside the microchannels proving the controlled regeneration of the nerve through the scaffold.

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CHAPTER I

INTRODUCTION

Injuries to the peripheral nervous system inducing severed neural connections can result in functional abnormalities [1]. Often times, axons of the severed nerve in the peripheral nervous system attempt to regenerate themselves. Within 24 to 48 hours of injuries, the axons undergo Wallerian degeneration to clear the debris such as cell organelles from the injury and to prepare for axonal sprouts on the surface of the Schwann cells or basal lamina [2-4]. However, a chance of natural recovery of neural connection is slim due to the incorrect axonal pathways being taken during the regeneration process. In the case of mixed nerves damage, the regenerating motor axons tend to project toward skin; whereas sensory axons grow towards muscle [5, 6]. The tendency of regenerating nerves to fasciculate due to the adhesive interactions between the recognition molecules on the surrounding axons and axons themselves serving as a scaffold for each other further complicates the process of axon recovery [7-9]. Therefore, correctional measures are necessary to repair peripheral nerve damages.

Currently, there are three main methods for correcting peripheral nerve damage: tensionless end-to-end repair, nerve grafts, and nerve conduits [10]. Tensionless end-to-end repair involves suturing severed ends of a nerve together, but it is very limited for injuries resulting in a very small nerve gaps [11]. Nerve grafting uses donor nerves to fill the gap between the severed nerves, and artificial nerve conduits facilitate axon regeneration by serving as a bridge between

the severed ends. Although both nerve grafting and conduits can be used to treat longer nerve gaps, nerve grafting is limited by the availability and compatibility of the donor nerves. The donor nerve, which is acquired mainly through the patient himself and is usually harvested from the sensory sural nerve, often does not match the dimensions of the severed nerves and necessitates the establishment of adequate vascularization to avoid necrosis or other potential complications [12, 13]. Although it can still be used, the geometrical irregularities of the axon result in delayed recovery and may possibly lead to a conduction failure due to the alteration of the action potential speed correlated to the change in axon diameter [14-17]. Additionally, acquiring donor nerves means having additional invasive surgeries, which may increase the chance of complications. Nerve conduits eliminate these issues posed by nerve grafting. However, existing nerve conduits lack control, are limited by their designs and are difficult or expensive to produce. The hollow design and the lack of control post-installation of nerve conduit allow regenerating nerves to become disorganized and intertwined in the conduit and run the risk of inappropriate target reinnervation [18-20]. The linear design of the nerve conduit also limits where this conduit may be placed and does not accommodate the fact that most peripheral nerve injuries involve multiple nerve paths.

To address these problems, handcrafted multi-branch microchannel scaffolds (HMMS) have been developed and are examined in this paper. Microchannels embedded within the HMMS allow better control of axonal organization and guidance for nerve regrowth by restricting the area for regeneration while increasing the surface area for support [21-24]. Additionally, these multi-branch microchannel scaffolds are made more accessible by being handcrafted and made of microwires, silastic tubes, and PDMS, a cheaper yet reliable and biocompatible alternative to 3D printing. Any biological laboratory would have the capability to develop microchannel scaffolds

without any background of micromachining technology and equipment. The designs can be modified to accommodate different numbers and sizes of neural branching. Specifically, the HMMS was fabricated and implanted in *Lewis* rat sciatic nerve model. Implanting the HMMS in the sciatic nerve, which branches out into the tibial, sural, and perineal nerves, allowed for the demonstration of single-to-three branch nerve regeneration in which the scaffold acts as a 'nerve hub', routing the sciatic nerve to its targets.

A previews design implemented a single line flat array of microchannels, that were stacked to form a three dimensional array structure of the scaffold. The artificial nerve conduit allowed for the regeneration of the nerve through a single path by connecting the distal and proximal end of the nerves to the sides of the conduit. This method has proven itself to be effective in nerve regeneration, but its inflexibility to treat multiple affected nerve paths can become problematic due to the increase of surgeries required to successfully repair the entire nerve. The single-branch conduit lacks the required control to redirect a complex nerve such as the sciatic nerve. A multi-branch micro-channel scaffolds would be ideal in these situations. It will reduce the chance of multiple surgeries to fix the multiple nerve paths and the branching will allow for a better analysis of the axons at the intersection point.

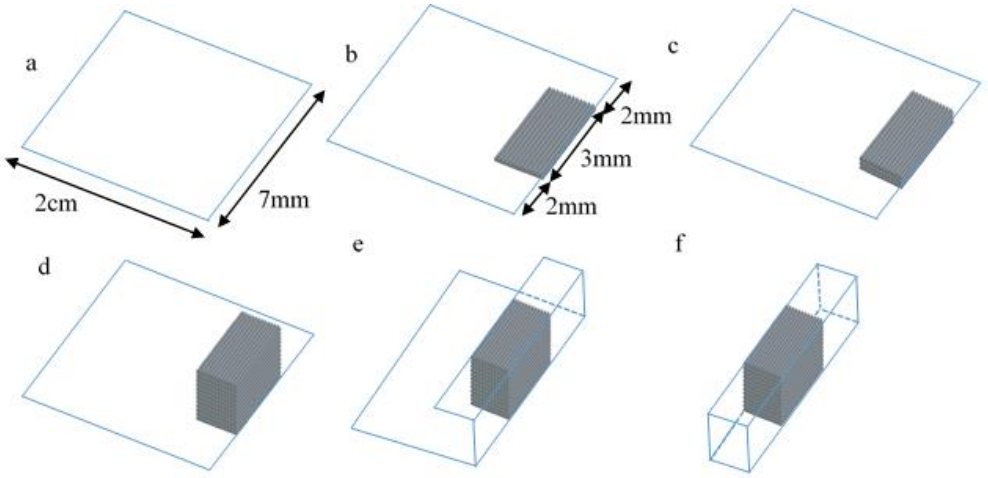

Figure 1.1 Single Line Microchannel Scaffold

CHAPTER II

FABRICATION PROCEDURE

To produce the extremely PDMS microchannel structures a master structure mold first needed to be fabricated. A three-inch diameter silicon wafer was first sprayed with isopropyl alcohol, dried with a nitrogen gun, and placed on a hotplate to prepare and clean the surface prior to the application of the photoresist and placed in the spinning machine. Two-thirds of the wafer was covered by the SU-8 2035 photoresist. The wafer was then spin-coated 900 rpm for 30 seconds. The sample was then placed on a leveled hotplate at 95º C for 20 minutes.

