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# Evaluating the Use of Engineered Nervous Tissue Constructs in the Repair of Peripheral Nerve Lesions and Amputations

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# Evaluating the Use of Engineered Nervous Tissue Constructs in the Repair of Peripheral Nerve Lesions and Amputations

## **Abstract**

Severe trauma to the limbs can often result in the lesioning, or even amputation, of the underlying peripheral nerves. In these cases, endogenous neural repair mechanisms are compromised and a path to the end target may be lost, resulting in the need for surgical intervention. Current repair strategies are incapable of maintaining this regenerative pathway, or providing a bridge to a surrogate end target, often resulting in incomplete repair.

This thesis describes the development and evaluation of a novel method of addressing peripheral nerve lesions and amputations that utilizes living tissue-engineered neural grafts. These grafts are created by the controlled mechanical separation of axons spanning integrated neuron populations in vitro, resulting in axon tracts spanning several centimeters in length. Techniques were developed to encapsulate and transplant these tracts, with the goal of providing structural and nutrient support, while minimizing macrophage infiltration. The efficacy of these constructs in the treatment of lesions and amputations was then assessed using a rat sciatic nerve transection model.

In the first study, the ability of neural constructs to (a) encourage host regeneration from the proximal stump, while also (b) attenuating distal pathway degeneration, was evaluated. At the 4-week time point, the axonal constructs were observed to promote more robust host axonal and tissue regeneration across the graft when compared to unstretched grafts. A measurement of nerve conduction velocities also revealed a statistically significant improvement in the stretch-grown group, correlating with the observed increased fiber regeneration. At the distal pathway, neural constructs were observed to prevent the atrophy of the support cells, and maintain the alignment of the Schwann cell columns for up to 4 months. These results suggest that the use of neural grafts may expand the time window within which successful nerve regeneration can occur.

The axon grafts were then shown to support and maintain regenerating host axon fibers for up to 4 weeks in the absence of a distal end target. Finally, axon grafts pre-attached to an implantable electrode substrate were shown to encourage host ingrowth to the vicinity of the substrate, showing promise for the development of a chronic brain-machine interface.

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Douglas H Smith, MD

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EVALUATING THE USE OF ENGINEERED NERVOUS TISSUE  
CONSTRUCTS IN THE REPAIR OF PERIPHERAL NERVE  
LESIONS AND AMPUTATIONS

Niranjan Kameswaran

A Dissertation

in

Bioengineering

Presented to the Faculties of the University of Pennsylvania in Partial Fulfillment  
of the Requirements for the Degree of Doctor of Philosophy

2010

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Dedicated to my parents and grandparents...

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## ABSTRACT

### EVALUATING THE USE OF ENGINEERED NERVOUS TISSUE CONSTRUCTS IN THE REPAIR OF PERIPHERAL NERVE LESIONS AND AMPUTATIONS

*Niranjan Kameswaran*

*Douglas H Smith, MD*

Severe trauma to the limbs can often result in the lesioning, or even amputation, of the underlying peripheral nerves. In these cases, endogenous neural repair mechanisms are compromised and a path to the end target may be lost, resulting in the need for surgical intervention. Current repair strategies are incapable of maintaining this regenerative pathway, or providing a bridge to a surrogate end target, often resulting in incomplete repair.

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# Chapter 1

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## Introduction

# Chapter 1: Introduction

## 1.1 Clinical Relevance

Peripheral nerve (PN) trauma is a serious condition affecting 2.8% of trauma patients annually [Chiono V et al, 2009]. This form of injury is primarily caused by traumatic accidents, tumor resection or iatrogenic effects of surgeries such as orthopedic procedures [Kretschmer T et al, 2001], and can often lead to lifelong pain and disability. Etiologies of PN trauma include penetrating injury, crush and ischemia [Robinson LR, 2004]. Upper limb injuries occur in over 70% of reported cases, with the ulnar nerve being most frequently damaged.

*Table 1.1 – The Sunderland Classification of Nerve Injury*

<b>Sunderland Scale</b>	<b>Description</b>
First degree	Neurapraxia – segmental demyelination
Second degree	Axonotmesis with intact endoneurium
Third degree	Axonotmesis with damaged endoneurium, intact perineurium
Fourth degree	Axonotmesis with damaged perineurium, intact epineurium
Fifth degree	Neurotmesis – disruption of entire nerve trunk

*Campbell WW, 2008*

The Sunderland classification is a widely used scheme for classifying peripheral nerve injury [Table 1.1] (Sunderland S, 1990). According to this scale, the mildest form of PN injury is neurapraxia, or segmental demyelination, which results in an inability of the nerve to conduct action potentials across the affected region. The loss of function persists until subsequent remyelination, and is typically resolved within 12 weeks. Axonotmesis, which is most often associated



with crush injuries, refers to the loss of continuity of the axon wherein the ensheathing structures remain at least partially undamaged. Repair typically occurs naturally, and the extent of recovery is thought to be a function of the location of the injury and the degree of disruption of the surrounding stroma. The most severe form of PN lesion is neurotmesis. Here, the entire nerve trunk is severed and the ability of the axons to functionally reconnect with their targets is severely compromised. This type of injury typically requires surgical intervention, and even minor functional recovery can take several months using current treatments [Campbell WW, 2008].

The most debilitating form of PN damage occurs as a result of limb amputation. Without presentation of a distal target, the proximal neurites frequently undergo extensive collateral branching, resulting in either inaccurate innervation of surrounding tissue or the formation of painful neuromas. Although the nerve remains electrophysiologically functional, there currently exists no chronic means of recording its activity.

## **1.2 Hypotheses and Goals**

By exploiting a newly discovered method of creating axonal tracts *in vitro* using mechanical tension, we have been able to create transplantable nerve constructs that can be used to repair damaged axonal tracts and nerves. In the following studies, the applicability of these nerve grafts in the repair of PNS

lesions and amputations is assessed. The study design was motivated by the following hypotheses: (1) neural constructs can provide regenerative support for the proximal stump while attenuating degeneration of the distal pathway, and (2) living axonal constructs can provide support for the extension and maintenance of regenerating neurites in the absence of a distal target.

In chapter 2, a synopsis of the stretch-growth mechanism and its adaptability for various therapeutic applications is provided. Chapter 3 describes the development of techniques to encapsulate and transplant nervous tissue constructs into a rat sciatic nerve lesion. Chapter 4 illustrates the beneficial effects of using these constructs for (a) repairing a short nerve lesion, and (b) maintaining the support architecture at the distal stump, potentially increasing the window for successful target reinnervation. Finally, chapter 5 describes how these axonal constructs can be used to (a) extend and maintain regenerating proximal axons in the absence of a distal target, and (b) encourage host axonal growth towards an electrode substrate, which may ultimately enable bi-directional communication with the peripheral nervous system. A synthesis of these findings is provided in chapter 6, as well as future work for this field of study.

## Chapter 2

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# Overview of Stretch-induced Axonal Growth

## Chapter 2: Overview of Stretch-induced Axonal Growth

The study of axon growth has traditionally comprised two distinct areas – growth cone extension and guidance during embryogenesis, and nerve regeneration following traumatic injury. In both these cases, the axon has a free end that advances towards its final target using chemotaxic and/or haptotaxic cues. However, we now know that there is a third form of axon growth that occurs *after* synaptic integration with the target, and is triggered by the application of mechanical tension. This form of growth, which we dub “stretch-growth”, is a newly discovered phenomenon that challenges long-held notions on axonal transport and protein assembly, and could potentially transform our understanding of post-embryonic nerve development.

### *Traditional concepts on axon growth*

Growth cone motility and axonal pathfinding during embryonic development has been extensively studied over the years [Dickson, 2002; Yu and Bargmann, 2001; Tessier-Lavigne and Goodman, 1996]. During this process, pioneer axons forge the initial path to the target, following which all other axons advance. The pioneer axons are influenced by the push-pull effect

of attractive and repulsive chemotaxic cues in the surrounding milieu, as well as the internal mechanical forces generated at the tip of the growth cone. Trailing axons, on the other hand, are believed to respond to a combination of these chemotaxic cues and contact-influenced guidance. Protein assembly has been shown to occur within the growing tip. Developing axons grow at a rate of approximately 1mm/day, and this phase of neurogenesis is typically complete within 8 weeks in humans.

The other form of axon growth that has been extensively researched is post-trauma regeneration. Here, the proximal segment of a transected axon attempts to rejoin its target by a process remarkably similar to that seen during development. After injury, the neuron shifts from a transmitting mode to a growth mode resulting in a growth cone lamelipodial formation at the tip (Dahlin, 2006). This growth cone is guided across the lesion primarily via chemoattractive and chemorepulsive cues until it reaches the distal support cells, after which the guidance is primarily contact-mediated (Mueller, 1999; Goodman, 1996). The response to injury can begin within hours of the insult and may persist for as long as 12 months [Campbell WW, 2008].

While both of these forms of axonal outgrowth are essential for the formation and maintenance of the nervous system, neither can account for the rapid growth of already-integrated axonal tracts in the fetus and thereafter. Since the axons no longer having growing tips, the rapid expansion must occur by a

completely different mechanism, a process of stretch-growth that we are only now beginning to understand.

*Stretch-growth – a newly discovered form of axonal growth*

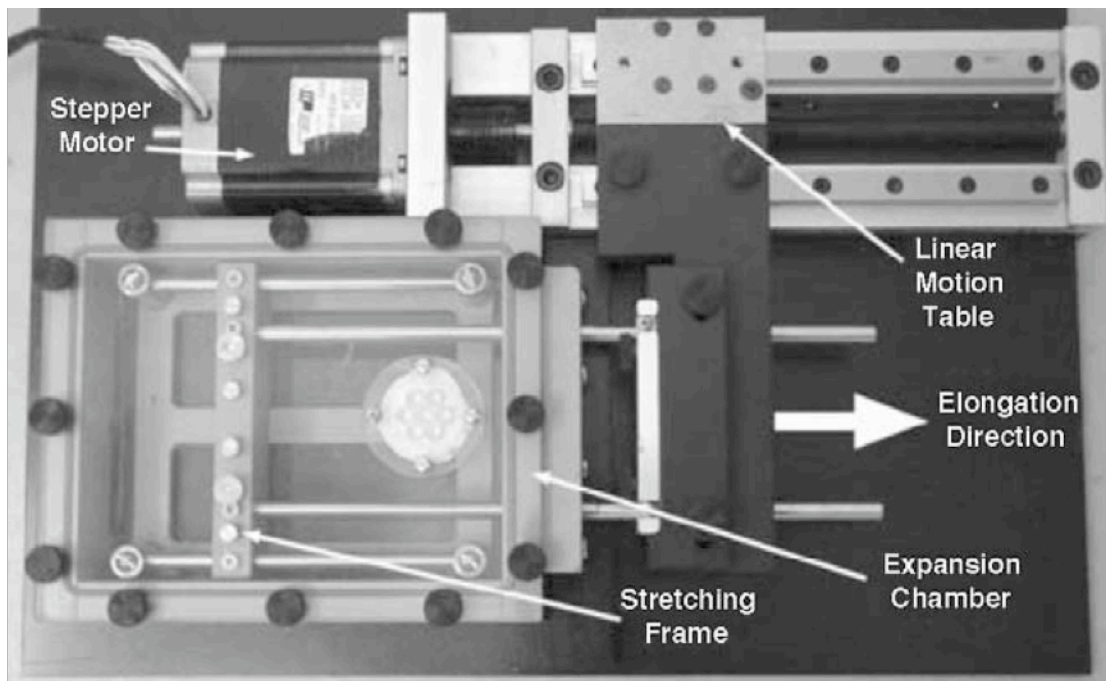
The use of mechanical tension to extend the tip of an axon was first demonstrated in 1984, where a movable substrate was used to tow chick sensory ganglion growth cones [Bray, 1984]. This work has expanded upon since, to induce transient lengthening of the growth cones of single axons *in vitro* [Dennerll et al., 1989; Zheng et al., 1991; Heidemann et al., 1995; Chada et al., 1997]. The interpretation of these findings was that tension generated at the growth cone (by internal cytoplasmic components) is a key factor for the growth of axons. However, the idea that external mechanical forces may play a role in the growth of *synaptically-integrated axon tracts* was suggested as far back as 1935 by RG Harrison and 1941 by Paul Weiss, who referred to it as “passive stretching” and “towing” respectively. They noted that at the peak growth of the human fetus, motor axons in the lower limb grow at rates approaching 1.2mm/day, which is well within the known rates of axonal transport. However, evidence of far more rapid axonal growth can be seen elsewhere in nature. Axon tracts in the giraffe neck have a peak growth rate of 2cm/day [Bannister et al, 1996; Dagg and Foster, 1982]. Axon tracts in the blue whale spine are estimated to grow at even more astonishing rates, potentially exceeding 3cm/day [Smith DH, 2009]. These observations challenge fundamental notions of neural protein

synthesis and transport. The rate of axonal growth has traditionally been thought to be limited by slow transport – indeed, neurofilament proteins have been shown to be shipped down the axon at the rate of a few millimeters per day [Brown, 2003; Nixon, 1998a,b; Roy et al, 2000], which is well below the rates described here. Even at fast transport rates of 300mm/day, it would take over three months for neurofilament proteins to be carried the 30m distance from the cell body down the length of a blue whale axon [Smith DH, 2009]. A possible explanation for this discrepancy could be local synthesis of protein within the axon itself [Alvarez J et al 2000; Brittis PA et al, 2002], which would preclude the need for such long-distance transport.

#### *Experimental verification of stretch-growth using rat primary cortical neurons*

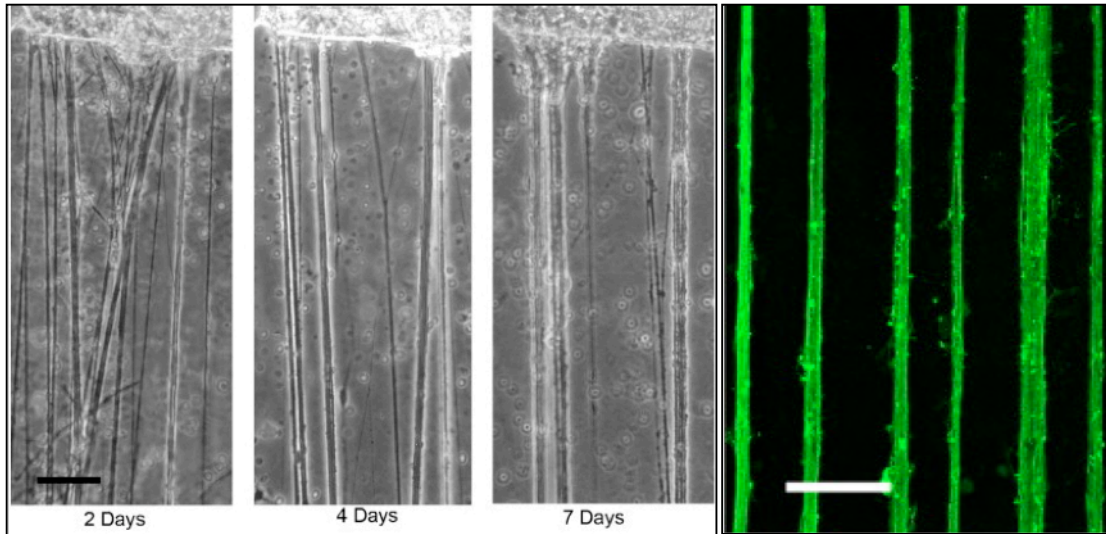
The development of techniques to create transplantable stretch-grown axon tracts evolved from *in vitro* experiments examining traumatic axonal injury [Wolf et al, 2001] using rat primary cortical neurons. In these experiments, tension was transiently applied to axons spanning two neural populations spanning a short gap with the goal of producing a deleterious effect. However, it was noticed that the axons demonstrated a resilience to stretch at less rapid strain rates. Expanding upon these findings, an *in vitro* stretch-growth model was developed wherein axons spanning two populations of either cortical or N-tera 2 cell line neurons were allowed to integrate over several days and then were slowly separated using a micro-stepper motor (Figure 2.1). In initial

studies, the populations were separated at the rate of  $3.5\mu\text{m}$  every 5 minutes, resulting in 1cm axon tracts after 10 days of stretch-growth (Figure 2.2, left). The axons were also seen to coalesce into highly organized fascicles (Figure 2.2, right), potentially replicating the mechanism of organized axonal tract formation during development [Smith et al, 2001].



**Figure 2.1:** Axon stretch-growth device. Integrated axon tracts in the stretching frame are mechanically separated in a controlled manner by the computer-controlled micro-stepper motor system.





**Figure 2.2:** Left – The stretch growth of cortical axon tracts as seen at 2, 4 and 7 days. Adjacent fibers gradually coalesce into aligned axon bundles. Right – Confocal image of cortical axon bundles after 7 days of stretch-growth.

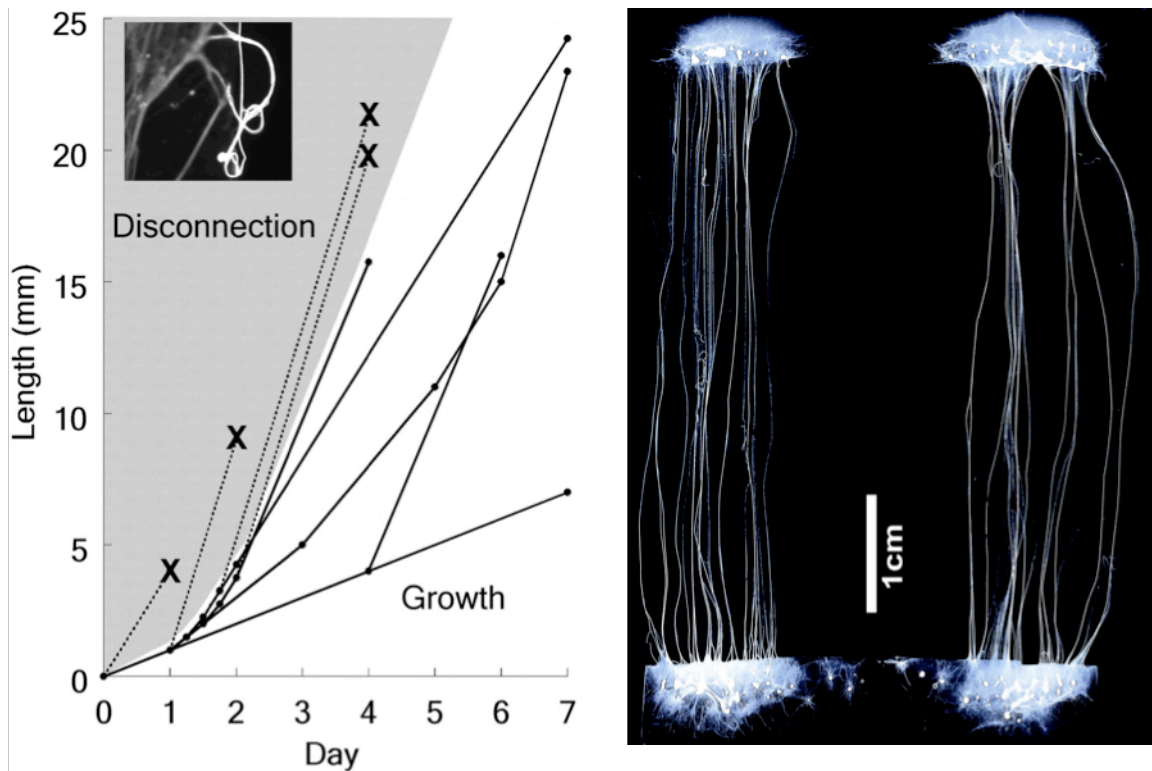
*Smith DH et al, 2001*

#### *2.1.4 Extreme stretch-growth of rat dorsal root ganglion axons*

With a view towards development of a transplantable axonal construct for eventual clinical usage, dorsal root ganglia (DRG) neurons were selected as our primary cell source. Adult human DRG neurons can be harvested from patients undergoing elective ganglionectomies, making them a viable autologous cell source. Human and animal DRG neurons are also known to be robust in culture, and can survive for extended periods of time while maintaining normal electrophysiological function [Pfister et al, 2006a,b].

Subsequent experiments revealed strain rate and acclimation to be the main determining factors for successful stretch-growth. Starting at the low rate of 1mm/day, the strain rate could be gradually increased, provided the axons were given a sufficient “rest period” in order to acclimate (Figure 2.3a). By employing

this strategy, growth rates of up to 1cm/day were reached within a few days of commencing stretch-growth [Pfister et al 2004]. In addition, integrated axonal tracts of up to 10cm length could be created in less than 2 weeks (5cm length shown in Figure 2.3b).