In order to provide guidance to the Sciatic nerve, a multi-branch channel pattern was designed in AutoCAD as presented in Figure 2.1. The channel patterned film was placed on a four-inch glass plate coated with chromium and AZ1518 photoresist (Figure 2.2), placed on the MJB4 Suss Microtec® mask aligner, and then exposed to UV light.

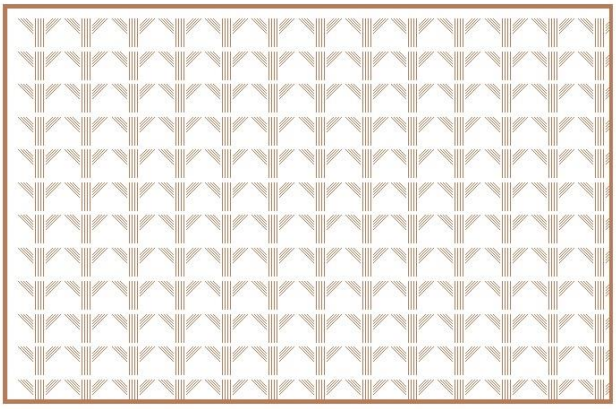

Figure 2.1 Microchannel pattern mask

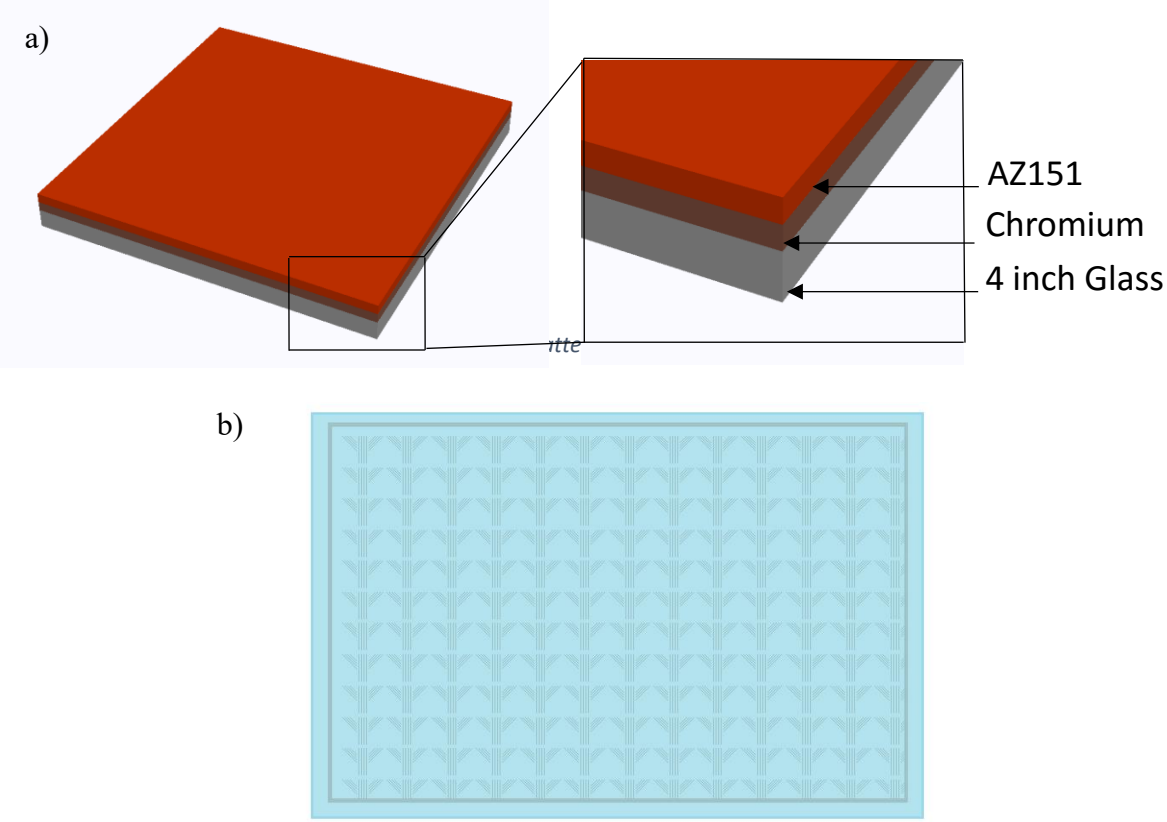

Figure 2.2 (a) Chromium and 4 inch Glass (b) Chromium glass with film

After exposure, the sample was placed in the 400K developer solution for two and a half minutes, rinsed in deionized water and dried with a nitrogen gun. The sample was then placed in a chromium etchant for three minutes, rinsed with deionized water and dried with a nitrogen gun. The sample was then washed with acetone and rinsed with deionized water to remove remaining AZ1518 photoresist and form the chrome mask.

The SU-8 covered silicon wafer was placed on the mask aligner with the chromium glass mask on top of it, aligned and then exposed to UV light with a light intensity of 250 mJ/cm2. Immediately after exposure, the silicon wafer was placed on the hotplate at 95º C for 10 minutes. The post exposure bake solidified the photoresist to a permanent structure. The sample was then placed in the SU-8 developer to remove the unexposed portion of the photoresist. Figure 2.3 represent the finalized master structure.