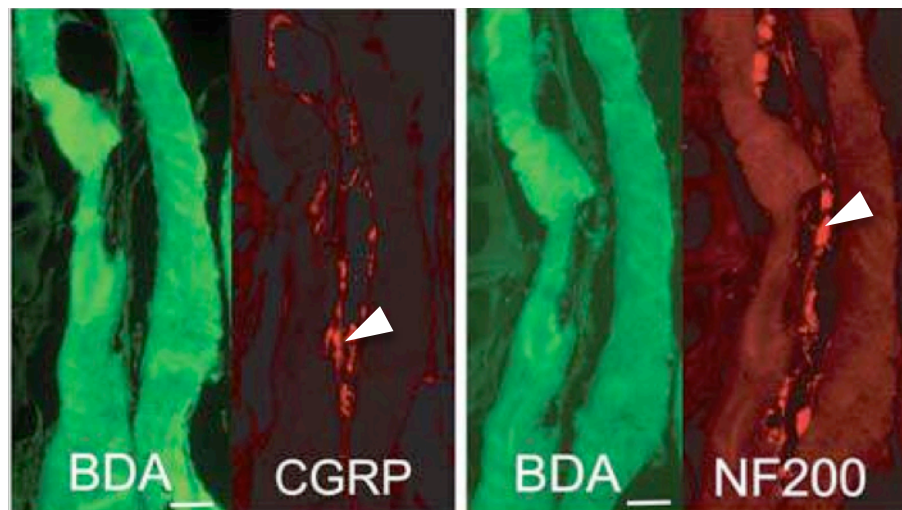
**Figure 2.3:** Left – Graphical representation of acceptable stretch-growth parameters for DRG neurons. Right – DRG axon tracts in culture spanning 5cm.

Despite these high-growth rates, the stretch-grown axons were seen to possess a normal morphology. However, the average diameter of these axons was measured to be 30% greater than non-stretched DRG axons, possibly in order to sustain increased stability and transport. Transmission electron microscopy revealed a normal complement of microtubules and neurofilaments per cross-section area. In addition, the axons were shown to be able to conduct

normal action potentials, although they did appear to possess an increased density of sodium and potassium channels [Pfister et al, 2006a,b].

#### *Using stretch-grown axon tracts to repair the nervous system*

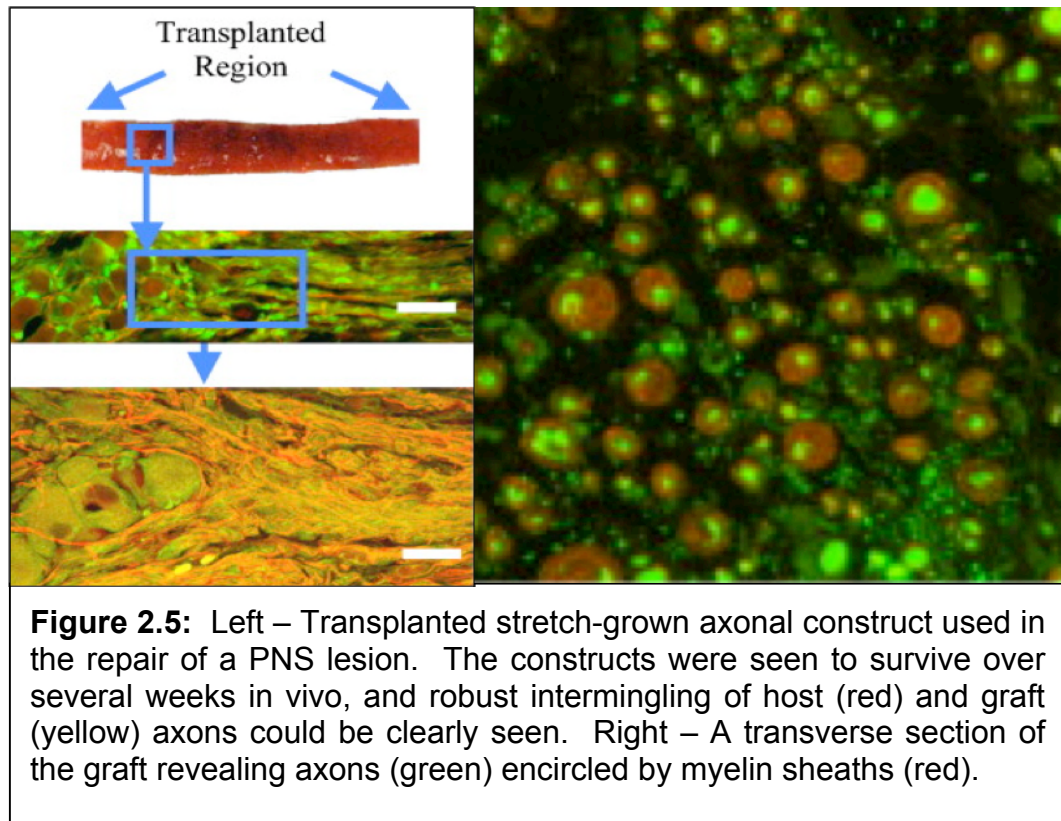
In addition to providing insight into the mechanisms of axon growth during development, stretch-grown axonal constructs may be exploited for nerve repair. Living nerve tracts can be rapidly created to any desired length *in vitro* and transplanted to bridge even extensive central and peripheral nervous system lesions. In the case of spinal cord repair, transplanted axonal tracts were used to bridge a 1cm defect spanning three vertebrae in the rat. The constructs were seen to survive and remain structurally intact one month post-transplantation



**Figure 2.4:** Survival of transplanted stretch-grown axons in the spinal cord at 1 month. The transplanted cells (indicated by the arrows) were found sandwiched between layers of collagen in the SCI cavity.

*Iwata A et al, 2006*

(Figure 2.4) [Iwata A et al, 2006]. Evidence of host axon penetration into the graft area, as well as graft neurite outgrowth into the host tissue, was also observed. It is still unclear whether the constructs form functional synaptic connections with the host – however, these results demonstrate the feasibility of this approach and merit further investigation.

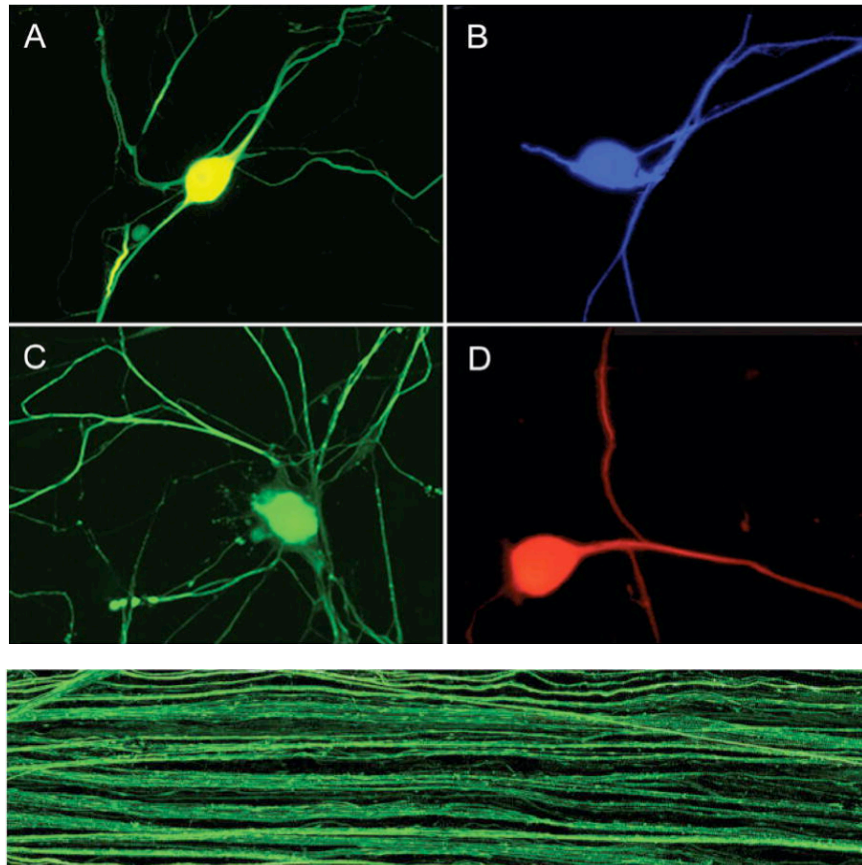


Axonal constructs have also been used to bridge 1cm lesions in the rat sciatic nerve (Figure 2.5). The graft tissue was seen to survive for at least 4 months without immunosuppression. Robust intermingling of host and graft axons was seen in the repaired lesion area as well as in the proximal and distal nerve segments, indicating graft neurite outgrowth into the host. Analysis of

cross-sections of these nerve segments revealed robust regeneration and myelination of the axons.

#### *Stretch-grown axons from Human dorsal root ganglia*

Ultimately in order to be clinically viable, axonal constructs will need to be generated from autologous tissue. As previously mentioned, human DRG neurons can be isolated from patients undergoing elective ganglionectomy procedures, and can be successfully maintained *in vitro*. We have been able to obtain ganglia from live human patients, as well as from organ donors, and have maintained the tissue in culture for at least 3 months, replicating the findings of other studies. We have also been able to create stretch-grown axonal constructs from these human DRGs, thus demonstrating the feasibility of this approach [Huang J et al, 2008].



**Figure 2.6:** Fluorescence micrographs of adult human DRG neurons and axons. The human DRG neurons were labeled using (A) CGRP or neurofilament proteins; (B) RMO-254; (C) SMI-31; and (D) neurofilament-200. Bottom – Aligned fascicles seen upon stretch-growth of human DRG axons.

*Huang J et al, 2008*

## Chapter 3

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Development of Nervous Tissue

Encapsulation Techniques

## Chapter 3: Development of Nervous Tissue Encapsulation Techniques

With the goal of creating transplantable axonal tracts that may be used to bridge CNS and PNS lesions, we have developed techniques to encapsulate and remove the constructs from the *in vitro* environment without disrupting their structural integrity and alignment. The two major components required for transplantation are an embedding hydrogel and an encapsulating nerve guidance channel. The primary criteria behind hydrogel selection were structural support offered, ability to incorporate nutrient supply and permissiveness to neurite outgrowth. While our initial design solely consisted of a simple collagen hydrogel, it has since evolved to include a variety of materials, each serving a unique and critical purpose.

### ***Initial design – collagen hydrogel***

The first material employed for construct encapsulation and removal was a simple collagen hydrogel (3.67mg/mL) in Dulbecco's modified Eagle's medium supplemented with nerve growth factor (2.5S, 10ng/mL). The collagen matrix was gently applied over the construct axons and allowed to polymerize in the incubator at 37°C. Upon gelation (typically 10-15 minutes) the cell cultures lift off the underlying Aclar membranes and become completely



immersed in the collagen. The embedded axons are then rolled along the axis of the tracts, and the entire hydrogel-tissue combination is deposited into a nerve guidance channel cut open lengthwise [Iwata et al, 2006].

This approach has several disadvantages. While the 80% collagen does provide a permissive environment for neurite growth and is seeded with growth factors, the structural support offered for the axons is minimal. Removal from the culture environment using this technique can be precarious – axon tracts are regularly damaged and discarded during the removal process, and there is no certain way of determining if they are intact even upon successful transference into the tube. With the goal of overcoming these limitations, and facilitating easier removal from the culture environment, a variety of additional hydrogel components were systematically evaluated.

### ***Incorporation of agarose hydrogel***

Agarose is a naturally occurring polysaccharide that has been extensively studied in PNS repair. It can be easily formulated to a wide range of stiffnesses, each of which has different effects on neurite outgrowth. Neither central nor peripheral neurons have any known receptors that interact with agarose side chains.

Our first goal was to evaluate the range of gel stiffnesses that could be applicable to the construct encapsulation and transfer procedure. It has been shown by other groups [Balgude AP et al, 2000] that DRG neurite outgrowth is

inversely proportional to the stiffness of the gel used, with minimal outgrowth at formulations exceeding 2% (wt/vol). Our initial design assessment, therefore, was limited to agarose formulations of 0.5-2.0% (wt/vol).

### **3.1 Materials and Methods:**

Agarose is water-soluble at temperatures exceeding 65°C and typically gels at a temperature range of 17-35°C. It is clear and stable once gelled, and does not re-liquefy until heated above 65°C again. The agarose gels used in this experiment were created in the range of 0.5% to 2.0% (0.25% increments, wt/vol) by heating and stirring agarose powder (Ultrapure; Invitrogen, Carlsbad, CA) in pH 7.4 1x Dulbecco's phosphate buffered saline (PBS; Gibco, Grand Island, NY).

Dorsal root ganglion neurons were isolated from embryonic day 15 fetuses from timed-pregnant Sprague-Dawley (Charles River, Wilmington, MA) rats. The DRG explants were suspended at  $5 \times 10^6$  cells/mL in Neurobasal® medium supplemented with 2% B-27, 0.4 mM L-glutamine, 1% penicillin/streptomycin, 2 mg/mL glucose (Sigma-Aldrich, St. Louis, Mo), 10 ng/mL 2.5S nerve growth factor (NGF) and 1% fetal bovine serum (FBS) (HyClone, Logan, UT), and a mitotic inhibitor formulation of 10 mM 5-fluoro-2'-deoxyuridine (FdU) (Sigma-Aldrich), and 10 mM uridine (Sigma-Aldrich) to encourage non-neuronal cell elimination.

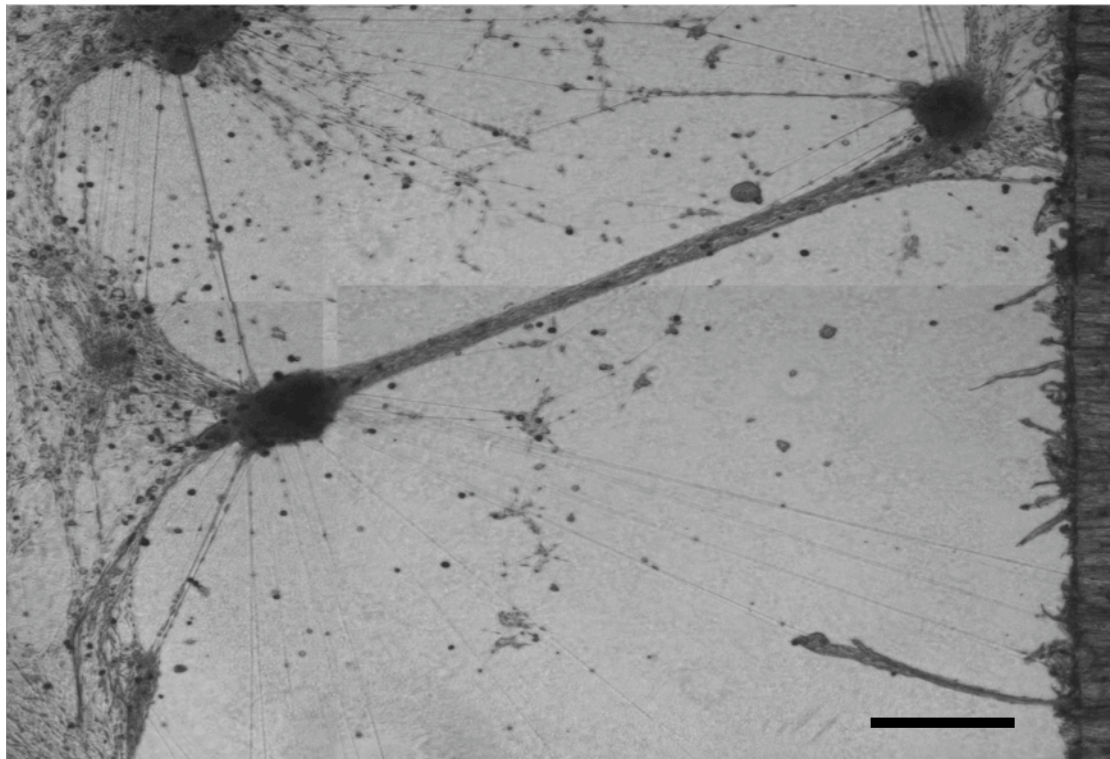
## **3.2 Results**

### *Evaluation of agarose as an encapsulating medium*

The isolated DRG explants were initially plated at the interface of two overlapping membranes of Aclar pre-coated with rat-tail collagen (3.67mg/mL) in order to approximate initial stretch-growth conditions. After allowing the populations to integrate over 5 days, liquefied agarose formulations at 37°C were added directly over the cultures and allowed to gel by cooling them to 4°C for 2 minutes, followed by placing them for 10 minutes in the incubator at 37°C. Using a cell scraper, the agarose was then gently separated from the underlying Aclar layers. At all formulations exceeding 1.5%, the cells remained preferentially adhered to the underlying collagen substrate and did not separate from the Aclar. Below 1%, however, the agarose was structurally fragile and transference into the tube repeatedly resulted in fragmentation of the gel and disconnection of the integrated axonal connections. Those instances of successful transfer also necessitated the length-wise opening of the tubes for insertion and its subsequent re-suturing. For these reasons the strategy of adding agarose to already integrated tracts was abandoned, and its use as an underlying substrate was evaluated.

### *Evaluation of agarose as a base substrate*

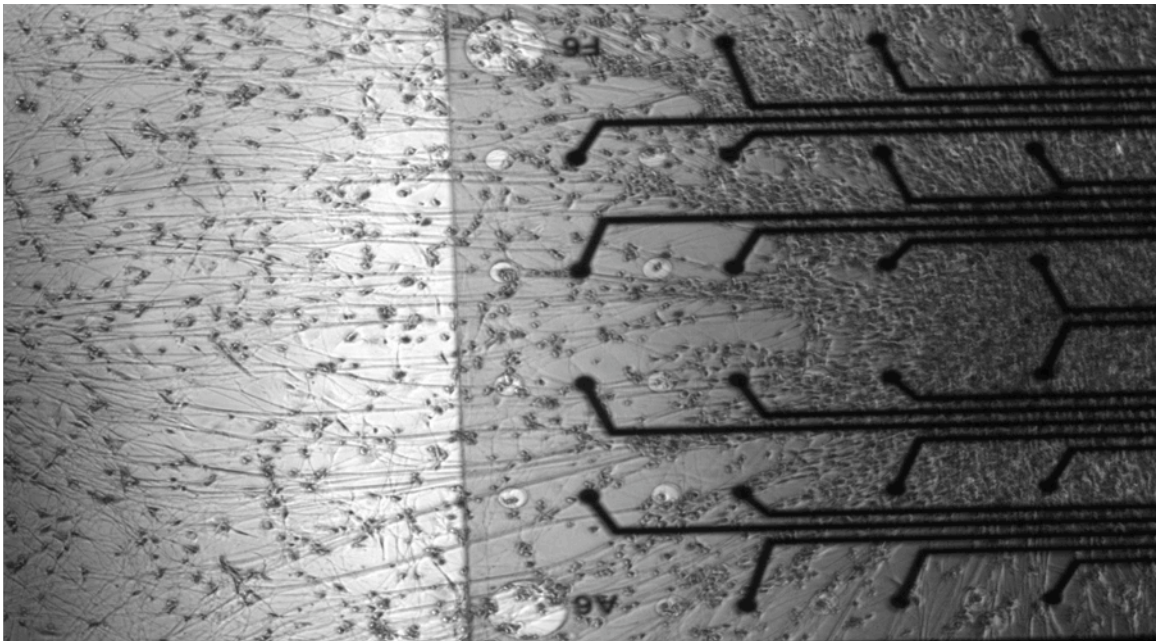
In the next design iteration, agarose was used as the base substrate on which the cells were directly plated. Following from our previous findings, the formulations were now limited to 1.0-1.5%. Liquefied agarose was added to the bottom Aclar substrate in the elongator chamber, and allowed to gel by cooling it to 4°C for 2 minutes followed by 10 minutes in the incubator at 37°C. The towing membrane was then placed over the agarose a thin layer of collagen (3.67mg/mL) was then coated over the interface and allowed to polymerize in the incubator overnight. Isolated DRG explants were then added at the interface and



**Figure 3.1:** Stretch-growth of axons spanning two DRG explants plated on a 1.25% agarose hydrogel base layer. Scale bar = 1mm

allowed to adhere and integrate over 5 days. In all 3 formulations (1%, 1.25% and 1.5% wt/vol) axons were seen to span the two populations (Figure 3.1). In order to assess ease of removal from the culture environment, matched concentrations of agarose were added to the neural populations and the towing membranes were gently separated using a cell scraper. The neurons were found to remain encapsulated within the hydrogel, and axon tracts could be easily cut out using a scalpel blade and directly inserted into the tube through the open ends.

*Adaptation of design to incorporate polyimide electrode array*



**Figure 3.2:** Axons spanning the interface of a polyimide electrode array and the 1,25% wt/vol agarose hydrogel base layer.