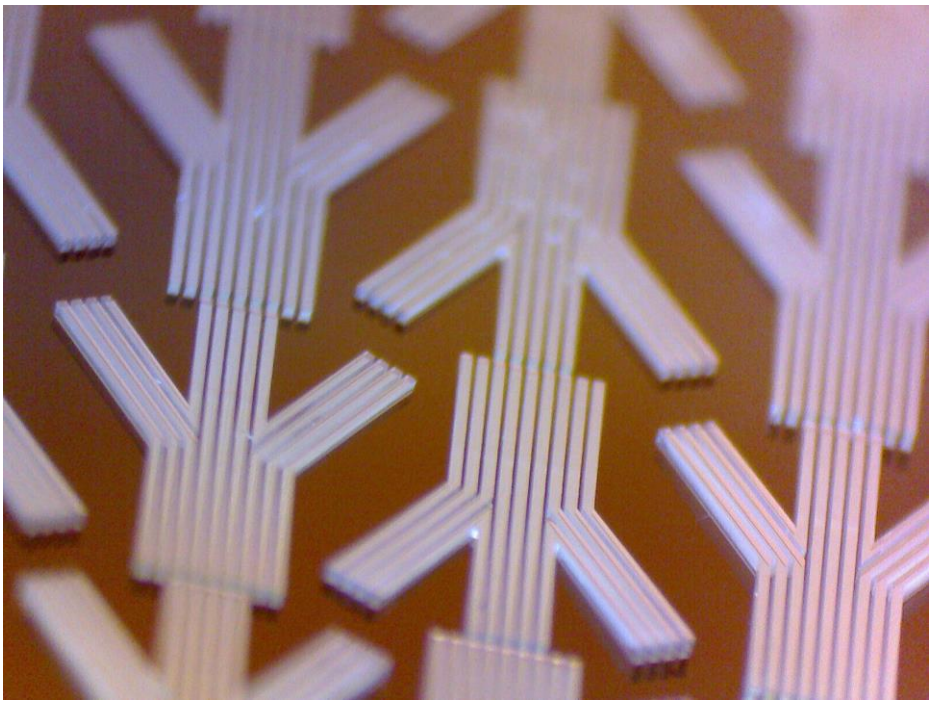

Figure 2.3 Master structure on silicon wafer 3 way channel

PDMS Microchannel Layer Fabrication: Master structure was covered by the 10:1 PDMS mixture (Sylgard® 184, Dow Corning®, MI) then spin-coated for 800 rpm for 30 seconds. It was then cured in an oven for 30 minutes at 95º C, removed and allowed to cool. The structure was then submerged in a chloroform solution, within which the PDMS solution expanded and detached from the SU-8 master structure. The PDMS layer was then submerged in an isopropyl alcohol solution until it retracted to its original size and simultaneously removed any chloroform remaining on it, removed from the solution, and allowed to air dry.

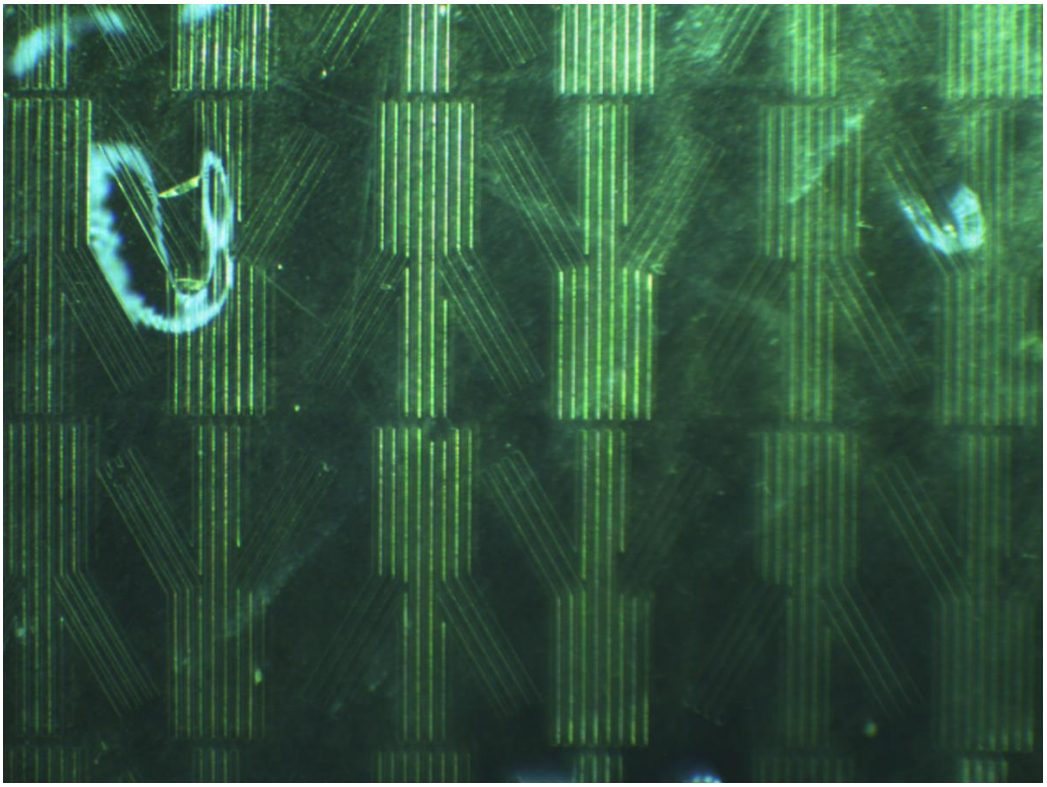

Figure 2.4 Final Film

The PDMS structure is sectioned into a series of appropriately dimensioned multichannel layers with a sharp blade. In order to have precise guidance in the microchannels, the re-dimension of the multichannel layers has to be as accurate as possible so that the stacks fit perfectly on top of each other. This can be seen in Figure 2.5. A single microchannel layer was bonded with 10:1 PDMS to curing agent mixture (Sylgard® 186, Dow Corning®, MI) to the thin film in order to secure a starting point for the stacks. After the PDMS cured, microchannel layers were stacked to approximately 1.5 mm so that the structure cross-section is approximately square (Figure 2.5 (d)).