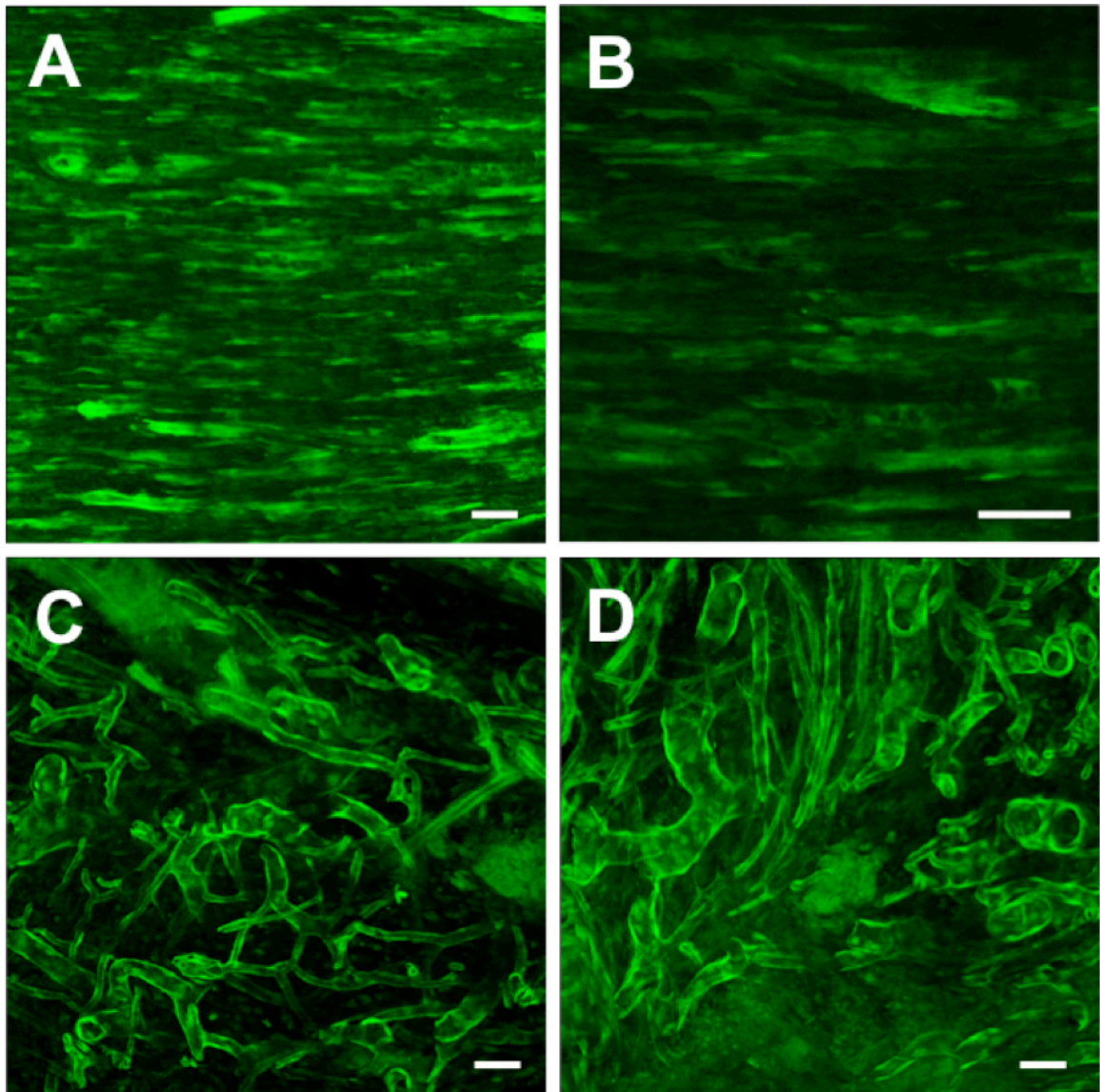
A polyimide electrode array was then incorporated into the design, in order to develop the optimal parameters for neural-interface encapsulation. After formation of an initial agarose base layer and placement of the towing membrane, a thin (1.5mm x 5mm) strip of polyimide was positioned length-wise over the agarose at the hydrogel-towing membrane interface. A thin layer of collagen (3.67mg/mL) was added at the towing membrane-polyimide junction, and more agarose was added to the other end of the polyimide strip to anchor it in place. As before, isolated DRG neurons were then plated at the interface, and allowed to integrate over 5 days (Figure 3.2).

During encapsulation and removal from the elongation device, it was found that the 1% agarose was unable to sufficiently secure the polyimide in place, resulting in frequent defragmentation of the encapsulated construct during removal and insertion into the tube. On the other hand, the 1.25% and 1.5% agarose formulations did not suffer from this structural disintegration. As a result of these findings, and with the goal of minimizing the agarose stiffness, a final agarose concentration of 1.25% wt/vol was selected.

#### *In vivo evaluation of encapsulation design*

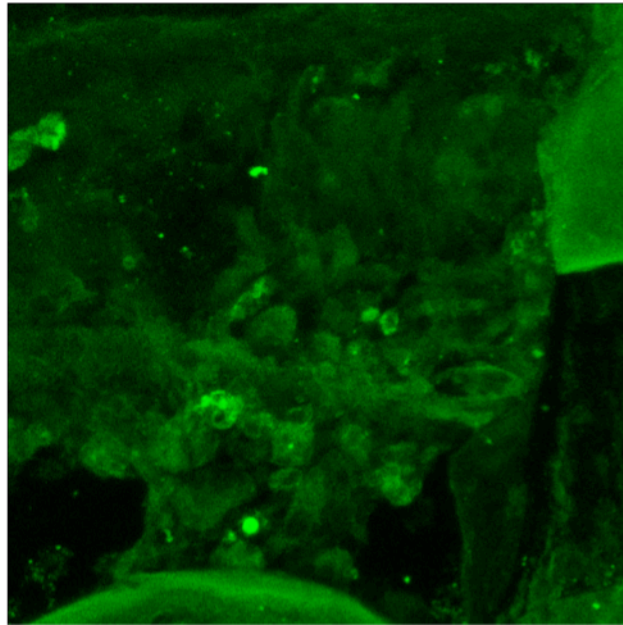
In order to assess the feasibility of this design *in vivo*, static neural constructs were generated, encapsulated in 1.25% agarose, inserted into a 1cm Neuragen™ tube and sutured to the proximal and distal stumps of a transected

sciatic nerve. The constructs were harvested at 2 weeks and examined for signs of nerve regeneration, vascularization and/or host immune response. The agarose was still predominantly intact at this time. Axon infiltration into the tube was observed, as were signs of vascular ingrowth (Figure 3.3). In addition however, ED-1 staining revealed substantial macrophage infiltration into the tube



**Figure 3.3:** Confocal reconstructions of the regenerating sciatic nerve (GFP<sup>+</sup>) within the transplanted construct. **(A-B)** Axon tracts running longitudinally along the nerve. **(C-D)** Revascularization was observed along the outer nerve perimeter. Scale bars = 50µm

(Figure 3.4), and in the general vicinity of the transplanted neurons. While macrophages are a necessary element in the degradation of agarose, their infiltration was perceived to be potentially disadvantageous considering the presence of the allograft tissue.



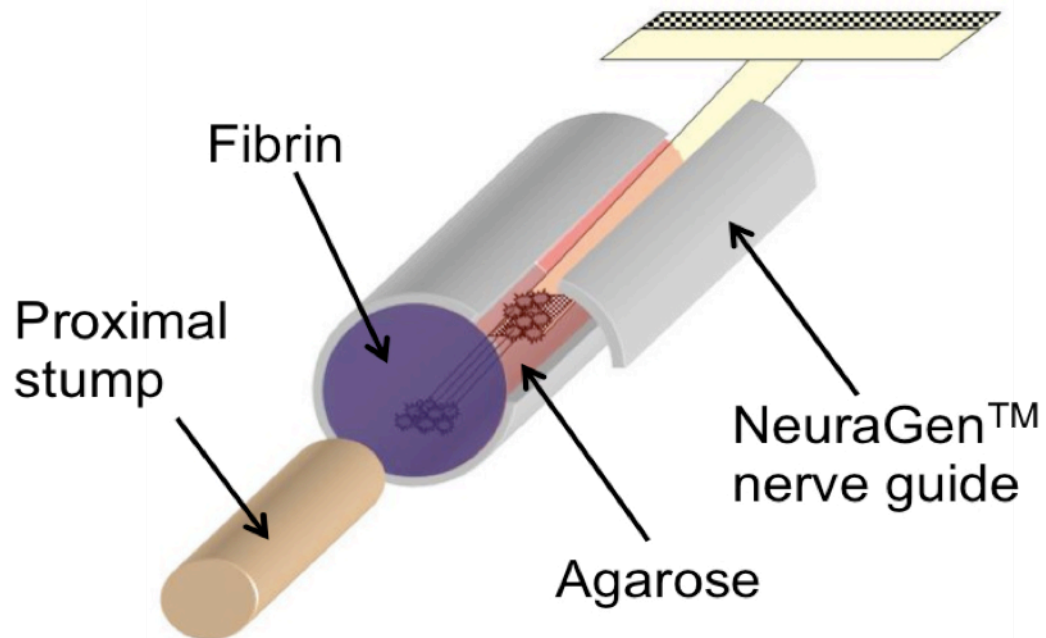
**Figure 3.4:** ED-1 positive cells adjacent to transplanted DRG neurons, indicating macrophage infiltration. Scale bar = 50 $\mu$ m

#### *Final encapsulation design*

With the goal of attenuating macrophage infiltration, salmon fibrin gel (5mg/ml fibrinogen, 5 NIH units/ml thrombin in Neurobasal) was incorporated into the final encapsulation design (Figure 3.5). Salmon fibrin has been shown to degrade more slowly than mammalian fibrin, and is resistive to glial and



macrophagic infiltration [Ju Y et al, 2007; Georges PC et al, 2006]. Fibrin is also extensively used as a surgical adhesive, thus making it a clinically appropriate choice of end sealant.



**Figure 3.5:** Schematic representing final construct encapsulation design. The stretch-grown axons, pre-attached to a polyimide electrode array, are encapsulated in 1.25% agarose hydrogel and inserted into a NeuraGen™ nerve guide. After suturing the tube to the proximal stump, the ends are sealed using fibrin gel.

### **3.3 Conclusion**

By exploiting the mechanism of axonal growth, we have been able to engineer living axonal tracts of several centimeter lengths in vitro. We have demonstrated our ability to utilize these constructs to repair lesions in the central and peripheral nervous system. By optimizing the encapsulation techniques, we have been able to create a stable method of removing the axonal tracts from the culture environment without disrupting their alignment and structural integrity. This encapsulation design also permits the incorporation of a thin flexible electrode substrate for the goal of creating a stable neural interface with the peripheral nervous system.

## Chapter 4

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# Repair of Lesions in the Peripheral Nervous System Using Stretch-Grown Axonal Constructs

# **Chapter 4: Repair of Lesions in the Peripheral Nervous System Using Stretch-Grown Axonal Constructs**

## **4.1 Overview**

The current 'gold standard' for repair of PN lesions are autologous nerve grafts. These grafts, typically of the sural nerve, provide a physical bridge across the lesion and supply endogenous biological cues to guide regenerating axons from the proximal nerve stump to the remaining distal nerve segment. However, this approach is plagued by permanent loss of harvested nerve function and the potential formation of painful neuromas [Lundborg, 2004; Sinis et al, 2007]. Furthermore, there are obvious limitations in the supply of donor nerves, making autografts inadequate to repair extensive nerve damage. Alternative clinical approaches to bridge PN lesions include synthetic tubes composed of poly(glycolic acid) (PGA) or collagen; however, these conduits by themselves have been only used for bridging relatively small peripheral nerve (PN) gaps (Dellon, 2006; Trumble et al., 2006). Moreover, no current strategy simultaneously addresses the steady degeneration of support cells in the nerve segment distal to the injury site. This degeneration ultimately severely limits recovery of function due to the eventual loss of cues to guide regenerating axons beyond the lesion to their final targets. This need is particularly important in cases of major nerve lesions where lost nerve segments reach greater than

several centimeters, or complex lesions to multiple nerves where conventional approaches are overwhelmed or unsuitable. In these instances, the rate of regeneration, ranging between 1-4 mm/day, may result in repair taking months or even greater than one year, depending on the location of the injury, over which time the distal support cells no longer remain. In addition, the end targets themselves will undergo degeneration and atrophy after a period of denervation, further impeding functional recovery [Lien SC et al, 2008]. Thus, there is clearly a need to address the issue of distal pathway degeneration in conjunction with regeneration of the proximal stump in order to enable complete functional recovery.

More recently, tissue-engineered solutions have been sought to overcome the limitations associated with autografts and nerve guidance channels. These approaches include creating combinations of permissive scaffolds (such as decellularized grafts or hydrogels), extracellular matrix (ECM), trophic factors, and glial or stem cells (Evans et al., 2002; Frerichs et al., 2002; Lee et al., 2003; Yu and Bellamkonda, 2003; Fansa and Keilhoff, 2004; Hu et al., 2005; Stang et al., 2005; Chalfoun et al., 2006; Keilhoff et al., 2006; Nie et al., 2007). The importance of anisotropy has been well recognized as an important spatial cue to direct axon growth, and is typically achieved via gradients (e.g., neurotrophic or ECM), longitudinally-aligned fibers, or tailored porosity (Matsumoto et al., 2000; Bellamkonda, 2006; Dodla and Bellamkonda, 2006; Bozkurt et al., 2007).

Typically, the optimization of these tissue-engineered constructs *in vitro* focuses on developing permissive environments for axonal growth cone extension.

Unfortunately, these approaches typically ignore the fact that axonal outgrowth *in vivo* occurs along Schwann cells and the basal lamina; thus, strategies that were optimized based on directly promoting axonal outgrowth *in vitro* may not translate mechanistically. In addition, all of these approaches are geared towards promoting regeneration of the proximal stump; none of these strategies can delay the eventual degeneration of the distal pathway.

We have recently begun investigating axon-mediated axon outgrowth as an alternative mechanism of regeneration. By exploiting the newfound mechanism of “stretch-growth”, whereby large axon tracts can be created by the controlled and continuous separation of integrated neural populations, we have created transplantable neural constructs that have been shown to effectively bridge a 1cm sciatic nerve lesion in the rat 4 months post-transplantation. However, the advantage of utilizing stretch-grown axonal constructs vis-à-vis a simple (or “static”) neural culture was unknown. We also observed evidence of graft axon outgrowth into the host nerve; this suggests the possibility of maintaining the distal pathway support architecture by providing a temporary axonal surrogate, a phenomenon often referred to as nerve “babysitting”. Therefore, the objectives of this study were to (1) determine if stretch-grown axons provide an advantage over static cultures in promoting of host

regeneration, and (2) assess whether axons emerging from the graft can attenuate the degeneration of the distal nerve segment.

## **4.2 Repair of a 1cm PNS lesion using stretch-grown axons**

### ***4.2.1 Relevant Background***

During development of the peripheral nervous system, axons known as “pioneer axons” prescribe the initial path for subsequent axons to follow in a process known as “selective fasciculation” [Dickson, 2002; Yu and Bargmann, 2001; Tessier-Lavigne and Goodman, 1996]. The pioneer axons are believed to enable this targeted axonal outgrowth by a combination of neurotrophic support and contact guidance. This phenomenon has inspired a variety of strategies in the area of peripheral nerve repair. Several groups are investigating the use of growth factors and/or Schwann and glial cell combinations to enhance nerve growth within nerve guidance channels [Evans et al., 2002; Frerichs et al., 2002; Lee et al., 2003; Yu and Bellamkonda, 2003; Fansa and Keilhoff, 2004; Hu et al., 2005; Stang et al., 2005; Chalfoun et al., 2006; Keilhoff et al., 2006; Nie et al., 2007]. Yet others have shown that axons appear to prefer longitudinally-aligned fibers [Kim et al, 2008; Bellamkonda, 2006] as a regenerative substrate, and attempts are being made to create trophic factor-eluting versions of these [Cao et al, 2009].

Building upon these findings, we hypothesize that the axonal portions of the tissue-engineered neural constructs contribute more to host PN regeneration than static cultures, as they may provide both trophic support, as well as a longitudinally aligned pre-established pathway that spans the entire lesion. In this study, we investigate the repair of a 1cm sciatic nerve lesion using transplants of stretch-grown axonal constructs as well as static neural (dorsal root ganglion) cultures.

#### **4.2.2 Materials and Methods**

##### *Overview of Study Design*

Building upon our previous findings on host nerve regeneration at 6 weeks and 4 months [Huang J et al, 2009], the initial study design required harvesting the nerve at the 2-week and 4-week time points in order to assess the progression of the nerve through the graft. However, an assessment of host regeneration at 2 weeks revealed similar results to the 4-week findings. This can be attributed to the early time point – within days of repair, the proximal nerve stump will undergo rapid regeneration and abundant collateral sprouting, both of which will be maintained for several weeks to months. As a result of these preliminary observations, the 2-week time point was subsequently discontinued.

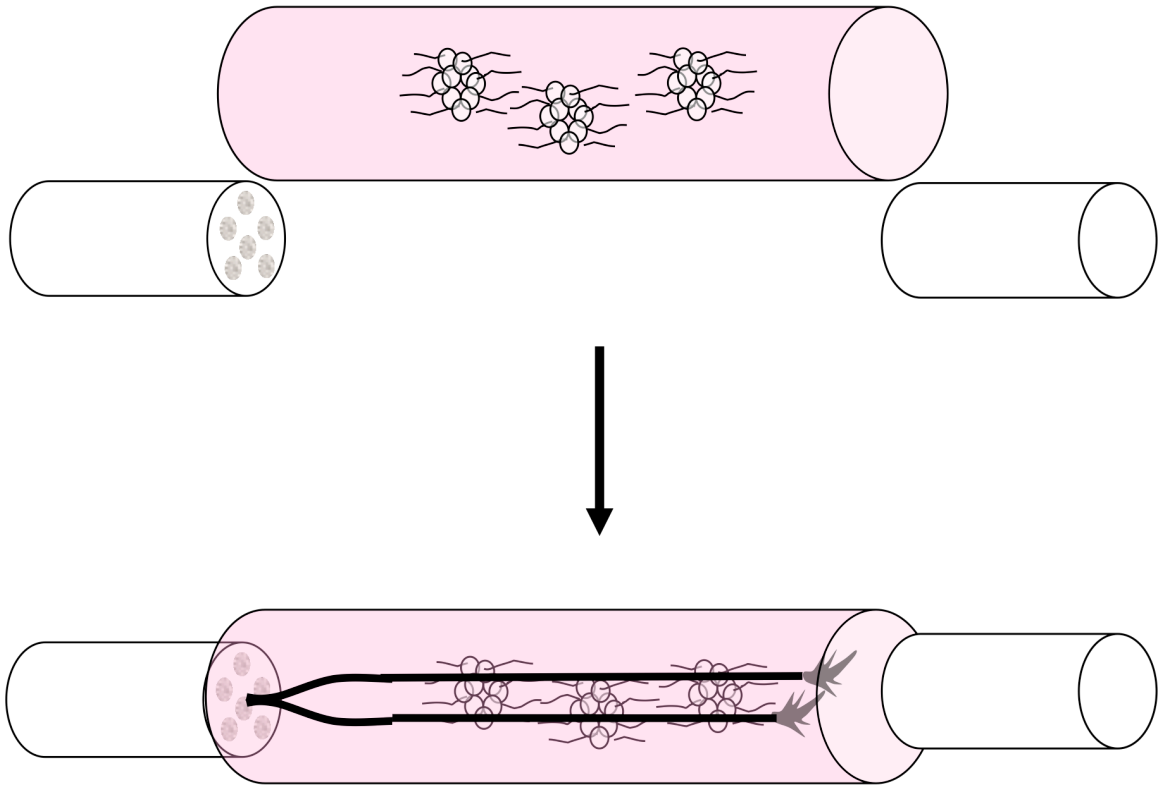


At 4-weeks post-transplantation, the nerves were re-exposed for electrophysiological evaluation and then immediately harvested for immunohistochemistry. This time-point was selected since the host nerve would have regenerated through the length of the transplant, allowing us to compare the extent (number of axons) and quality (nerve conduction velocity and latency) of the repair.

#### *Dorsal root ganglion neuron isolation*

Dorsal root ganglion (DRG) neurons were isolated from embryonic day 15 fetuses from timed-pregnant Sprague-Dawley (Charles River, Wilmington, MA) rats. The DRG explants were suspended at  $5 \times 10^6$  cells/mL in Neurobasal® medium supplemented with 2% B-27, 0.4 mM L-glutamine, 1% penicillin/streptomycin, 2 mg/mL glucose (Sigma-Aldrich, St. Louis, Mo), 10 ng/mL 2.5S nerve growth factor (NGF) and 1% fetal bovine serum (FBS) (HyClone, Logan, UT), and a mitotic inhibitor formulation of 10 mM 5-fluoro-2'-deoxyuridine (FdU) (Sigma-Aldrich), and 10 mM uridine (Sigma-Aldrich) to encourage non-neuronal cell elimination.

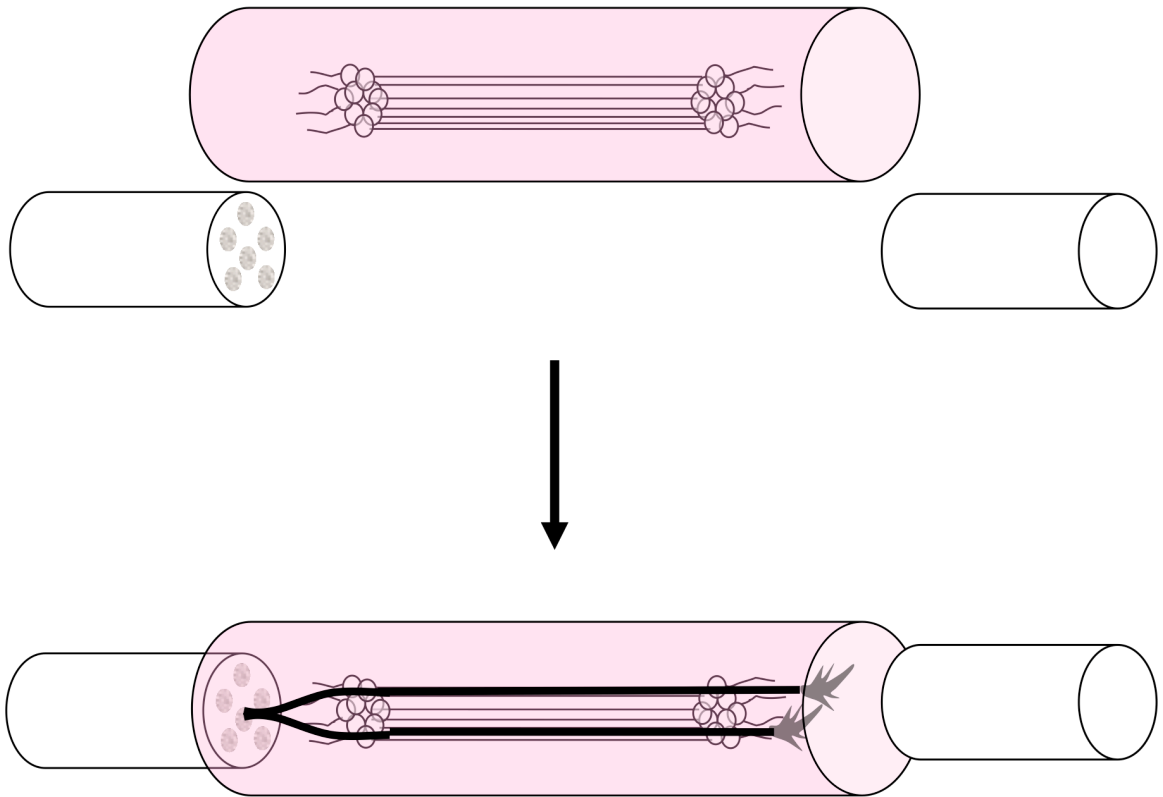
## Static Nervous Tissue Constructs



**Figure 4.1:** Schematic representation of the bridging of a 1cm sciatic nerve lesion using static DRG neuron cultures

The explants were plated into a collagen-filled (rat tail type 1, 3.67mg/mL) agarose (1.25% in 1X PBS; Ultrapure, Invitrogen, Carlsbad, CA) trough measuring 2mm x 1cm. The cell cultures were allowed to form axonal connections over 12 days *in vitro*. The neural growth medium described above was replaced on post-isolation days 1, 3, 5 and 12.

### *Stretch-grown Nervous Tissue Constructs*



**Figure 4.2:** Schematic representation of the bridging of a 1cm sciatic nerve lesion using stretch-grown axons

The explants were plated into mechanical elongation chambers custom-fabricated for the stretch-growth procedure. The neurons were plated in two populations along the interface of a collagen-coated (rat tail type 1, 3.67mg/mL)

aclar “towing” membrane and a base layer of agarose (1.25% in 1X PBS; Ultrapure, Invitrogen, Carlsbad, CA), resulting in a separation of 50-100 $\mu$ m. Over 5 days *in vitro*, axonal connections were formed between these two populations. The populations were then gradually separated over the course of 7 days using a stepper motor system until the axons spanning them reach a length of 1cm.

### *Neural Construct Encapsulation*

On post-isolation day 12, the neural cultures (both static and stretch-grown) were removed from the incubation chambers and embedded in a collagen-based matrix (3.0 mg/mL) in Dulbecco’s modified Eagle’s medium supplemented with NGF (2.5S, 10 ng/mL). After gelation at 37°C, embedded cultures were gently removed and placed within a 1cm absorbable collagen nerve guidance channel (3mm inner diameter, NeuraGen<sup>TM</sup>, Integra LifeSciences Corp, Plainsboro, NJ).

### *Peripheral Nerve Surgery and Tissue Construct Implantation*

Stretch-grown (n=10) as well as static (n=8) constructs were implanted into GFP<sup>+</sup> rats (adult male, 400-450mg, strain TgN(act-EGFP)OsbCZ-004). The animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.)

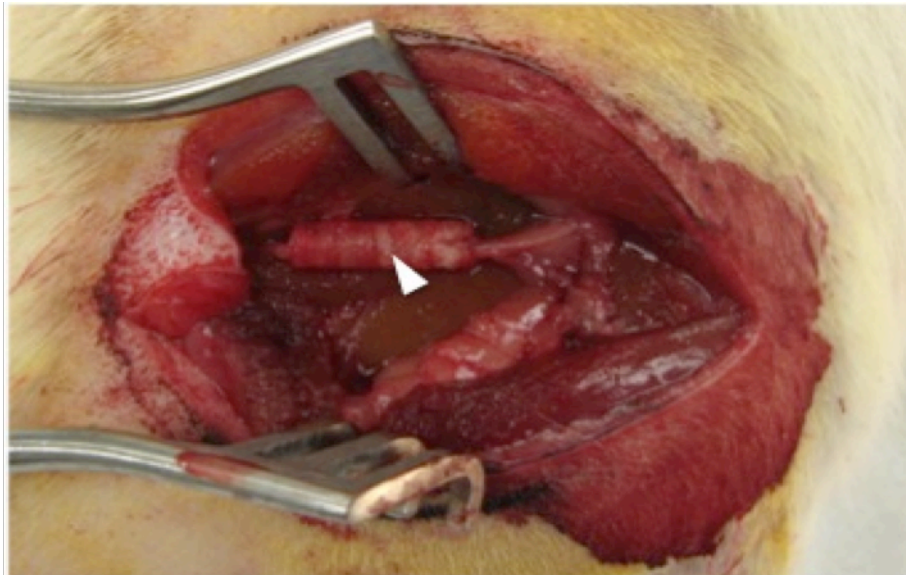
prior to surgery. A longitudinal skin incision was made in the hind leg from the sciatic notch proximally to the region of the popliteal fossa distally. The gluteal muscle was freed up to expose the sciatic nerve and its posterior tibial branch. A 1cm segment of sciatic nerve was transected immediately proximal to the trifurcation. The proximal and distal stumps were carefully inserted into the ends of the NeuraGen™ tube with an overlap of 1mm, and the epineurium was secured to the tube using 8-0 absorbable sutures. The ends of the tube were sealed with salmon fibrin (5mg/ml fibrinogen, 5 NIH units/ml thrombin) to provide additional stability, and to attenuate macrophage infiltration into the tube. Finally, the muscle and skin layers were sutured over the graft with absorbable chromic gut 4-0 sutures.

### *Nerve Electrophysiology*

The sciatic nerve was re-exposed and 4 weeks (n=6) using the aforementioned procedure. The animal was placed on a heating pad to maintain body temperature at 37°C. A stimulating electrode was placed 2mm proximal to the transection site, and the recording cuff was placed 2mm distal to the graft. All recordings were obtained at a sampling frequency of 4000 samples/s for 20s (MP-150; Biopac Systems Inc, Goleta, CA). Stimulation was provided using a 50µA, 100µs, constant current pulse train (SIU-91A; Cygnus Technology, Delaware Water Gap, PA); the current pulse train was controlled by a gating command from the recording unit in order to accurately calculate the conduction

velocity. The procedure was repeated on the contralateral side for each animal, and the ratio of conduction velocity of the operated side to the non-operated nerve was determined. All related data analyses were performed using standard software (Acknowledge 3.9.1; Biopac Systems Inc, Goleta, CA).

### *Nerve Harvest and Histological Analyses*



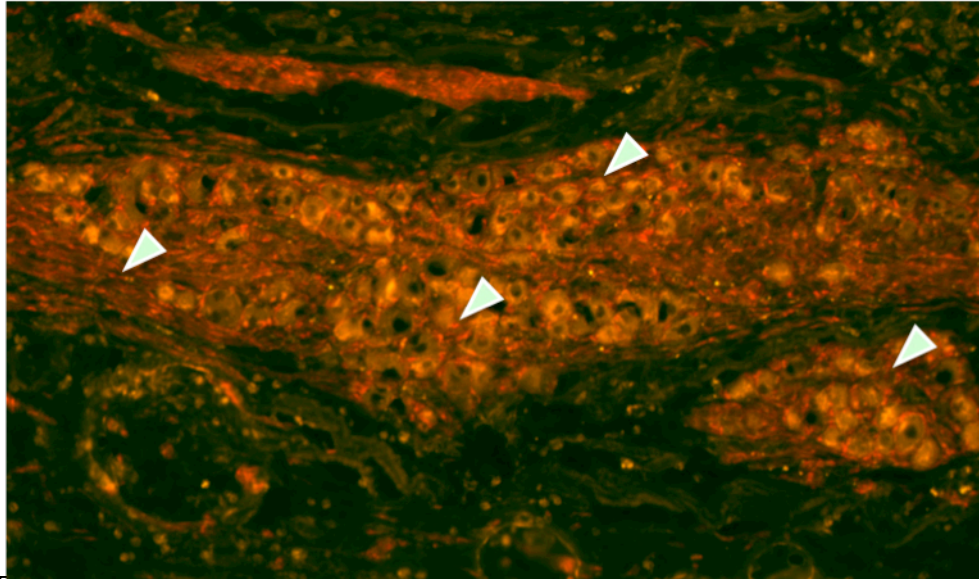
**Figure 4.3:** The grafted neural construct (indicated by the arrow) being harvested at the 4-week time point. The collagen tube can be seen to be securely fastened to the proximal (left) and the distal (right) nerve segments. Note the negligible fibrotic response around the tube.

Immediately following electrophysiological evaluation, the constructs were harvested by transecting the nerve 2mm proximal and 2mm distal to the graft (Figure 4.3), and were placed in 4% paraformaldehyde. The animals were euthanized by an overdose of sodium pentobarbital.

Following overnight fixation in paraformaldehyde, the excised tissue was removed and immersed in 30% sucrose solution. The tissue was then cryosectioned longitudinally (15-25 $\mu$ m), and immunostained for (i) neurofilament-200kDa (NF-200; N0142, 1:200; Sigma-Aldrich), (ii) myelin basic protein (MBP; SMI-94R, 1:500, Covance Research Products, Princeton, NJ), (iii) S-100 (1:500; Dako, Carpinteria, CA), or (iv) calcitonin gene-related peptide (CGRP; C8198, 1:1000 Sigma-Aldrich). The appropriate secondary fluorophore-conjugated antibodies (TRITC- or AMCA-conjugated IgG; Jackson ImmunoResearch, West Grove, PA) were used. The sections were examined under an epifluorescent microscope (Eclipse E600; Nikon, Melville, NY) and the images were digitally captured (Spot RT Color; Diagnostic Instruments, Sterling Heights, MI).

### 4.2.3 Results

#### *Stretch-grown and static neural construct survival*



**Figure 4.4:** Static cultures of dorsal root ganglion neurons were clearly identified in the tube. Clusters of cell bodies and their associated axonal connections (indicated by the arrows) were recognized using NF-200 staining (red), and could be distinctly identified against the host tissue (GFP, green). Note that no co-localization could be seen in their vicinity, indicating the lack of host and graft axon intermingling in the area.

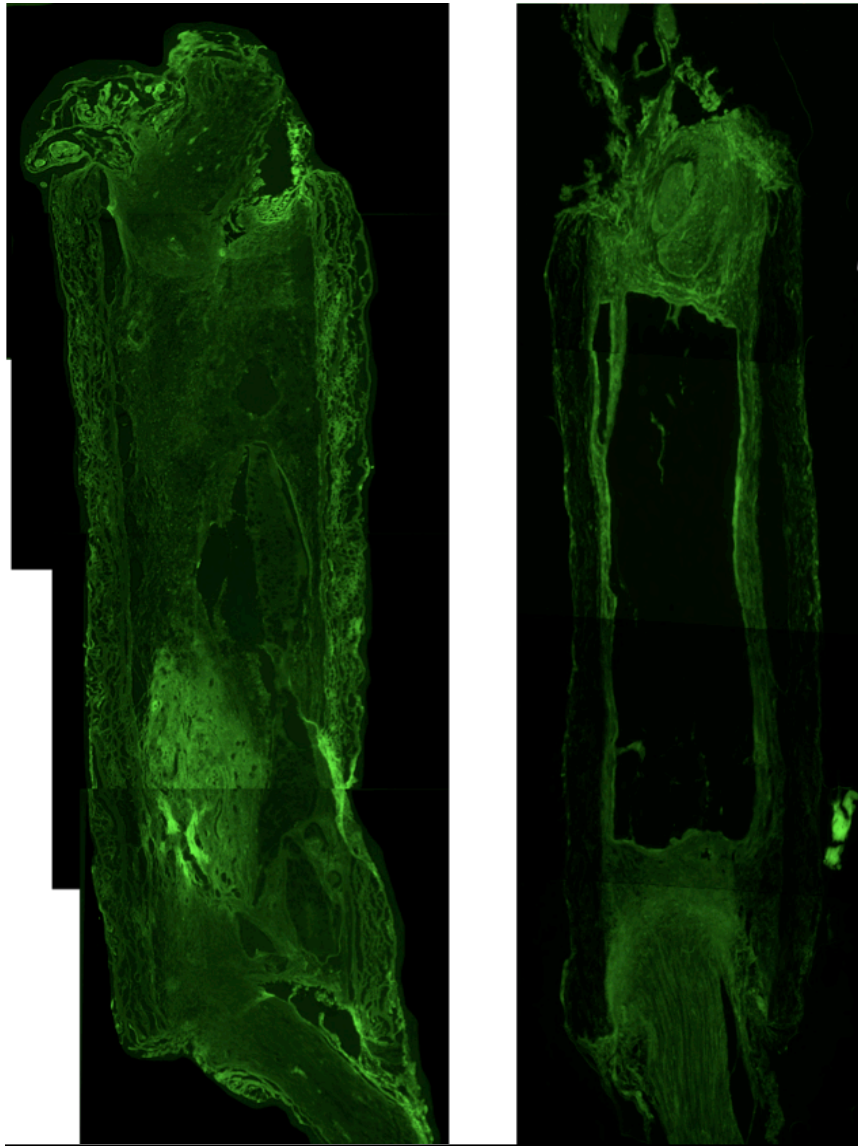
Stretch-grown constructs were harvested at the 4-week (n=6) and 8-week (n=2) time points. All static constructs (n=6) were harvested 4-weeks post-transplantation. At both time-points the transplanted area could be clearly identified as the collagen tube was still un-resorbed and maintained its structural



integrity. Within the tubes the transplanted ganglia and their axonal connections were identified using the neuronal/neuritic cytoskeletal protein NF-200; these areas did not co-localize with green fluorescent protein, indicating non-native tissue (Figure 4.4). Examination of the grafts also revealed robust vascularization along their entire lengths, which is a necessary requirement for the survival and maintenance of host and graft tissue. Also, as observed in our previous studies, there was no overt sign of immunologic response as denoted by infiltrating macrophages and neutrophils despite the lack of immune suppressant usage.

#### *Host regeneration across stretch-grown constructs*

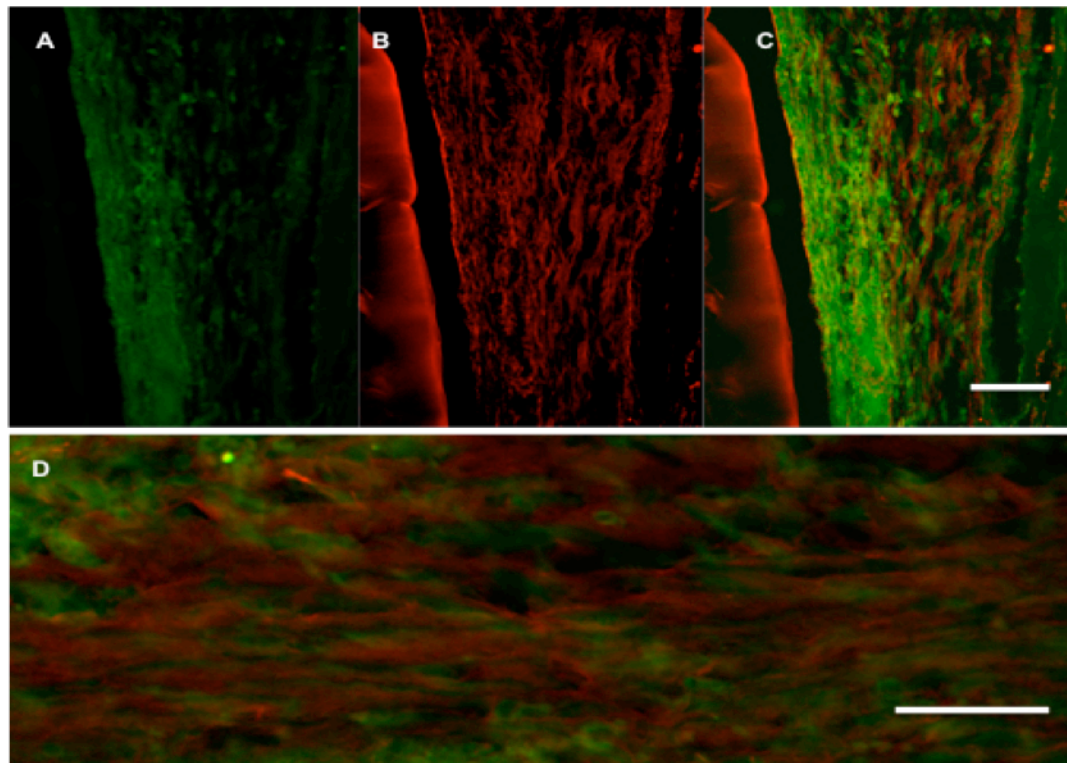
Host nerve fibers were seen to extend across the length of the 1cm lesion in the stretch-grown as well as the static neural transplants (Figure 4.5). However, far most extensive host infiltration (neural and vascular) could be seen in the stretch-grown group, whereas the static group had preferential neurite growth along the walls of tube. The host fibers were identified as those expressing NF-200 co-localized with GFP (Figure 4.6 A-C). Remnants of the agarose were still present at 4 weeks; the axons were seen to grow between the agarose and the walls of the collagen tube in both cases, presumably due to the relative impermeability of the still-disintegrating agarose fragments. After 8 weeks *in vivo*, however, the nerve and accompanying vasculature was distributed through the width of the tube and the agarose could not be detected.



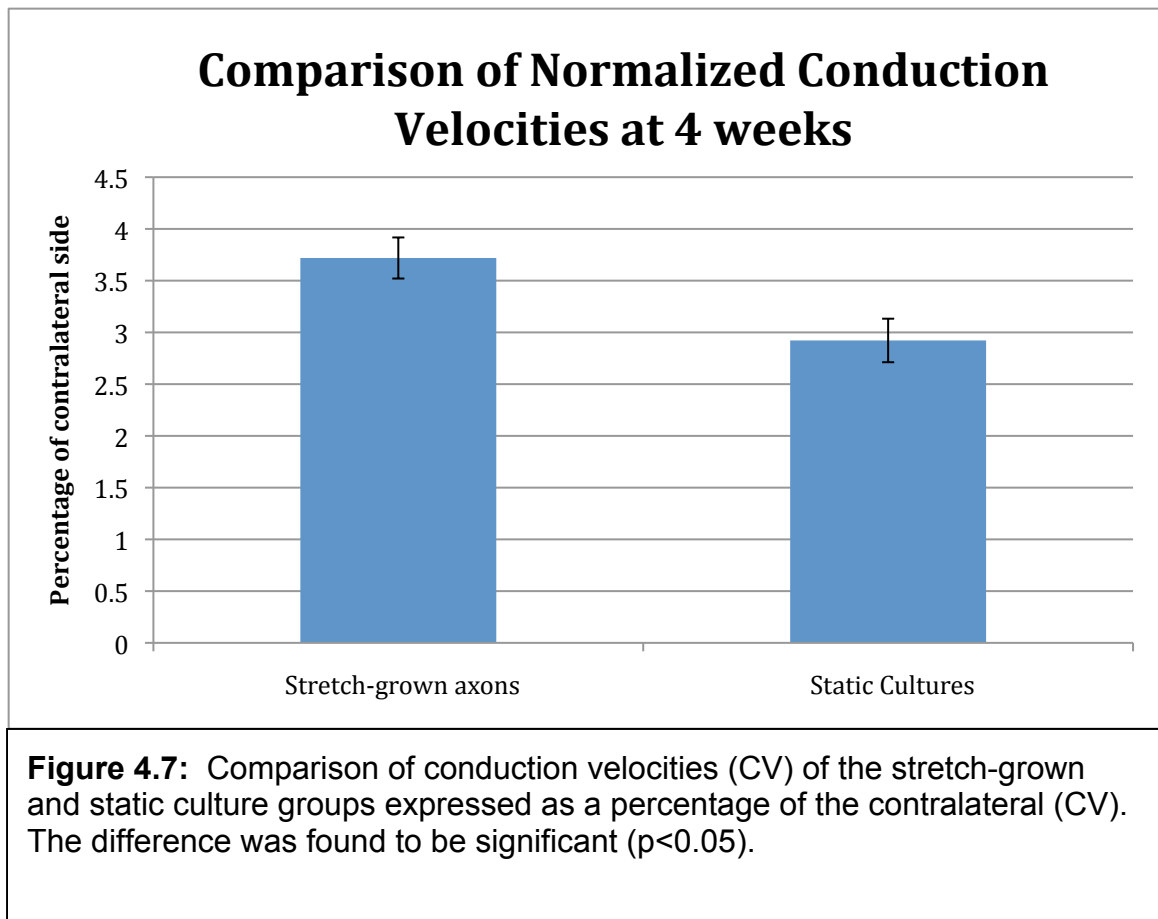
**Figure 4.5:** Comparison of host nerve regeneration through stretch-grown axon group (left) and static culture group (right). In the static group, host tissue appeared to preferentially regenerate along the walls of the collagen tube, whereas tissue distribution was more uniform in the stretch-grown axon group.