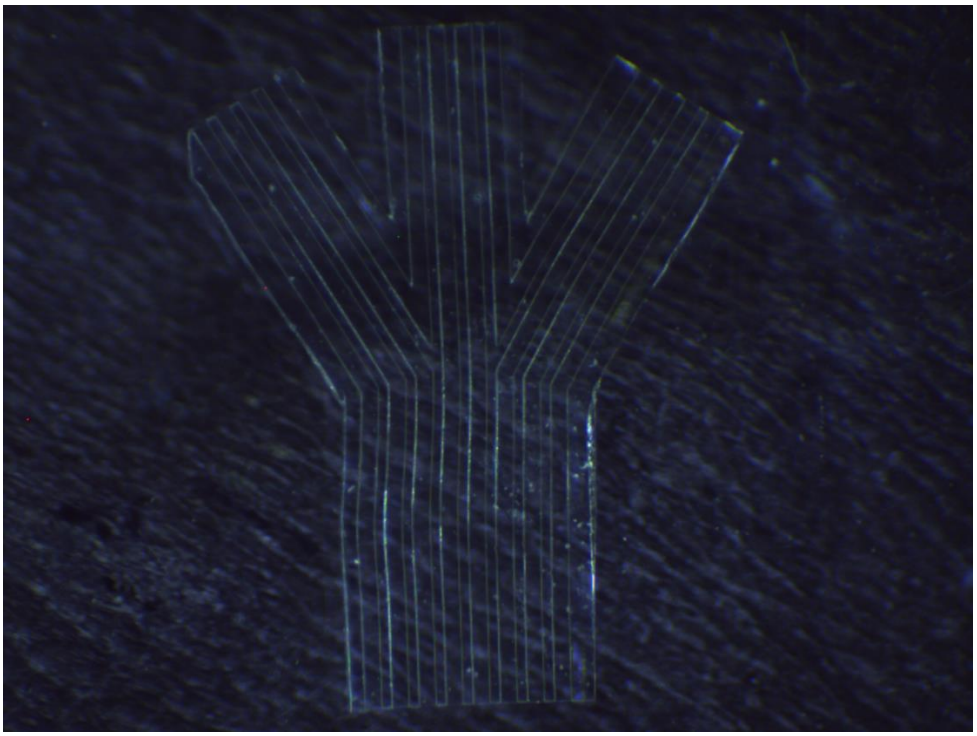

Figure 2.5 Multi-branch microchannel

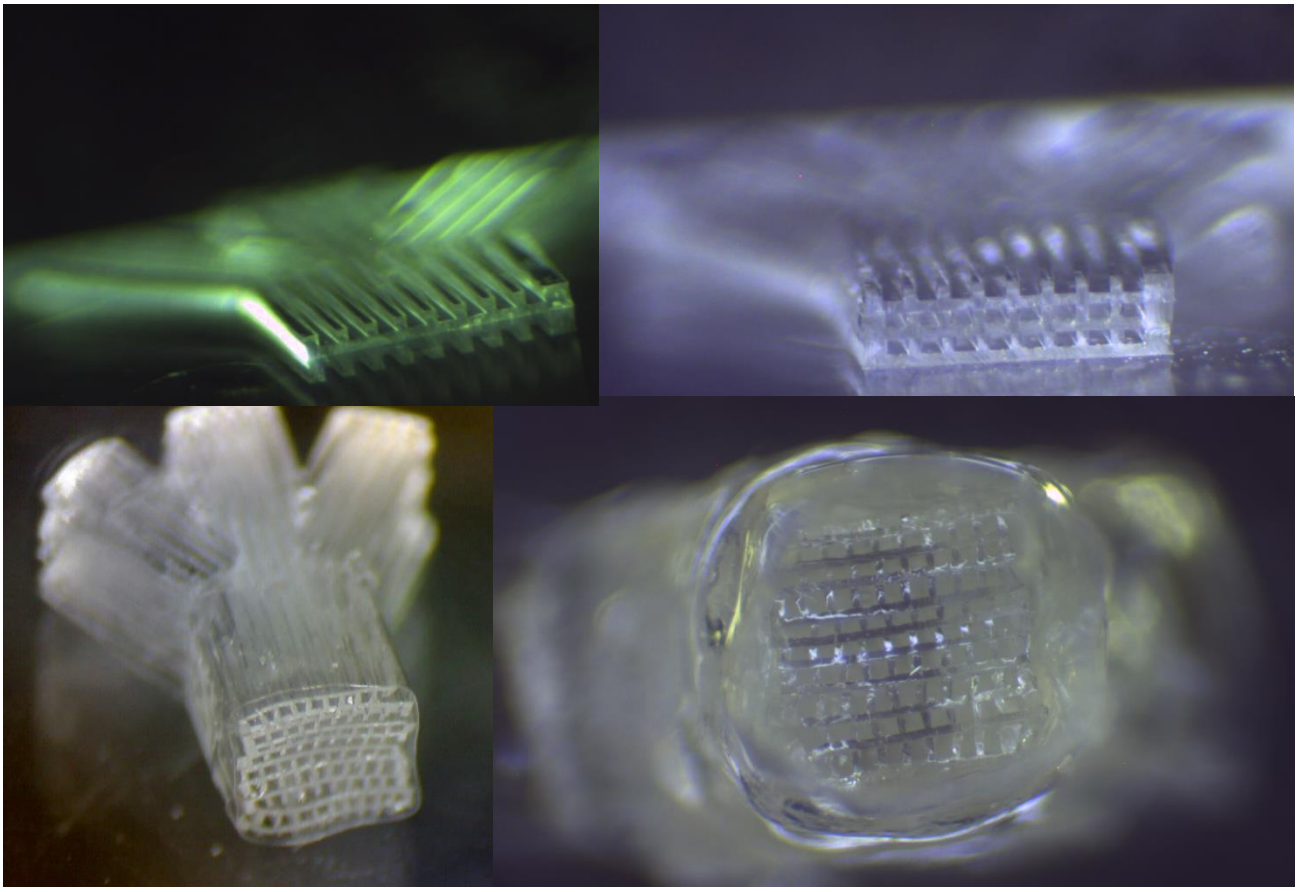

Figure 2.6 A) 1 Layer B) 3 Layers C) Full Device D) 10 Layers

The thin film was wrapped around the microchannel scaffold structure twice in each of the branches (Figure 2.7 and 2.8). And 10:1 PDMS to curing agent mixture (Sylgard® 186, Dow Corning®, MI) was applied to the thin film on top and bottom of the structure. The PDMS curing agent has to follow the shape of the Microchannels to connect all four branches together. This will ensure the stability of the device during the stitching of the nerves.

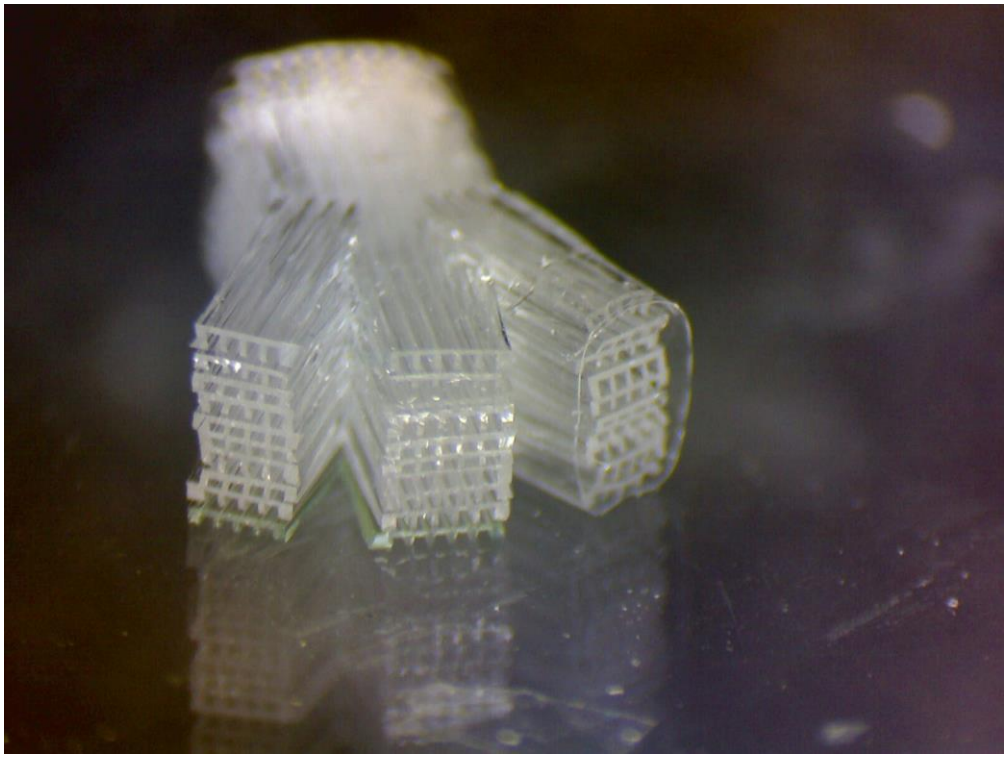

Figure 2.7 Thin film was wrapped around the microchannel scaffold in the branch

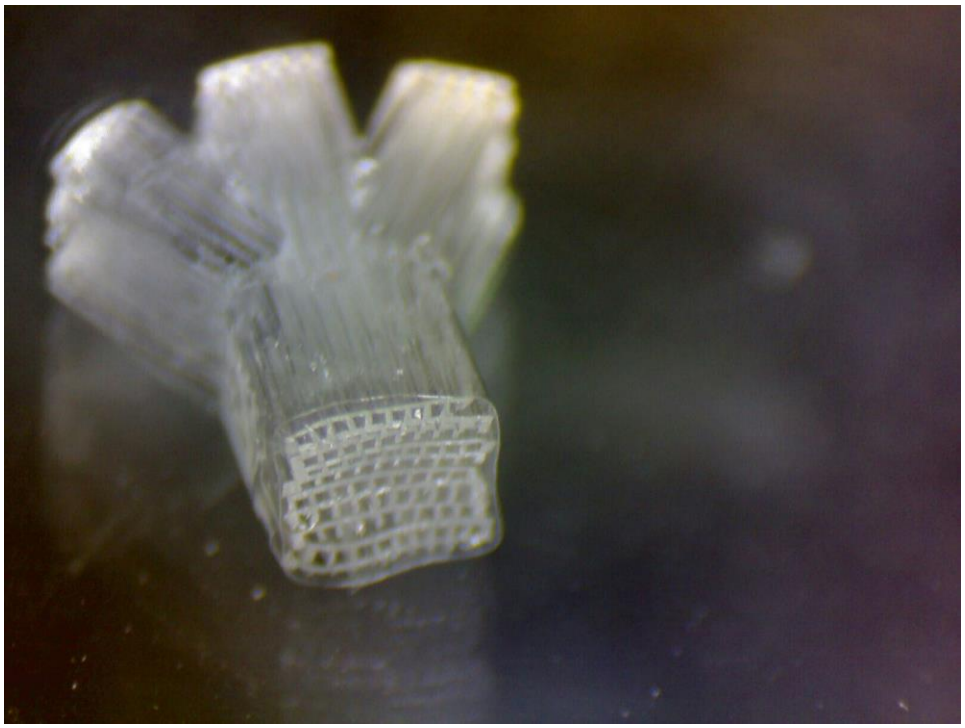

Figure 2.8 Thin film was wrapped around the microchannel scaffold in the trunk

CHAPTER III

SURGICAL PROCEDURE

All surgical procedures were performed under aseptic conditions at the UTRGV Animal facility. The *Lewis* rat was placed into an induction chamber to be subjected to gas anesthesia (5% Isoflurane with oxygen) until unconscious; then a gas mask was hooked to its maxillary central incisors to continue delivering small doses of 2% Isoflurane anesthesia. It was secured to a surgery table and its body temperature was regulated with a heat pad. Its right thigh was shaved and cleaned with betadine scrub and isopropyl alcohol. Incisions were made along the right thigh to expose the sciatic nerve (Figure 3.1(a)). The exposed sciatic nerve was severed, proximal to the sural, tibial, and common peroneal nerves (Figure 3.1(b)). In the severed ends of the nerve, the HMMS was sutured (Figure 3.2). All surgical procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC, 1996) and were reviewed and approved by the Institutional Animal Care and Use Committee UTRGV.