At 4 weeks, all constructs were also seen to contain GFP<sup>+</sup>/S-100<sup>+</sup> Schwann cell bodies in the vicinity of the host axon fibers (Figure 4.6D). These same cell bodies did not positively express myelin basic protein (SMI-94), suggesting that they had not yet converted into a myelinating phenotype.

As previously mentioned, the constructs were also evaluated at 2 weeks (n=2 per group). However, as no measurable differences were observed when compared with the 4-week time point, evaluation at 2 weeks was discontinued.



**Figure 4.6:** (A-C) Host regeneration within the construct. NF-200 staining (red) was expressed by both host and graft axons. Areas of co-localization (white arrow) indicate host axon fibers. (D) Schwann cells expressing S-100 (green) surrounding host axons (red) Scale bar = 100µm.



Evaluation of the electrophysiology of the repaired nerves at 4 weeks revealed clear differences between the stretch-grown and static groups. The ratio of the conduction velocities of the treatment and contralateral control sides were calculated, and nerves with stretch-grown axons demonstrated a statistically significant improvement over static cultures. This correlates with observed differences in regenerated axonal fiber density. It should be noted that the recorded conduction velocities are less than twenty times that of normal

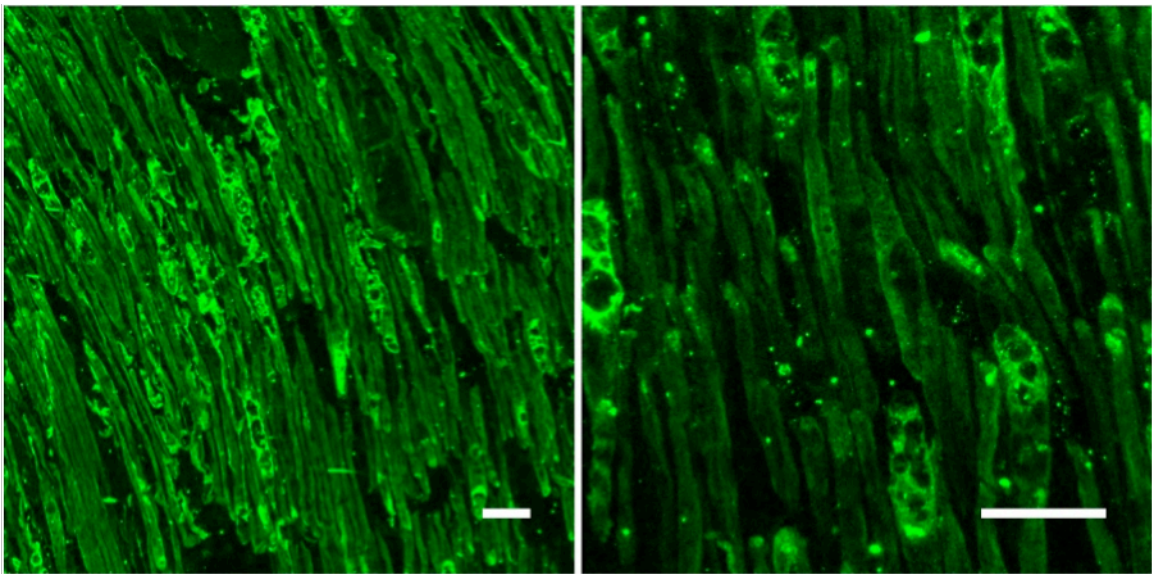
nerves. This could be attributed in part to the still-incomplete regeneration as well as the previously noted absence of myelination.

### **4.3 Attenuation of distal degeneration using a neural construct**

#### ***4.3.1 Relevant Background***

Following the transection of a nerve, distal axons – which have been physically cut off from their neural cell bodies – undergo a gradual degenerative process known as Wallerian degeneration. Within a few hours of transection, the axolemma fuses and seals the end; the axons themselves begin to disintegrate within a few days. Myelin and other cellular debris are phagocytosed by macrophages within 6 weeks. Concurrent with the loss of the axons, Schwann cells begin to proliferate at the site of the injury and organize themselves into aligned columns known as bands of Bungner (Figure 4.8). These Schwann cells upregulate their synthesis of neurotrophic factors including NGF, BDNF and NT-3 [Heumann, 1987; Thoenen et al, 1988; Funakoshi et al, 1993; Fu and Gordon, 1995; Raivich and Makwana, 2007] in order to attract regenerating axons of the proximal stump into the aligned columns. If regeneration is successful, axonal sprouts will advance through the bands of Bungner and ultimately innervate their appropriate distal targets within a few (3-6) months of the injury. If reinnervation does not occur within this timeframe, however, the recovery is likely to be incomplete [Fu and Gordon, 1995; Gutmann and Young, 1944]. A progressive

increase in the collagen content of the endoneurium and perineurium can lead to an inhospitable environment for regenerating axons [Salonen et al, 1985]. However, the predominant factor contributing to this poor recovery is the gradual reduction of neurotrophic support and eventual disintegration of the Schwann cell basal lamina [Sunderland, 1950], which is almost entirely complete within 4-6 months.



**Figure 4.8:** Example of columnar alignment of Schwann cells (S-100, green) in the sciatic nerve 2 weeks post-transection. Scale bar = 50 $\mu$ m.

*Image courtesy Cullen DK, 2008*

A variety of strategies have been investigated with the goal of delaying this distal atrophy and improving functional outcome. In the case of proximal nerve injuries or long nerve gaps, clinicians regularly perform distal motor nerve transfers in order to hasten muscle reinnervation [Mackinnon, Roque and Tung, 2007]; however, this results in impaired function at the donor site. Moreover, in many situations a suitable motor nerve candidate is simply not available.

Sensory nerve innervation of the distal stump has been shown to maintain a favorable neurotrophic milieu for at least 6 months post-transection [Michalski et al, 2008; Bain et al, 2001]. Here too, however, the sacrifice of an otherwise healthy nerve is required for improvement of outcome at the graft site.

Building upon these approaches, and our observation of graft axons extending into the host nerve stumps [Huang J et al, 2009], we hypothesize that axonal processes extending from a transplanted neural construct may maintain the support architecture of the distal segment, potentially leading to improved functional recovery even after delayed reinnervation. In this study, we compare the extent of degeneration of the distal stump over 16 weeks after treatment with a transplanted static neural construct vis-à-vis no repair.

#### **4.3.2 Materials and Methods**

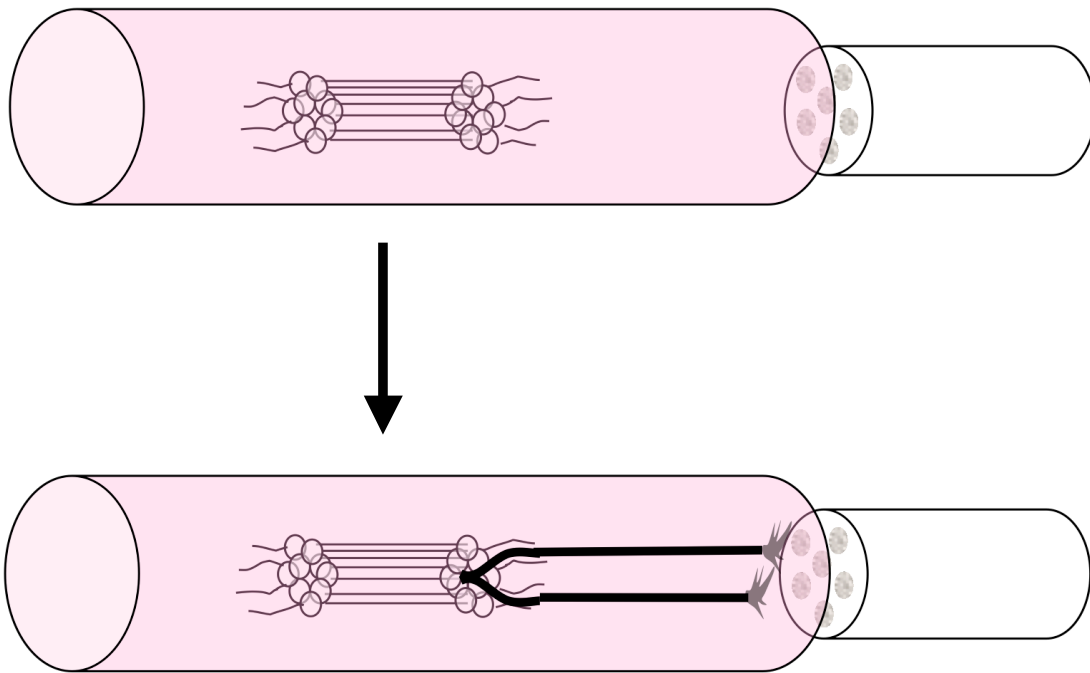
##### *Dorsal root ganglion neuron isolation*

Dorsal root ganglion (DRG) neurons were isolated from embryonic day 15 fetuses from timed-pregnant Sprague-Dawley (Charles River, Wilmington, MA) rats. The DRG explants were suspended at  $5 \times 10^6$  cells/mL in Neurobasal® medium supplemented with 2% B-27, 0.4 mM L-glutamine, 1% penicillin/streptomycin, 2 mg/mL glucose (Sigma-Aldrich, St. Louis, Mo), 10 ng/mL 2.5S nerve growth factor (NGF) and 1% fetal bovine serum (FBS) (HyClone, Logan, UT), and a mitotic inhibitor formulation of 10 mM 5-fluoro-2'-

deoxyuridine (FdU) (Sigma-Aldrich), and 10 mM uridine (Sigma-Aldrich) to encourage non-neuronal cell elimination.

#### *Static Nervous Tissue Constructs*

The explants were plated into a collagen-filled (rat tail type 1, 3.67mg/mL) agarose (1.25% in 1X PBS; Ultrapure, Invitrogen, Carlsbad, CA) trough measuring 2mm x 1cm. The cell cultures were allowed to form axonal connections over 12 days *in vitro*. The neural growth medium described above was replaced on post-isolation days 1, 3, 5 and 12.



**Figure 4.9:** Schematic representation depicting the use of neural constructs to innervate and protect the transected distal stump



### *Neural Construct Encapsulation*

On post-isolation day 12, the neural cultures were removed from the incubation chambers and embedded in a collagen-based matrix (3.0 mg/mL) in Dulbecco's modified Eagle's medium supplemented with NGF (2.5S, 10 ng/mL). After gelation at 37°C, embedded cultures were gently removed and placed within a 1cm absorbable collagen nerve guidance channel (3mm inner diameter, NeuraGen™, Integra LifeSciences Corp, Plainsboro, NJ).

### *Peripheral Nerve Surgery and Tissue Construct Implantation*

All procedures were performed using GFP<sup>+</sup> rats (adult male, 400-450g, strain TgN(act-EGFP)OsbCZ-004). The animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) prior to surgery. A longitudinal skin incision was made in the hind leg from the sciatic notch proximally to the region of the popliteal fossa distally. The gluteal muscle was freed up to expose the sciatic nerve and its posterior tibial branch, and the nerve was transected 1cm proximal to the trifurcation. For construct transplantations (n=6), the distal stump was carefully inserted into one end of the NeuraGen™ tube with an overlap of 1mm, and the epineurium was secured to the tube using 8-0 absorbable sutures. The ends of the tube were sealed with salmon fibrin (5mg/mL fibrinogen, 5 NIH units/ml thrombin) to provide additional stability, and to attenuate macrophage infiltration into the tube. For both neural construct transplantations and controls (n=6), the proximal stump was capped and the distal stump/tube was covered in a non-resorbable nylon mesh – these steps were taken in order to prevent

inadvertent regeneration of the proximal stump into the transection site. Finally, the muscle and skin layers were closed using absorbable chromic gut 4-0 sutures.

### *Nerve Harvest at 16 weeks and Histological Analyses*

The surgical site was re-exposed at the 16-week time point (with the exception of one neural construct graft and one control nerve that was left *in vivo* for 8 months), and the distal stumps were harvested by transecting the nerve as far as could be exposed distal to the graft (typically 1cm). The nerves and were immediately placed in 4% paraformaldehyde and the animals were euthanized by an overdose of sodium pentobarbital.

Following overnight fixation in paraformaldehyde, the excised tissue was removed and immersed in 30% sucrose solution. The tissue was then cryosectioned longitudinally (15-25 $\mu$ m), and immunostained for (i) neurofilament-200kDa (NF-200; N0142, 1:200; Sigma-Aldrich), (ii) S-100 (1:250; Dako, Carpinteria, CA), or (iii) calcitonin gene-related peptide (CGRP; C8198, 1:1000 Sigma-Aldrich). The appropriate secondary fluorophore-conjugated antibodies (TRITC- or AMCA-conjugated IgG; Jackson ImmunoResearch, West Grove, PA) were used. The sections were examined under an epifluorescent microscope (Eclipse E600; Nikon, Melville, NY) and the images were digitally captured (Spot RT Color; Diagnostic Instruments, Sterling Heights, MI).

### **4.3.3 Results**

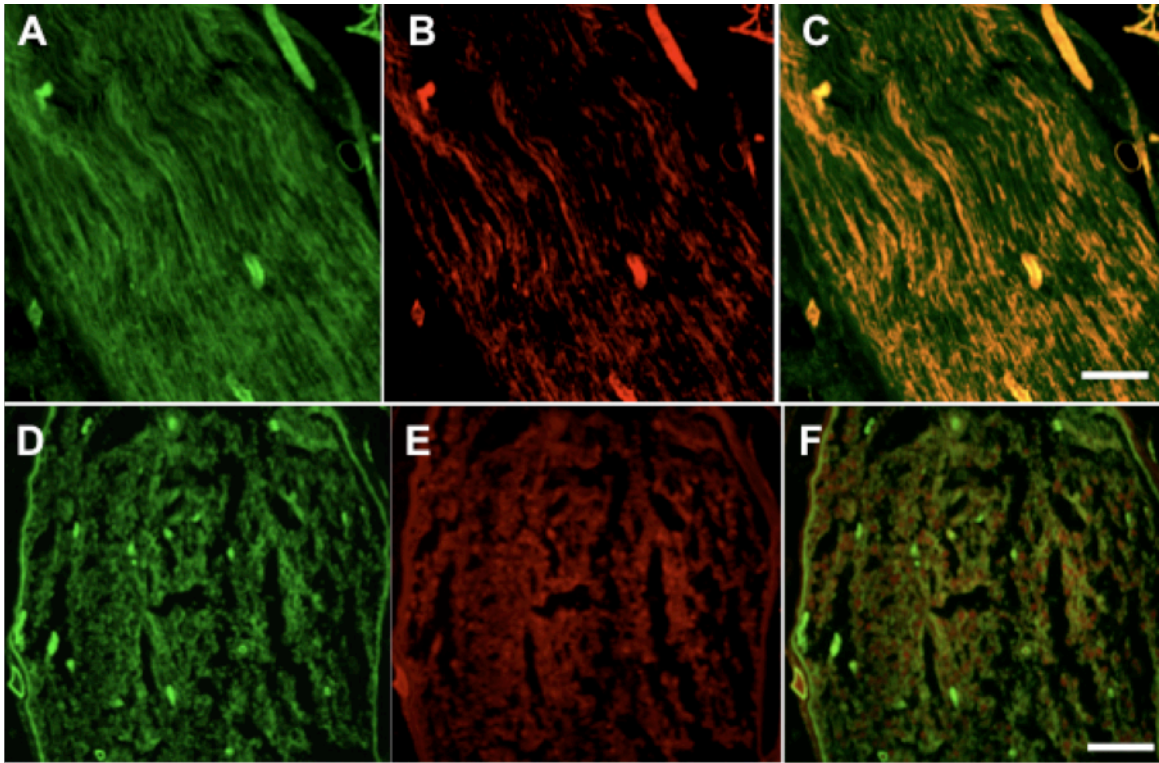
#### *Distal stump harvest and gross assessment at 16 weeks*

Distal stumps from both groups (neural constructs and controls) were evaluated at the 16-week time point. One non-treated control nerve could not be harvested, as the distal pathway appeared to have completely disintegrated. Aberrant regeneration of the proximal stump into the distal pathway was not seen in any of the animals. The neural constructs could be easily identified by means of the collagen tubes, which had still not been completely resorbed and maintained their structural integrity; the nerve stumps remained securely attached to the tubes. The remaining control nerves could be readily located beneath the non-resorbable nylon mesh protective sheaths. A pre-histological comparison of the nerves revealed narrower diameters for the control group in relation to treated nerves. On average, control nerves were 13.6% smaller in diameter than the treated group, and 20.8% narrower than uninjured nerves.

#### *Basal lamina alignment and neural construct survival*

At the 16-week time point, all distal stumps from both groups contained intact epineuria and living Schwann cells within them. Schwann cells were identified as those expressing S-100 co-localized with GFP. Within nerves attached to static neural constructs, the Schwann cells were aligned in columnar

formations similar to those observed within 2 weeks of nerve transection (Figure 4.10 A-C). In contrast, control nerves did not exhibit this organized Schwann cell alignment (Figure 4.10 D-F). Despite this disparity, neither dorsal root ganglia nor host/graft axons could be detected within these columns using NF-200, GAP-43 and CGRP staining, suggesting that the neural populations were no longer present at this time point.



**Figure 4.10:** The distal nerve stump at the 4-month time point. The treated nerve (A) was stained for S-100 (red, B) and the super-imposed image (C) revealed columnar alignment of Schwann cells (yellow) similar to that seen within a few weeks of transection. On the other hand, control nerves (D), when stained for S-100 (E) revealed a disorganized arrangement of Schwann cells (yellow, F), as is typically expected at this time point. Scale bar = 100 $\mu$ m.

## 4.4 Discussion

In order for peripheral nerve regeneration to be considered successfully, axons must grow across the lesion, through the distal pathway and finally integrate functionally with the appropriate end targets. If this does not occur promptly, the distal pathway will disintegrate and the regeneration will be incomplete. In this project, living tissue-engineered neural constructs, created using a novel stretch-growth paradigm, were used to (1) bridge a 1cm peripheral nerve lesion, and (2) delay the atrophy of the distal stump support architecture.

### *Repair of a PNS lesion:*

The ability of stretch-grown axons to bridge nerve lesions has already been shown in previous experiments [Huang et al, 2009; Iwata et al, 2006]. In this study, the regenerative assistance provided by these stretch-grown constructs was compared to that provided by non-elongated cultures of dorsal root ganglion neurons. This was performed in order to confirm that it is indeed the presence of elongated axon bundles that contribute to enhanced regeneration, and not the cell bodies. The repaired nerves were evaluated at 4 weeks and 8 weeks. By 4 weeks, the nerves had regenerated in all surviving animals. At this time-point, the agarose in the tubes was still largely undigested; indeed the axons appeared to flank the agarose portions and grow along the

edges of the tubes. The persistence of the agarose at 4 weeks was surprising as it contradicts previously published reports where agarose was used as a regenerative substrate [Balgude et al 2001]. A possible explanation is the use of salmon fibrin as a sealant at the ends of the tube. Salmon fibrin has been shown to attenuate the infiltration of macrophages, which are known to contribute to the enzymatic degradation of agarose. In any event, the agarose is not seen at 8 weeks and the tubes solely include host and graft axons as well as accompanying vasculature.