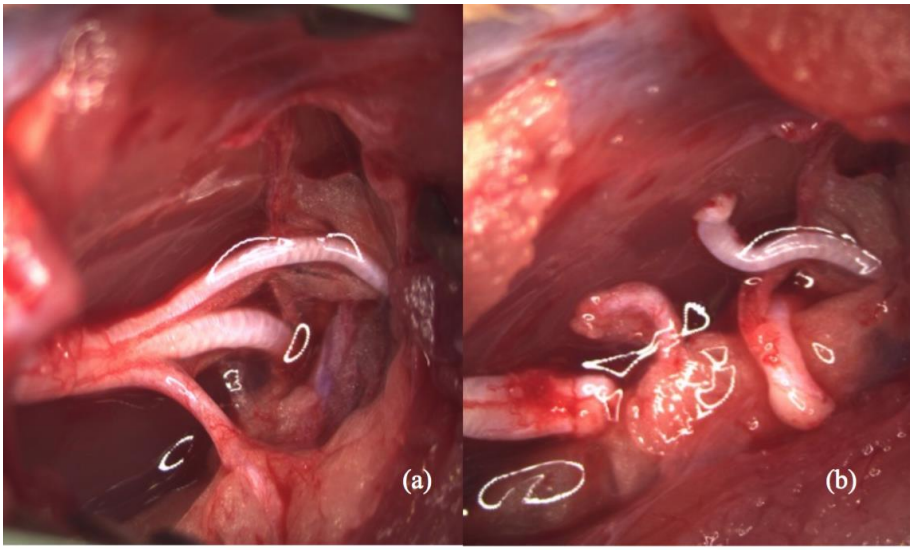

Figure 3.1 (a) complete sciatic nerve with sural, tibial, and common peroneal nerves branches (b) severed nerve connections

Histology. Four weeks after the implantation, the *Lewis* rat was euthanized and perfused transcardially with saline then with 4% paraformaldehyde in 1 x PBS. The HMMS and the sciatic nerve and three distal branches were harvested and post-fixed for 24 hours in 4% paraformaldehyde (Sigma-Aldrich). The sciatic nerve was rinsed in 1 x PBS and transferred to 30% sucrose solution in phosphate buffer, pH 7.4. and frozen at -80 °C for cryosectioning. Then the general IHC protocol for frozen sections was performed: the sections were reacted for an immunofluorescent demonstration of a marker on axons, neurofilament 160 target by a primary antibody (NF160, 1:250 dilution, Sigma-Aldrich) and by a secondary antibody, goat anti-mouse IgG1 Alexa 488. Nuclei were labeled with DAPI, Invitrogen. IHC procedure was started by thawing and cleaning the frozen tissue sections using 1x PBS. The sections were incubated for one hour at room temperature in a blocking solution of 4% goat serum, Invitrogen, in 1 x PBS

containing 0.5% Triton X-100 (Sigma-Aldrich). The sections were again incubated but overnight at 4 °C in a mixture of primary antibody and blocking solution. Then they were washed and incubated in a solution of secondary antibody diluted 1:220 in 0.5% Triton in PBS for one hour at room temperature. The sections were washed once more and then dried. Mounting medium (Fluoromount-GTM with DAPI, eBioscience) was applied on the section; and the section was placed on a coverslip for evaluation. Figure 3.2 shows the harvested tissue with a white colored regenerated nerve in the microchannel scaffold.

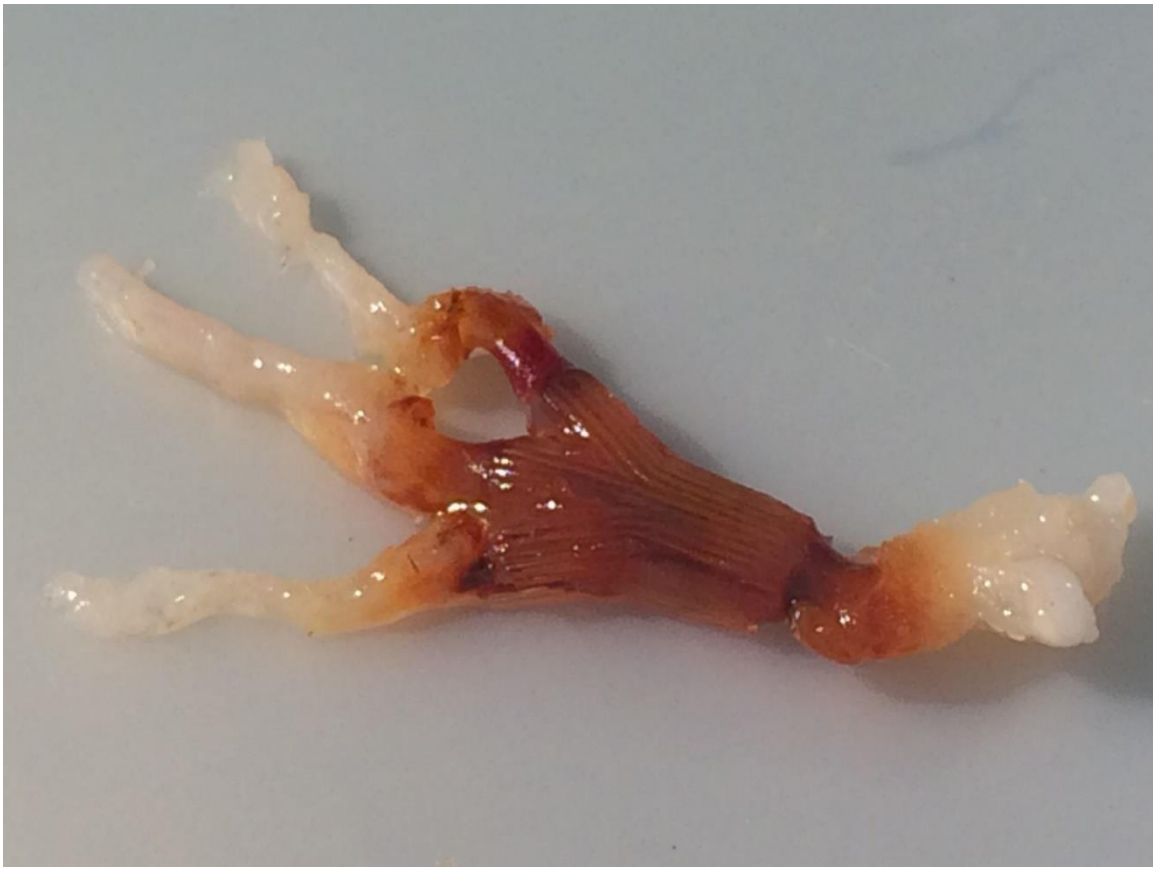

Figure 3.2 Harvested Device

CHAPTER IV

RESULTS

A total of 32 devices were developed, eight devices were successfully implanted into the *Lewis* rats. The harvested devices were submitted to a IHC analysis which revealed robust nerve regeneration of transected sciatic nerves. All 90 channels of each of the multi-branch nerve scaffolds were occupied by the regenerated nerves, which presented patterns of axonal growth. The data from 3D confocal imaging confirmed the distribution of axons seen with Alex Fluor® 633 stained on NF160, showing further details of regenerated axonal growth such as a more thorough analysis of individual axon morphologies [25][26] as well as general axon growth within each constricted 6 mm scaffold channels and the quantification of axons. The z-stack images of layer sections removed after the fourth week showed an average of 30 axons per 120μm diameter channel and robust nerve regeneration in all devices at the right and left turning points of the microchannel (Figure 4.1 and 4.3). Axon modality was difficult to identify at this point due to the motor and sensory axon sizes' being indistinguishable. Typically, some sensory axon types tend to be considerably thinner than most motor axons; and somatic nerves tend to be thicker than autonomic nerves [27-29]. Growth cones were found throughout the whole micro-channel structures but concentrated mostly at the axonal ends. This is seen in Figure 4.2 by the presence of neurofilament shown in red, which, along with the microtubules, is specific to axonal structures and localizes the end of the regenerating axons and growth cones for the sustenance of axon elongation and branching [30-32].