Despite the uniformly obstructive nature of the agarose at 4 weeks, cross-sections of the harvested constructs revealed a greater number of host axons in stretch-grown constructs than in the non-elongated group. A likely explanation for this is the preference for regenerating neurites to coalesce around and grow along longitudinally-aligned fibers within a regenerative matrix [Clements IP et al, 2009; Kim YT et al, 2008]; indeed, these axons appear to be intertwined with graft fibers. It is well established that regenerating neurites project an abundance of collateral branches that are pruned once the distal target is reached [Witzel et al, 2005; Jenq and Coggeshall, 1985]. The ultimate success of any graft is highly dependant on the generation of these surplus branches [Madison et al, 2007], and thus the observed axon density within the stretch-grown constructs is promising.

Clusters of Schwann cells were also identified adjacent to the host axon fibers. Intriguingly, these cells stained positively for S-100 but not for SMI-94.

Unlike S-100, SMI-94 is specific to myelin and does not identify non-myelinating or neoplastic cells (Clark HB et al, 1985) suggesting that these Schwann cells had not converted to a myelinating phenotype at 4 weeks. However, myelination of the regenerated axons was observed by the 16 week time point [Huang J et al, 2009], indicating that the phenotypic conversion occurs somewhere within this time frame. Indeed, regenerated axon myelination has been typically reported at 2 months, and tends to steadily increase from 2-12 months [Mackinnon S et al, 1991].

Electrophysiological evaluation was the clearest indicator of the differences between the two groups. The conduction latency across each transplant in response to a stimulus pulse train was measured from which the nerve conduction velocities (NCV) were evaluated. The NCV was then normalized to contralateral nerve for each animal. This particular metric was chosen as a consequence of the size disparity between the animals. A comparison between the groups revealed a statistically significant improvement in animals with stretch-grown transplant in relation to the static group. This finding can be attributed to the observed difference in the axonal fiber distributions between the two groups. It should be noted that the observed velocities are approximately 20 times less than those of the non-injured nerves. This can be attributed to a number of reasons, principal of which is the absence of myelination at 4 weeks - saltatory conduction can often occur at up to a hundred times the speed of a simple action potential. Axon fiber caliber also plays a

significant role. Regenerating fibers are known to be of a narrower diameter until they integrate with their end targets, after which they significantly increase in girth.

The use of living axon grafts may enable the creation of alternative pathways for communicating motor and sensory signals. Indeed, stretch-grown axons *in vitro* have been shown to be electrophysiologically functional including intra-culture synaptic integration [Pfister BJ et al, 2006]. However, preliminary work evaluating the presence of synaptophysin, a protein concentrated in pre-synaptic specializations, did not reveal evidence of host/graft synaptic integration. Alternative analyses techniques, such as morphological identification of putative synapses via electron microscopy, may further illuminate this important consideration.

#### *Attenuation of Distal Degeneration:*

The eventual atrophy of the distal stump is a confounding factor when nerve lesions are either too large or too proximally located. Efforts have been made to delay this atrophy using sensory nerve innervation; so far, however, no one method is capable of both promoting regeneration and attenuating distal degeneration.

In this study, cultures of dorsal root ganglion neurons were encapsulated and affixed to a transected sciatic nerve distal stump, in order to determine if



axonal outgrowth and its associated neurotrophic support could maintain neural support cells over a period of 4 months. The increased degree of atrophy in control nerves was immediately apparent during nerve harvest – while their epineuria were still intact, untreated nerves were of considerably narrower caliber than those attached to the neural constructs. Control nerves also appeared structurally less robust, making them difficult to manipulate and harvest. In contrast, the treated distal stumps were noticeably more robust.

Histological analysis of the nerves revealed clear differences in the alignment of survival Schwann cells in both groups. Nerves affixed with neural constructs contained aligned columns of Schwann cells, similar to the bands of Bungner observed in the initial weeks following axotomy. In contrast, Schwann cells in control nerves were distributed in a haphazard manner. The columnar alignment of Schwann cells is essential for accurate guidance of the regenerating neurites to their appropriate targets; without this guidance, axons may occasionally grow along the epineuria directly onto the denervated muscle surface, resulting in poor reinnervation [Fu and Gordon, 1995]. Absent this path as well, uncapped axons will innervate surrounding muscle targets, resulting in the phenomenon of phantom pain.

Despite these observed differences, the neural constructs could not be detected within the collagen tubes at 4 months, preventing a thorough assessment of its' contributions. This was surprising, since neural constructs used for lesion repair were observed within the transplanted area at the same

time point. However, a critical difference between these two results is the attachment of the proximal stump and subsequent regeneration. The milieu surrounding the regenerating nerve is flush with neurotrophic support; however, on the distal side this support is transient, after which the support structures undergo aggressive macrophagic degradation [Hoke et al, 2003]. Immune suppressants, which were not employed in this study, might be a potential means of prolonging grafted neuron survival.

## **4.5 SUMMARY OF FINDINGS**

### Conclusions

From these results, we infer that the stretch-grown axonal constructs provide a degree of physical and neurotrophic support to the regenerating proximal stump that is absent when using populations of neural cell bodies alone. Neural cell bodies do not exist outside of the spinal cord and its immediate vicinity, suggesting that they may be physiologically irrelevant to outgrowing PNS neurites. Living axons, on the other hand, have been shown to play a critical role during embryonic development and in regeneration (such as after crush injury), and thus may be the essential element of any tissue-engineering approach that utilizes neural cellular components. At the distal end, infiltrating neurites from the transplanted neural constructs may provide a measure of trophic support that can

maintain the existing architecture for several months. While this support appears to be temporary, it may be sufficient for all but the most extensive of lesions.

#### Limitations and Future Studies

While the results are suggestive of a beneficial effect from the transplanted neural constructs at both the proximal and distal ends, a larger study encompassing more acute and long-term time points is needed to support these findings. In particular, a more thorough quantification of axon proliferation, myelination and electrophysiology at various time points must be performed. In addition, the distal stump maintenance must be evaluated (a) at an earlier time point in order to assess the effect of the neural constructs, and (b) using immune suppressants in order to evaluate their ability to promote construct survival over longer periods.

## Chapter 5

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# Development of a Neural Interface Platform with the Peripheral Nervous System Using Stretch-Grown Axonal Constructs

# **Chapter 5: Development of a Neural Interface Platform with the Peripheral Nervous System Using Stretch-Grown Axonal Constructs**

## **5.1 Overview**

Technological advances of the past several decades have now made interfaces with the nervous system a reality. The cochlear implant, the first device to truly interface the nervous system with the external environment, was developed in the 1960's. However, the direct neural control of prosthetic limbs is a far more nascent field. In 1999, a seminal study described the control of a robotic arm using signals directly derived from the rat motor cortex [Chapin et al, 1999]. Since then, there have been a number of exciting advancements in the field, in both central- (CNS) and peripheral nervous system (PNS) based approaches.

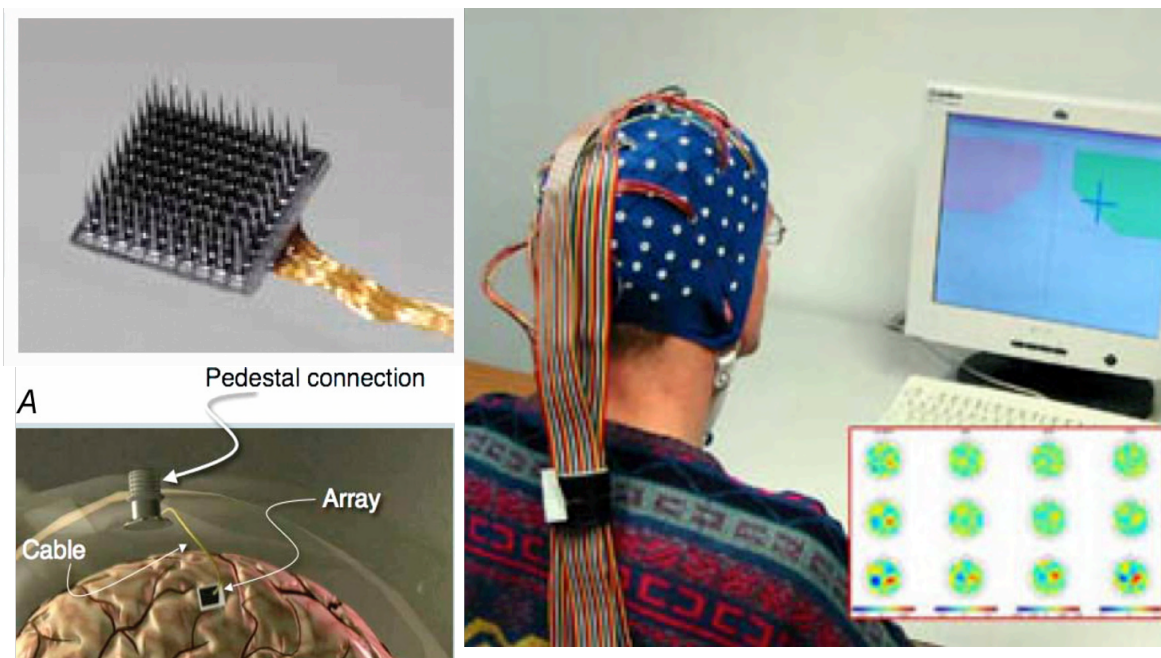
The ideal neuroprosthesis should be a functional facsimile of the amputated limb, i.e. it must facilitate continuous bi-directional communication between the central nervous system and the external environment. Currently, the vast majority of efforts in this area focus exclusively on the re-establishment of motor control, relying on visual feedback to guide the movement of the prosthesis. However, in order to achieve truly "normal" interaction with the

surroundings, tactile feedback is vital. Additionally, from a clinical and rehabilitation standpoint, it is important to have an architecture that minimizes surgical complexity and recovery time, provides a hospitable environment for nerve survival, and lends itself to rapid learning.

Over the past several decades a variety of architectures that target both the central and peripheral nervous systems have been developed. CNS-based approaches attempt to restore motor function by directly deriving commands from the patient's motor cortex. Two major strategies have emerged to accomplish this. The first is a non-invasive technique to obtain movement intent via surface (scalp) electrodes over the motor cortex [Guger C et al, 1999; Wolpaw JR and MacFarland DJ, 2004]. Using this approach, which entirely avoids the risks associated with surgery, patients have demonstrated the ability to perform such tasks as cursor manipulation and even basic word processing (Figure 5.1, right).

However, the poor information transfer rates associated with this technique makes its translation to the control of more sophisticated systems immensely challenging in the near future [Wolpaw JR et al, 2002; Lebedev MA et al, 2006]. To produce complex signal integration, more invasive methods have been developed, such as chronically implanted micro- electrodes into the motor cortex (Figure 5.1, left) or spinal cord to locally record activity from a select population of neurons [Palmer C, 1978; Maynard EM et al, 1999; Donoghue JP, 2002; Taylor DM et al, 2002; Hochberg LR et al, 2006]. Neuronal population decoding algorithms are then used to decipher the recorded signals in real-time.

This approach has yielded considerable success in re-enabling motor control; indeed, a version of this system is currently the subject of a pilot clinical trial. Nonetheless, this approach has a number of drawbacks, including substantial computational complexity, significant clinical risk arising from the chronic implantation of electrodes in healthy neural tissue, and signal attenuation and/or remapping due to scar formation. Moreover, findings from functional magnetic resonance imaging (fMRI) studies indicate that there is extensive and dynamic overlap of the cortical representations of different limb regions, adding additional

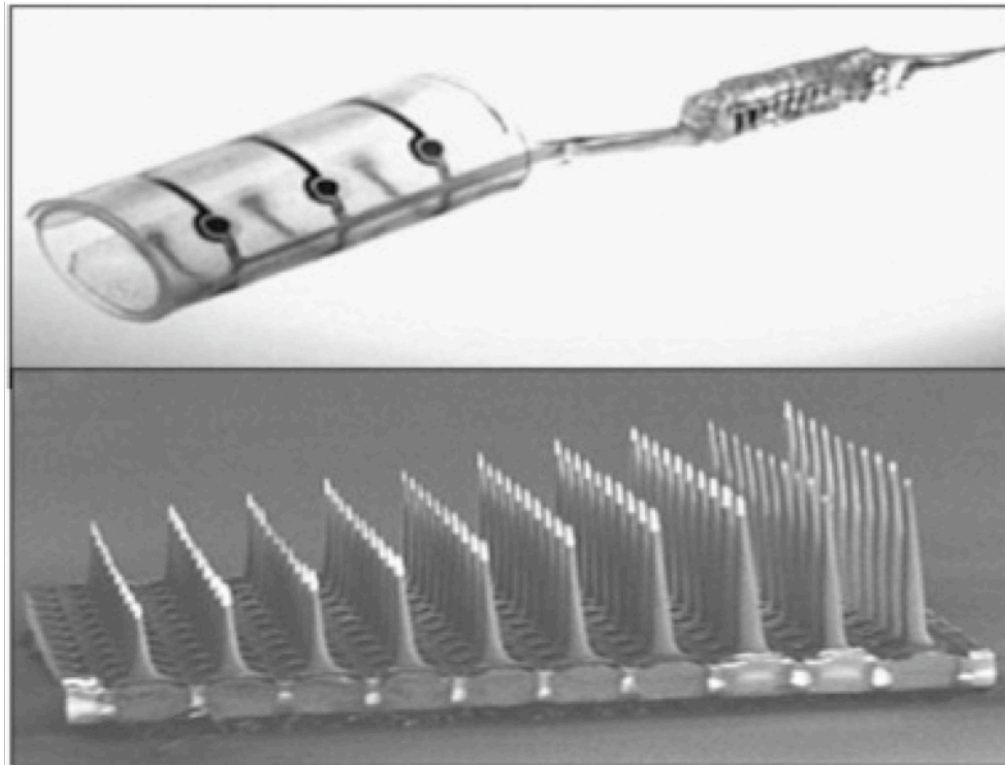


**Figure 5.1:** Examples of CNS-based brain-machine interface systems. **Top left** – a penetrative 10x10 cortical electrode array. **Bottom left** – after removal of the skull bone, the electrode array is inserted into the motor cortex tissue. Recorded cortical activity is transmitted to a pedestal connection implanted in the skull bone, which is then interpreted by an external computer. **Right** – EEG-derived neural interface system using a sensor array mounted on the user’s scalp.

*Donoghue JP, 2002; Wolpaw JR et al, 2004*

difficulty to implant positioning and signal decoding [Rao SM et al, 1995]. Additionally, there is still no clear approach to relay sensory signals/feedback.

Alternatively, interface approaches outside the CNS typically take one of two forms – (1) electrodes that are implanted in or around the damaged peripheral nerve, or (2) electrodes that are implanted within, or on the surface of, skeletal muscle [Navarro X et al, 2005]. Nerve electrodes, the form of which can vary from encircling nerve cuffs [Grill WM and Mortimer JT, 1998] to intra-



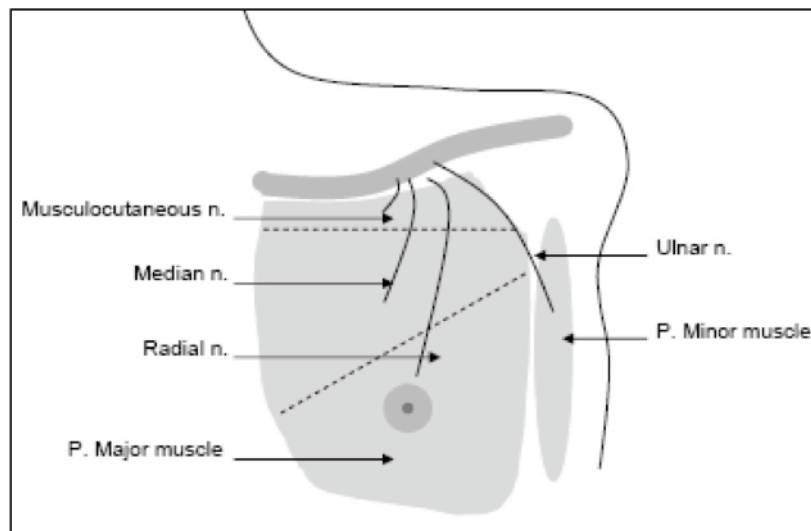
**Figure 5.2:** *Top* – Non-penetrative nerve cuff electrode. The electrode is wrapped around the nerve, and can be tightened to create a more secure connection. *Bottom* – a 10x10 penetrating intrafascicular electrode array. The array is inserted into the nerve, and the varying electrode lengths enable recording from fascicles at varying depths.

*Navarro et al, 2005 and Branner et al, 2001*



fascicular penetrating electrode arrays [Warwick K et al, 2003; Branner A et al, 2004] exhibit both enhanced signal selectivity and high signal-to-noise ratio. However, this approach still has its drawbacks – the materials used are often of poor biocompatibility, the electrodes themselves can be extremely damaging to the already traumatized nerves, and there is a reduced likelihood of chronic interface due to nerve degeneration.

EMG-based myoelectric prosthesis systems have met with remarkable success in recent years. In particular, the targeted innervation of the brachial plexus nerves into the pectoral muscles has allowed for the real-time control of multi-jointed prosthetic limbs [Kuiken TA et al, 2004; Kuiken TA et al, 2007a], as

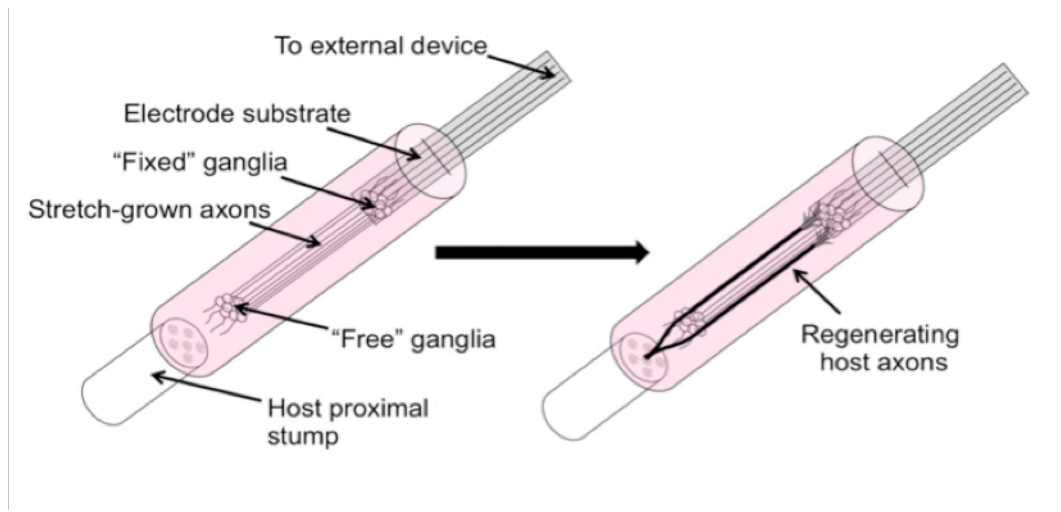


**Figure 5.3:** Brain-machine interface using targeted innervation of the brachial plexus nerves. The pectoral muscle is separated in four distinct regions, and each brachial plexus nerve is allowed to innervate a separate area. Surface electrodes placed over the muscle tissue detect the movement intention.

*Kuiken et al, 2007*

well as the transmission of sensory modalities including touch and pain [Kuiken TA et al, 2007b] to the CNS (Figure 5.3). Although with extremely beneficial practical applications for patients in the near future, this approach has clear limits; not only must healthy muscle tissue be compromised in order to provide a target for regeneration, but also the recovery time and first indications of reinnervation can be in the order of several months.

While tremendous advancements have been made, there is no approach to date that directly integrates with the nervous system while leveraging the processing abilities of the brain and spinal cord. In contrast to all other strategies for neural interface development, we propose to exploit a novel method of engineering nervous tissue constructs as a means of interfacing a multi-electrode array (MEA) with regenerating peripheral nerves. The use of living neural tissue, which may be coupled to the MEA to form a stable interface prior to transplantation, provides an enticing target for host axon ingrowth and synaptic integration (Figure 5.4). By directly accessing the transected nerve, we eliminate the need for interpreting computationally complex neural signals in the CNS. Instead, upon integration, simple operant conditioning should allow the implantee to control the prosthesis with ease [Dhillon GS et al, 2004]. Thus, our approach builds upon current interface architecture capabilities and holds enormous promise to provide both motor control and sensory feedback for normal function.



**Figure 5.4:** Schematic representation describing the use of stretch-grown axonal constructs pre-attached to an electrode array in a PNS-based neural interface system

## 5.2 Extension and maintenance of host neurites in the absence of a living distal target, using stretch-grown axons

### 5.2.1 Relevant Background

When a short segment of a nerve is lesioned, regenerating fibers will regenerate across the gap and along the distal segment until the end target is reached. However, if a living distal target is absent, or if the regenerative path is occluded, the axons will retract and sprout collateral branches, each attempting to forge a course to the end organ. If this too is not possible, the axons will either inaccurately innervate surrounding tissue (resulting in the phenomenon of

phantom pain, which occurs in approximately 10% of patients [Herndon JH et al, 1976]) or will form a painful bulbous enlargement at the site known as a neuroma [Fried K et al, 1993]. This entire process can occur within weeks, and neuromas can often persist for months to years.

In general, the fate of a nerve and its end target are considered to be interlinked – a severed nerve is continuously attempting to reach the target, and denervated end targets require subsequent reinnervation for their long-term survival. In order to create a viable neural-electrical interface with a peripheral nerve, it is crucial that the nerve fascicles be prevented from retracting from the vicinity of electrode sensors and/or forming a neuroma at the site. In this study, we evaluate the ability of a stretch-grown neural construct to sustain regenerating axons for up to 4 weeks *in vivo* in the absence of a distal target, vis-à-vis static neural constructs.

## **5.2.2 Materials and Methods**

### *Dorsal root ganglion neuron isolation*

Dorsal root ganglion (DRG) neurons were isolated from embryonic day 15 fetuses from timed-pregnant Sprague-Dawley (Charles River, Wilmington, MA) rats. The DRG explants were suspended at  $5 \times 10^6$  cells/mL in Neurobasal® medium supplemented with 2% B-27, 0.4 mM L-glutamine, 1% penicillin/streptomycin, 2 mg/mL glucose (Sigma-Aldrich, St. Louis, Mo), 10

ng/mL 2.5S nerve growth factor (NGF) and 1% fetal bovine serum (FBS) (HyClone, Logan, UT), and a mitotic inhibitor formulation of 10 mM 5-fluoro-2'-deoxyuridine (FdU) (Sigma-Aldrich), and 10 mM uridine (Sigma-Aldrich) to encourage non-neuronal cell elimination.

### *Static Nervous Tissue Constructs*

The explants were plated into a collagen-filled (rat tail type 1, 3.67mg/mL) agarose (1.25% in 1X PBS; Ultrapure, Invitrogen, Carlsbad, CA) trough measuring 2mm x 1cm. The cell cultures were allowed to form axonal connections over 12 days *in vitro*. The neural growth medium described above was replaced on post-isolation days 1, 3, 5 and 12.

### *Stretch-grown Nervous Tissue Constructs*

The explants were plated into mechanical elongation chambers custom-fabricated for the stretch-growth procedure. The neurons were plated in two populations along the interface of a collagen-coated (rat tail type 1, 3.67mg/mL) aclar "towing" membrane and a base layer of agarose (1.25% in 1X PBS; Ultrapure, Invitrogen, Carlsbad, CA), resulting in a separation of 50-100 $\mu$ m. Over 5 days *in vitro*, axonal connections were formed between these two populations. The populations were then gradually separated over the course of 7 days using a stepper motor system until the axons spanning them reach a length of 1cm.

### *Neural Construct Encapsulation*

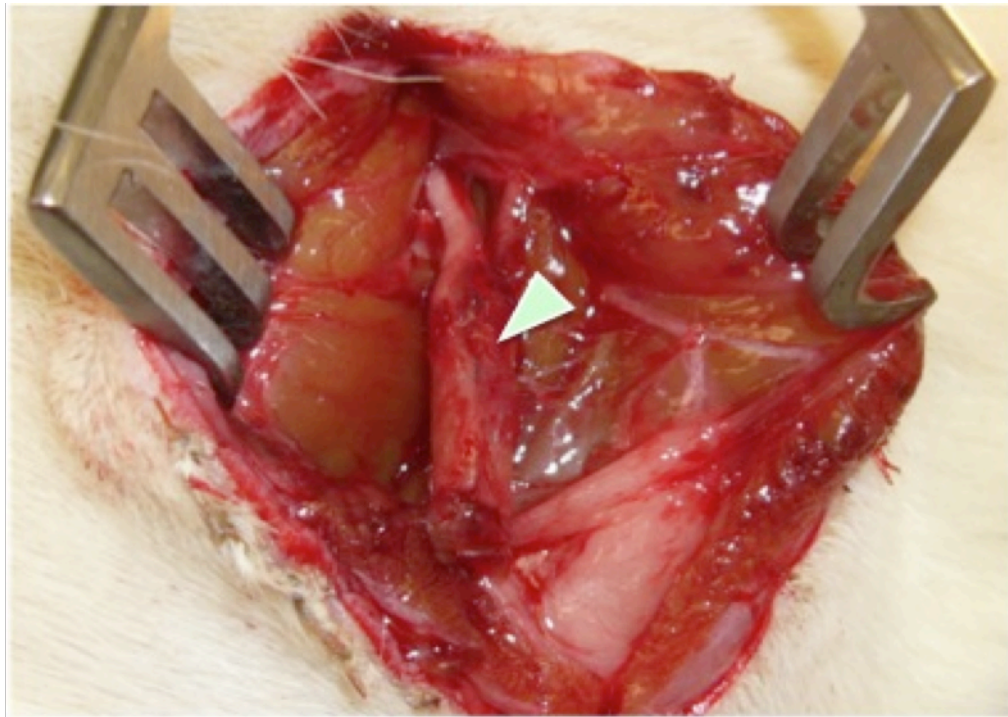
On post-isolation day 12, the neural cultures (both static and stretch-grown) were removed from the incubation chambers and embedded in a collagen-based matrix (3.0 mg/mL) in Dulbecco's modified Eagle's medium supplemented with NGF (2.5S, 10 ng/mL). After gelation at 37°C, embedded cultures were gently removed and placed within a 1cm absorbable collagen nerve guidance channel (3mm inner diameter, NeuraGen™, Integra LifeSciences Corp, Plainsboro, NJ).

### *Peripheral Nerve Surgery and Tissue Construct Implantation*

Stretch-grown (n=6) as well as static (n=6) constructs were implanted into GFP<sup>+</sup> rats (adult male, 400-450mg, strain TgN(act-EGFP)OsbCZ-004). The animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) prior to surgery. A longitudinal skin incision was made in the hind leg from the sciatic notch proximally to the region of the popliteal fossa distally. The gluteal muscle was freed up to expose the sciatic nerve and its posterior tibial branch, and the nerve was transected 1cm proximal to the trifurcation. The proximal stump was carefully inserted into the NeuraGen™ tube with an overlap of 1mm, and the epineurium was secured to the tube using 8-0 absorbable sutures. Both ends of the tube were sealed with salmon fibrin (5mg/ml fibrinogen, 5 NIH

units/ml thrombin) to provide additional stability. A sterilized strip of polyimide was then placed over the distal end of the tube and secured to the surrounding muscle in order to prevent axonal growth out of the tube. Finally, the muscle and skin layers were sutured over the graft with absorbable chromic gut 4-0 sutures.

### *Nerve Harvest and Histological Analyses*



**Figure 5.5:** The transplanted neural construct (indicated by the arrow) being harvested at the 4-week time point. The construct could be seen securely connected to the proximal stump. Evidence of fibrosis and vascularization could be seen along the outside walls of the collagen tube.

At 4 weeks post-transplantation, the constructs were harvested by transecting the nerve 2mm proximal to the graft (Figure 5.5), and were placed in 4% paraformaldehyde. The animals were euthanized by an overdose of sodium pentobarbital.

Following overnight fixation in paraformaldehyde, the excised tissue was removed and immersed in 30% sucrose solution. The tissue was then cryosectioned longitudinally (15-25 $\mu$ m), and immunostained for (i) neurofilament-200kDa (NF-200; N0142, 1:200; Sigma-Aldrich), (ii) myelin basic protein (MBP; SMI-94R, 1:500, Covance Research Products, Princeton, NJ), (iii) S-100 (1:500; Dako, Carpinteria, CA), or (iv) calcitonin gene-related peptide (CGRP; C8198, 1:1000 Sigma-Aldrich). The appropriate secondary fluorophore-conjugated antibodies (TRITC- or AMCA-conjugated IgG; Jackson ImmunoResearch, West Grove, PA) were used. The sections were examined under an epifluorescent microscope (Eclipse E600; Nikon, Melville, NY) and the images were digitally captured (Spot RT Color; Diagnostic Instruments, Sterling Heights, MI).

### **5.2.3 Results**

#### *Host axonal regeneration into stretch-grown constructs*

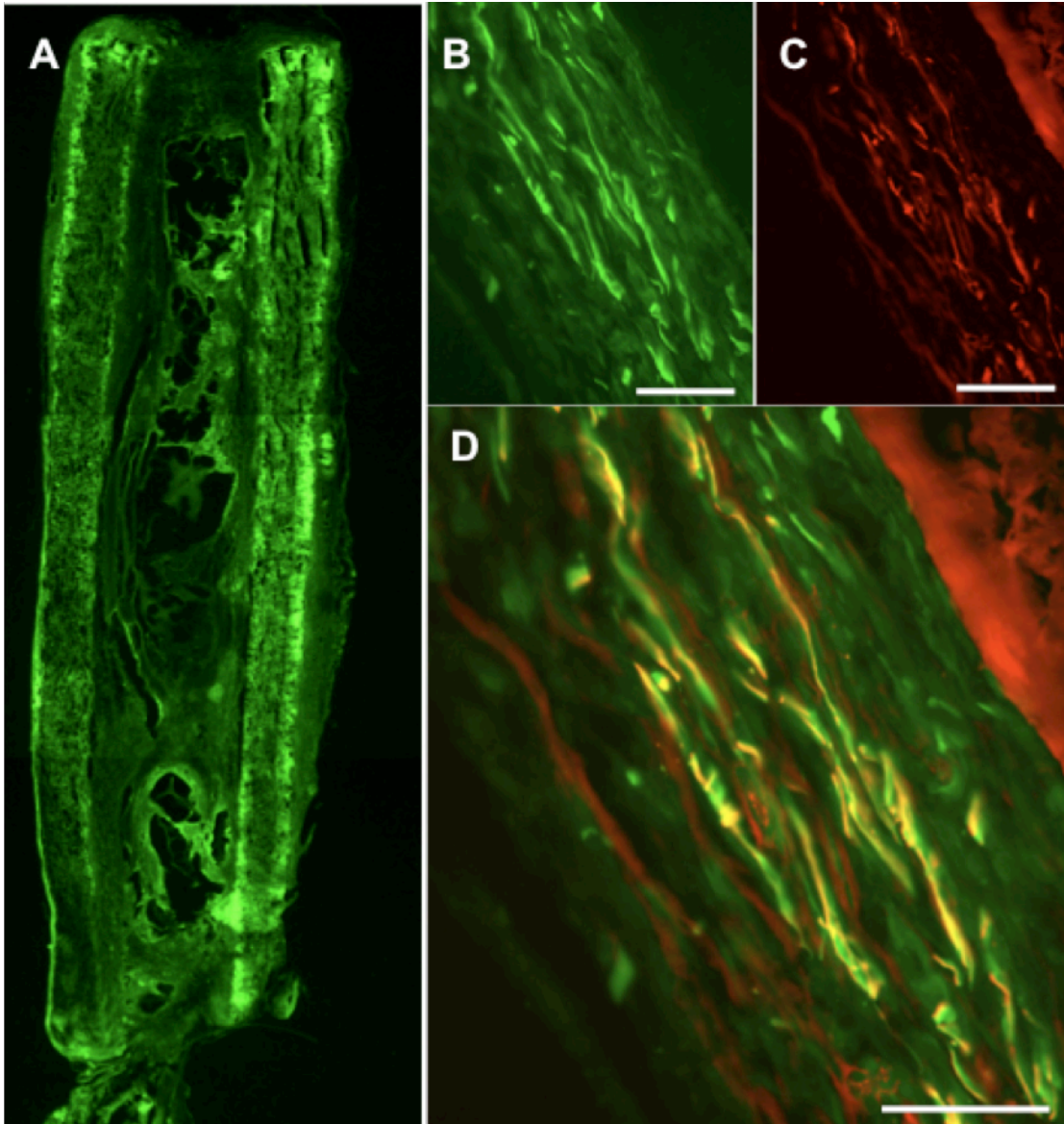
Host axon fibers were seen to extend into the construct tubes and could be found along their entire length. These axons were identified as those



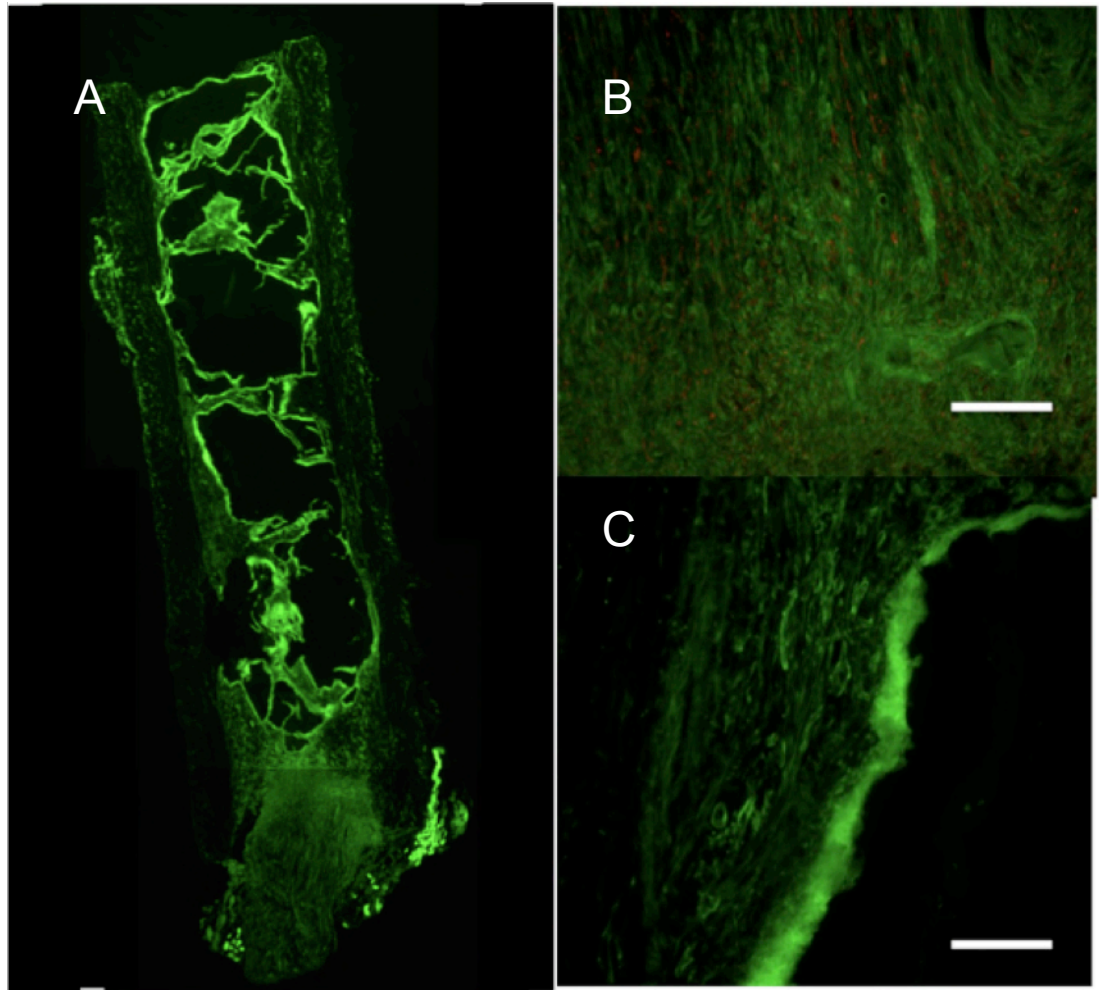
expressing NF-200 co-localized with GFP. Similar to observations in the previous study, the axons were observed to preferentially grow between the still-disintegrating agarose segments and the walls of collagen tube, although in n=4 of 6 cases they did not cross the distal end of the tube. Robust vascularization could be seen throughout the tubes and alongside the axons. In regions with axonal sprouting, co-mingling of host and graft (NF200<sup>+</sup>/GFP<sup>-</sup>) axonal fibers could be observed, indicating survival of the graft axons (Figure 5.6). There was no overt sign of immunologic response as denoted by infiltrating macrophages and neutrophils despite the lack of immune suppressant usage.

#### *Neuroma formation in static constructs*

In the case of static neural constructs (n=6 of 6), the attached nerves appeared to have undergone excessive collateral sprouting resulting in neuroma-like formations by the proximal opening (Figure 5.7). The axons within the nerve bundles could be clearly identified morphologically, and were also seen to express GAP-43 co-localized with GFP, a commonly observed marker in neuromas. Sparse vascularization was seen within the tube. Neither NF-200, nor GAP-43 nor CGRP were expressed in these vascularized areas or in any other area of the tube, suggesting that the transplanted cell bodies were no longer present. Here too, there was no sign of macrophage or neutrophil infiltration at this time point.



**Figure 5.6:** Cross section of the stretch-grown axonal construct in the collagen tube (A). The arrow indicates the proximal side. Robust axonal and vascular infiltration can be seen within the tube, as well as empty pockets containing un-disintegrated agarose. No axonal outgrowth was observed leaving the distal end of the tube. GFP tissue can be seen flanked between the tube walls and agarose (B). Staining for NF-200 (red) revealed axons in these areas (C). Superimposition of these images revealed robust intermingling of host (yellow) and graft axons (red). Scale bars = 100 $\mu$ m.



**Figure 5.7:** Cross section of the static neural construct in the collagen tube (A). The arrow indicates the proximal side. Large empty regions could be seen throughout the tube. Note the neuroma formation at the proximal stump, as characterized by the excessive localized collateral sprouting and positive identification for GAP-43 (red, B). No axonal ingrowth or graft cell survival could be seen within the tube, as evidenced by the lack of positive staining for NF-200. However, vascular infiltration could be clearly observed (C). Scale bars = 100 $\mu$ m.

## **5.3 Regeneration and maintenance of host axons in the vicinity of a flexible electrode substrate**

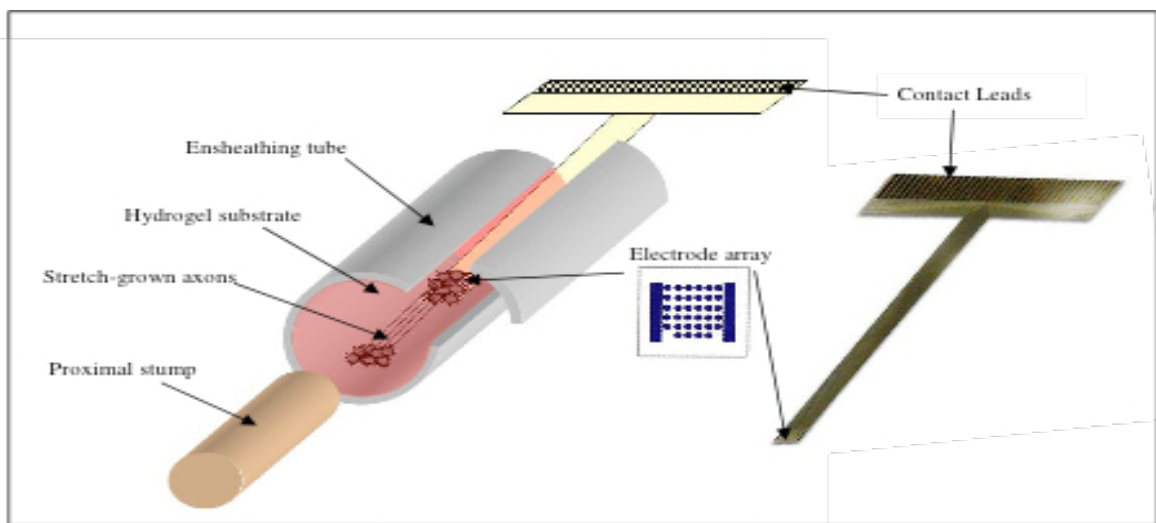
### ***5.3.1 Relevant Background***

Currently, direct stimulating/recording techniques from peripheral nerves can be broadly grouped into two categories. The first are nerve cuffs, which have been in clinical usage since the 1960's and have been implanted in some patients for as long as 15 years [Glenn WWL et al, 1985; Waters et al, 1985]. These are typically made of inert materials such as platinum-iridium or stainless steel, encircle the nerve externally and thus do not inflict any direct penetrative damage to the underlying axonal cytoskeletal structures [Heiduschka and Thanos, 1998; Naples et al, 1990]. They are also available in a variety of geometries such as helices [McCreery et al, 1992] and spiral cuffs [Rozman, 1991] that optimize the contact between the nerve and electrode. However, this design has inherent drawbacks – the electrodes can often impose stretching and compressive forces on the nerve, the materials can degrade over time, and there is a reduced likelihood of chronic interface due to nerve degeneration [Krarup et al, 1989; Larsen et al, 1998].

The second category of nerve-electrodes is intra-fascicular arrays. These electrodes penetrate at various depths into the nerve depending upon the degree of sensitivity and control desired [Branner et al, 2004; Lawrence et al, 2003;

Aoyagi et al 2003]. In the short term, they have been to exhibit enhanced signal selectivity and high signal-to-noise ratio. However, similar to nerve cuffs, this approach also has its disadvantages – the materials used are often of poor biocompatibility, the electrodes themselves can be extremely damaging to the already traumatized nerves, and nerve degeneration ultimately results in a loss of the neural-electrical interface.