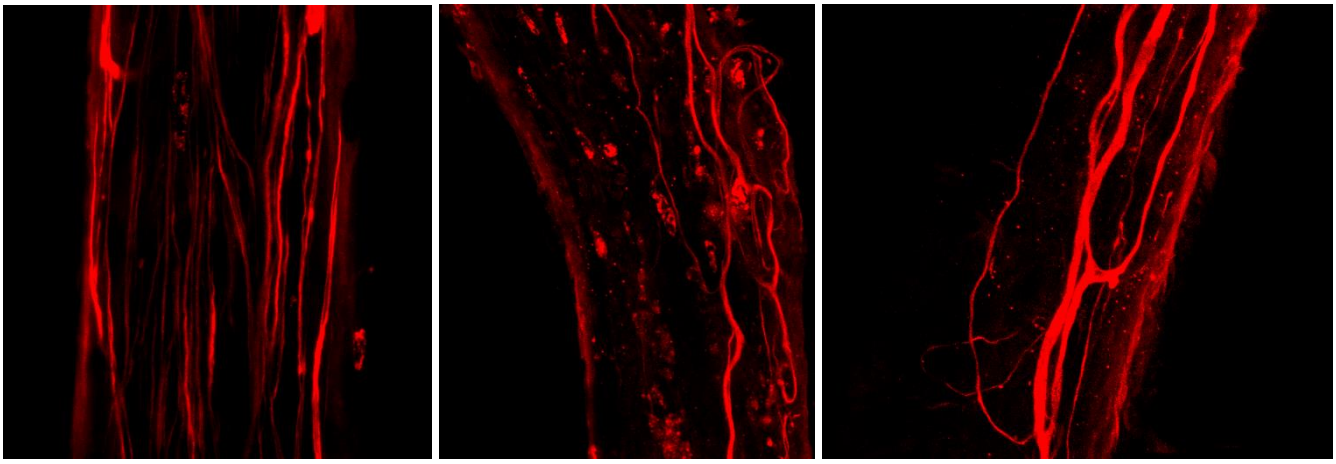

Figure 4.1 Nerve branching shown on z-stack imaging

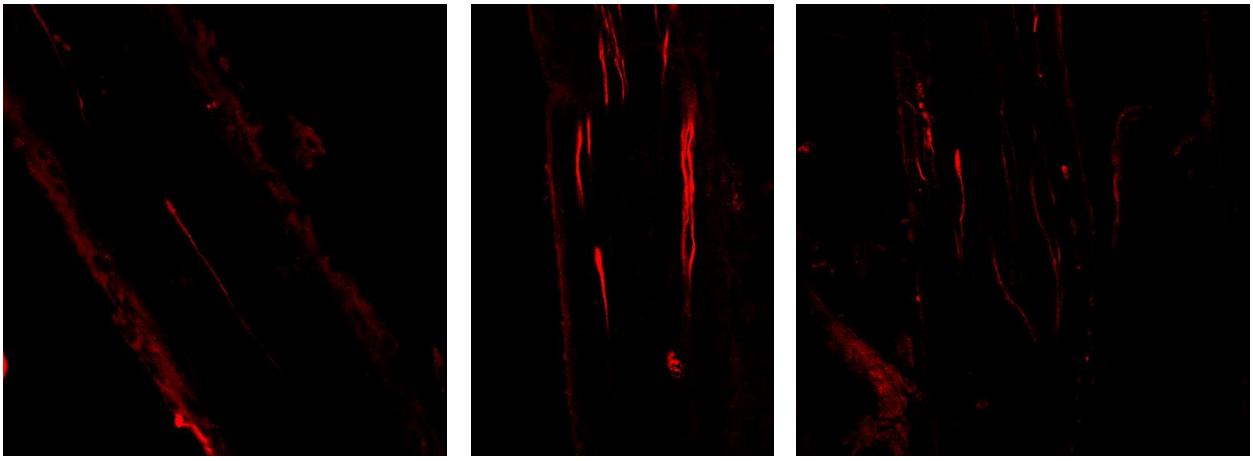

Figure 4.2 Presence of neurofilaments indicating axon regeneration

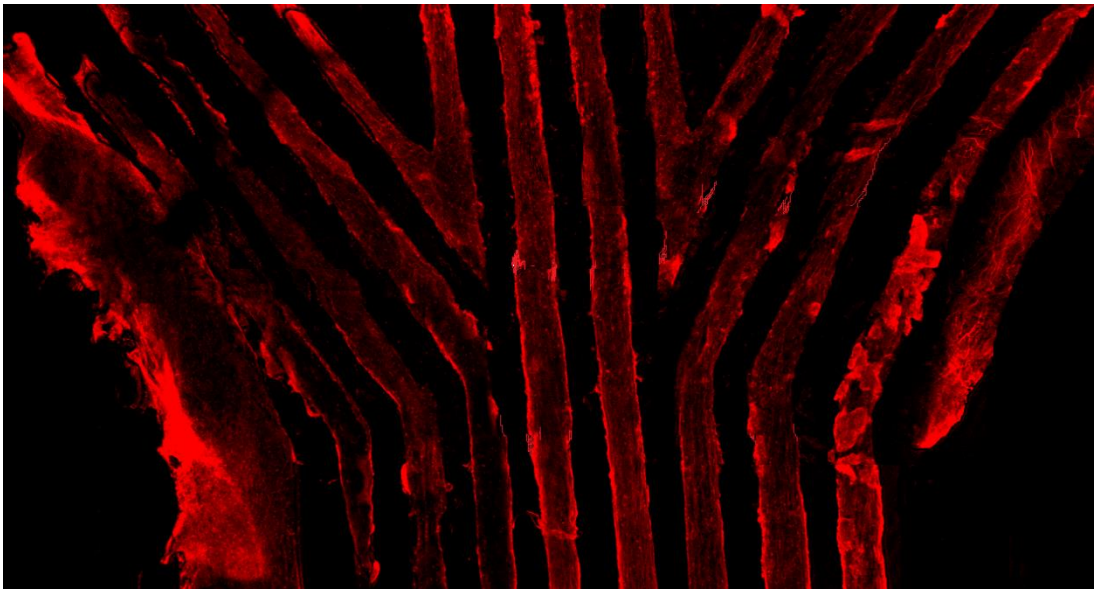

Figure 4.3 Presence of axons in 3 way channel

CHAPTER V

DISCUSSION AND CONCLUSION

The multi-branch microchannel nerve scaffold has been handcrafted and successfully demonstrated the effectiveness in the rat sciatic nerve. There was axon growth and branching to the distal ends of the tibial, sural, and peroneal nerves from the sciatic nerve with the presence of growth cones in all six *Lewis* rats. In the future, the success of nerve regeneration in the *Lewis* rats may be replicated in people, allowing for the repair of multiple nerve branches with fewer surgeries and a better chance of regaining functionality due to a more accurate direction of axonal growth. Moreover, the scaffold may be combined with a neural interface to record and stimulate neural signals for the prosthetic development [33, 34]. There has already been a success of recording efferent motor potentials in awakened mice using a single-branch microchannel neural interface [35-37]. Embedding the electrodes of the neural interface within the microchannel facilitates the identification of neural signals due to an increase in the voltage amplitude of the neural signals from the axon insulation by microchannels. It may also overcome the challenges encountered in extraneural and penetrating electrodes by providing direct axon-to-electrode contact and secure position within the sieve or scaffold [38][39]. Overall, HMMS provides a stable, selective, and proximal environmental for the electrodes [40][41]. The microchannel may also be coated with various chemicals and factors to investigate and compare the effects of stimulation or inhibition of axonal growth to further increase the control over axon regeneration and branching as studies show

that pathways taken by axonal growth cones can be influenced by chemorepulsion and chemoattraction [42-46]. On the other hand, the biocompatible PDMS may be substituted with biodegradable materials such as collagen and fibrin in the fabrication of nerve scaffold [47]. Chitin has also been shown to be an effective biodegradable material for the conduits because it allows the diffusion of neurotrophic factors [48]. Further studies should be done to compare different biodegradable materials to select the one that optimizes axonal growth. However, the biodegradable nature may restrict the extent of the study.

In summary, the handcrafted multi-branch scaffold enables efficient nerve repairs of complex nerve damages with a high chance of success in regaining nerve functions due to the control of axonal growth and directions allowed by the microchannels [49]. It can also provide insights into nerve regeneration and signaling processes which can be applied to various fields and development of better prosthetic interfaces to restore lost functionalities due to peripheral nerve damage [50].

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BIOGRAPHICAL SKETCH

Luis Enrique Castañuela was born in Saltillo Coahuila, México on November 17, 1989. He spent his first 17 years in Mexico under his father's tutelage, also a professor in the field of Computer Science, developing a fascination for Science and technology. He became an exchange student in 2007 to learn the language and started college in 2008 at the University of Texas Pan-American, now called University of Rio Grande Valley. He finished his Computer Engineering degree in 2013 and started his Masters in Electrical Engineering in 2014. He will graduate in August 2016. He is a Research Assistant for the Department of Electrical Engineering and has partaken in several publications in journals such as Biomedical Microdevices (BMMD). His primary research focus is on the regeneration of the peripheral nerve interface for data acquisition and installation of external systems such as prosthetics. Upon graduation from the University of Rio Grande Valley, Luis Enrique is looking forward to beginning his Ph.D. program in the United States or Europe.

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