Building upon the previous study, here we assess axonal growth towards a thin, flexible polyimide electrode array substrate pre-attached to the axonal constructs (Figure 5.8), and determine the proximity of regenerating axons to the substrate surface. From these observations, we can make a preliminary assessment of the feasibility of recording electrophysiological activity from the



**Figure 5.8:** Schematic of proposed neural interface design. The electrode array is attached to one population of neurons prior to axonal elongation. After stretch-growth to 1cm, the construct is embedded in a hydrogel substrate, placed in a resorbable Neuragen™ tube for structural support, and sutured to the proximal nerve stump.

*Image of FlexMEA array, Multi Channel Systems*

nerve using this approach.

### **5.3.2 Materials and Methods**

#### *Dorsal root ganglion neuron isolation*

Dorsal root ganglion (DRG) neurons were isolated from embryonic day 15 fetuses from timed-pregnant Sprague-Dawley (Charles River, Wilmington, MA) rats. The DRG explants were suspended at  $5 \times 10^6$  cells/mL in Neurobasal® medium supplemented with 2% B-27, 0.4 mM L-glutamine, 1% penicillin/streptomycin, 2 mg/mL glucose (Sigma-Aldrich, St. Louis, Mo), 10 ng/mL 2.5S nerve growth factor (NGF) and 1% fetal bovine serum (FBS) (HyClone, Logan, UT), and a mitotic inhibitor formulation of 10 mM 5-fluoro-2'-deoxyuridine (FdU) (Sigma-Aldrich), and 10 mM uridine (Sigma-Aldrich) to encourage non-neuronal cell elimination.

#### *Stretch-grown Nervous Tissue Constructs*

The explants were plated into mechanical elongation chambers custom-fabricated for the stretch-growth procedure. The neurons were plated in two populations along the interface of a collagen-coated (rat tail type 1, 3.67mg/mL) aclar “towing” membrane and a base polyimide strip layered on an agarose bed (1.25% in 1X PBS; Ultrapure, Invitrogen, Carlsbad, CA), resulting in a separation of 50-100 $\mu$ m. Over 5 days *in vitro*, axonal connections were formed between these two populations. The populations were then gradually separated over the

course of 7 days using a stepper motor system until the axons spanning them reach a length of 1cm.

### *Neural Interface Encapsulation*

On post-isolation day 12, the polyimid/neural cultures (both static and stretch-grown) were removed from the incubation chambers and embedded in a collagen-based matrix (3.0 mg/mL) in Dulbecco's modified Eagle's medium supplemented with NGF (2.5S, 10 ng/mL). After gelation at 37°C, embedded cultures were gently removed and placed within a 1cm absorbable collagen nerve guidance channel (3mm inner diameter, NeuraGen™, Integra LifeSciences Corp, Plainsboro, NJ).

### *Peripheral Nerve Surgery and Interface Implantation*

The stretch-grown constructs (n=4) were implanted into GFP<sup>+</sup> rats (adult male, 400-450mg, strain TgN(act-EGFP)OsbCZ-004). The animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.) prior to surgery. A longitudinal skin incision was made in the hind leg from the sciatic notch proximally to the region of the popliteal fossa distally. The gluteal muscle was freed up to expose the sciatic nerve and its posterior tibial branch, and the nerve was transected 1cm proximal to the trifurcation. The proximal stump was carefully inserted into the NeuraGen™ tube with an overlap of 1mm, and the

epineurium was secured to the tube using 8-0 absorbable sutures (Figure 5.9A). Both ends of the tube were sealed with salmon fibrin (5mg/ml fibrinogen, 5 NIH units/ml thrombin) to provide additional stability. A sterilized strip of polyimide was then placed over the distal end of the tube and secured to the surrounding muscle in order to prevent axonal growth out of the tube. Finally, the muscle and skin layers were sutured over the graft with absorbable chromic gut 4-0 sutures.

### *Neural Interface Harvest and Histological Analyses*

At 4 weeks post-transplantation, the constructs were harvested by transecting the nerve 2mm proximal to the graft, and were placed in 4% paraformaldehyde. The animals were euthanized by an overdose of sodium pentobarbital.

Following overnight fixation in paraformaldehyde, the excised tissue was removed and immersed in 30% sucrose solution. The tissue was then cryosectioned longitudinally (15-25 $\mu$ m), and immunostained for (i) neurofilament-200kDa (NF-200; N0142, 1:200; Sigma-Aldrich), (ii) myelin basic protein (MBP; SMI-94R, 1:500, Covance Research Products, Princeton, NJ), (iii) S-100 (1:250; Dako, Carpinteria, CA), or (iv) calcitonin gene-related peptide (CGRP; C8198, 1:1000, Sigma-Aldrich). The appropriate secondary fluorophore-conjugated antibodies (TRITC- or AMCA-conjugated IgG; Jackson ImmunoResearch, West Grove, PA) were used. The sections were examined under an epifluorescent



microscope (Eclipse E600; Nikon, Melville, NY) and the images were captured digitally (Spot RT Color; Diagnostic Instruments, Sterling Heights, MI).

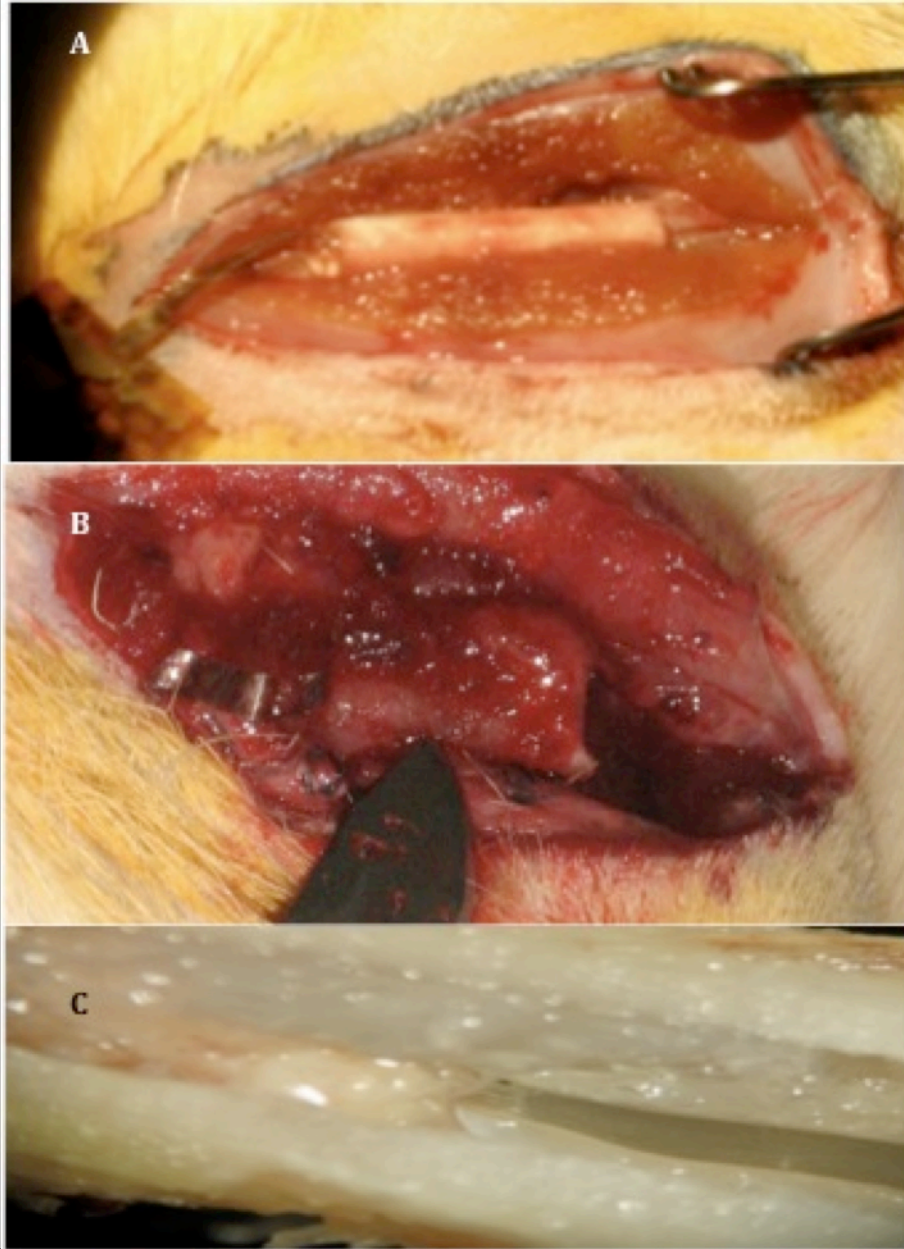
### **5.3.3 Results**

#### ***Gross assessment of neural interface at 4 weeks***

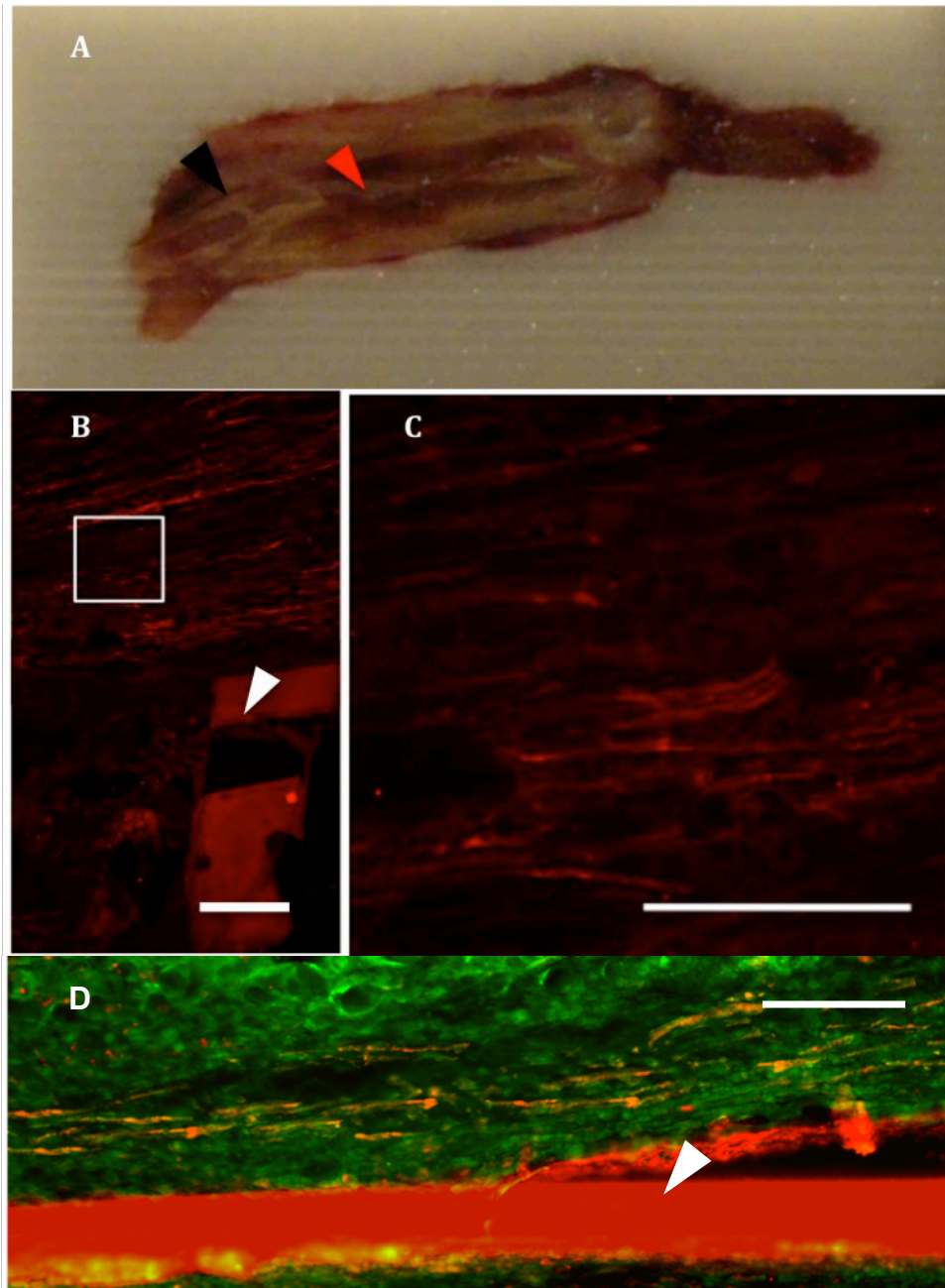
At the 4-week time point, the surgical site was re-exposed and the neural interface was harvested. Fibrosis could be seen surrounding the collagen tube, but surprisingly the polyimide strip was spared. The polyimide strip/electrode array was still securely contained within the collagen tube, despite normal ambulatory movement by the animal during this time (Figure 5.9B). Within the tube, the proximal stump was seen to have grown into intimate contact with the electrode array, and both the nerve and electrode array were still secured in place by the surrounding agarose hydrogel [Figure 5.9C].

#### ***Axonal outgrowth in the vicinity of the electrode substrate***

A histological evaluation of the constructs revealed host axonal outgrowth in the immediate vicinity of the polyimide strip. NF-200<sup>+</sup> (Figure 5.10D) and tau-positive (Figure 5.10B,C) staining was observed at distances ranging from 100-200um from the substrate. Vascular ingrowth was also observed in the region, portending well for continued survival of these axons.



**Figure 5.9:** (A) The neural interface, consisting of the stretch-grown axons attached to the polyimide substrate (indicated by the arrows) within a NeuraGen™ tube being sutured to the proximal stump of the rat sciatic nerve. (B) The neural interface being harvested at the 4-week time point. Note the fibrosis surrounding the tube, but minimal response by the polyimide strip. (C) Visual assessment within the tube revealed the proximal stump in close physical proximity to the end of the polyimide strip.



**Figure 5.10:** (A) Cross section of the harvested neural interface at 4 weeks. The polyimide strip (black arrow) and the proximal stump (red arrow) can be clearly seen. (B-C) Axon fibers, identified by tau-positive staining (red) can be clearly seen in close proximity to the polyimide electrode array (white arrow). (D) Regenerating axons, identified by NF-200 staining (yellow) can be seen within host tissue (GFP, green) running closely alongside the polyimide strip (white arrow). Scale bar = 100 $\mu$ m.

## 5.4 Discussion

This project demonstrates a means of extending and maintaining a transected nerve segment in the absence of a distal pathway, with the goal of ultimately recording electrophysiological signals from the host axons using an implanted electrode array. While the results are admittedly preliminary, they provide support for further study and development of this novel PNS-based neural interface platform.

### *Extension and maintenance of regenerating neurites*

A confounding factor in the development of peripheral nerve-based neural interface systems has been the inability to preserve electrical contact with the remaining nerve segment over the long-term. The primary reason for this is the unfavorable, and often physically damaging, environment created around the nerve fascicles when rigid metal electrodes are placed in their immediate vicinity [Navarro X et al, 2005]. The presence of the electrodes invariably triggers a robust immunologic response and connective tissue buildup, rendering the neural-electrical coupling ineffectual over the long-term [Stieglitz T, 2004; Larsen et al, 1998]. In addition, the electrodes themselves can cause compressive and/or penetrative stress on the neurites, further harming already impaired nerves [Rodriguez FJ et al, 2000; Grill WR and Mortimer JT, 1998].

A successful PN interface design must therefore incorporate components that actively encourage neurite outgrowth towards them, in addition to being compliant enough to prevent damage during the course of normal activity. Since Schwann cells play a pivotal role in regeneration, they have been extensively used in so-called hybrid interface designs. The most sophisticated of these are regenerative sieve electrodes – in this design, axons are induced to grow across a finely perforated barrier into a chamber containing a short segment of distal nerve and Schwann cell cultures [Lago N et al, 2009]. Axons have been shown to grow into the chamber and survive for several months. However, there are a number of disadvantages with this approach including the limited regeneration across the barrier, frequent axotomy that occurs during normal ambulatory movement, neuroma formation over the short-term, competition between neurites and proliferating Schwann cells for volume, and the eventual atrophy and retraction of the axons [Navarro X et al, 2005].

Building upon these studies and our previous results where it was shown that living stretch-grown axonal constructs provide regenerative support for transected fascicles, their ability to maintain axons in the absence of a distal target was initially evaluated in this study. Neural constructs (stretch-grown, static and control) were transplanted against a transected proximal nerve segment, and the extent of axonal ingrowth was measured. At 4 weeks, extensive axonal sprouting and neuroma formation was seen in the static groups at the site of suture of the stump. This is comparable to the response seen after

post-transectional nerve capping [Galeano M et al, 2009]. The animals had suffered extensive automutilation, presumably as a result of the neuroma formation [Zeltser R et al, 2000]. Neurofilament staining did not reveal the presence of axons nor graft cell bodies within the tubes, indicating that the static cultures may not have survived at this time point. It is unclear whether the axonal regeneration occurred at all, or after nerve ingrowth and subsequent retraction. Since the distal end of the tube was sealed, all vascularization seen must have originated at the proximal end of the tube. This form of vascularization typically accompanies nerve regeneration [Best TJ et al, 1999], however, suggesting that abortive reinnervation may indeed have occurred. The stretch-grown construct group showed evidence of neurite ingrowth, albeit sparse, and graft axon survival in those areas. No neuromas were evident in this group, and automutilation was much less pronounced.

#### *Evaluation of the neural interface in vivo*

Following from these results, a commercially available flexible electrode array made of polyimide PI12611 was incorporated into the pre-stretch design, enabling the creation of a fully-formed neural-electrical interface *in vitro*. Polyimide was selected as the base material as it has been shown to be durable, flexible, capable of being implanted over several years, and inciting only minimal fibrotic response [Lago N et al, 2007; Klinge et al, 2001; Navarro et al, 1998]. This interface was transplanted against the transected sciatic nerve, and left *in*

*vivo* for 4 weeks. A gross evaluation of the neural interface upon removal showed that interface design is capable of withstanding normal ambulatory movement of the animal, at least in the acute phase. Fibrous tissue was seen surrounding the collagen conduit, which paradoxically may have provided additional stability. The polyimide strip, however, was surprisingly devoid of fibrous tissue even in this acute time frame, suggesting that it may be capable of withstanding host rejection within this design over a longer period.

Careful exposure of the tube revealed a proximal stump that was now in the close contact with the polyimide strip. Unlike in the previous study where only sparse host ingrowth was seen, here the nerve appeared to be of a much more robust girth. This may be a result of the pre-coated polyimide strip presenting a favorable 2-dimensional regenerative surface for the nerve to grow along [Bellamkonda RV, 2006]. It should be noted that although the agarose gel kept the nerve and polyimide in close contact, manipulation of the nerve-electrode interface revealed a weak adhesion. This may be a result of minimal fibrotic infiltration due to the use of the salmon fibrin sealant – chronically implanted polyimide nerve cuffs maintain their neural connection only upon encapsulation by fibrous tissue [Rodriguez FJ et al, 2000]. It remains to be seen if a beneficial fibrotic response occurs after enzymatic degradation of the agarose and fibrin.

Histological evaluation of the nerve-electrode contact revealed axonal growth in close proximity to the polyimide surface. The separation ranged from

100-200 $\mu$ m, as demonstrated by NF200 and Tau staining. In comparison, polyimide nerve cuff electrodes can consistently stimulate and record axonal activity at distances of up to 400 $\mu$ m [Naples GG et al, 1990], suggesting that the axon-electrode separation observed here is electrophysiologically viable.

Finally, while the stretch-grown axons have been shown to be electrophysiologically functional [Pfister BJ et al, 2006], there was no evidence of host synaptic integration with either of the graft cell body populations. Although the design goal of this project was simply to coax axonal growth into the vicinity of the electrode substrate, such synaptic integration could also provide a mechanism for stable neuro-electrical interfacing over the long-term, and thus warrants further investigation.

## **5.5 SUMMARY OF FINDINGS:**

### *Conclusions*

In the absence of the appropriate distal target with which to integrate, regenerating axons will retract and sprout numerous collateral branches that then either inaccurately innervate surrounding tissue or coalesce into a neuroma at the site. Here, we demonstrate a method of extending and maintaining these axons for at least 4 weeks with retraction, despite the absence of an end target, using living stretch-grown axonal tracts. We then harnessed this phenomenon to



entice host neurite growth into close contact with an implanted electrode substrate, potentially enabling recording and/or stimulation from these fascicles. host axonal growth into close contact with an implanted electrode substrate. While our findings are preliminary, they represent an important initial step in evaluating the feasibility of this novel bio-electrical hybrid platform for long-term interfacing with the peripheral nervous system.

### *Limitations and Future Studies*

The principal limitations of this study are two-fold. First, while the findings are suggestive of the advantages offered by stretch-grown axons, a clearer understanding can only be reached by using a larger sample size per group. In particular, a quantification of the number of axon fibers in the vicinity of the electrode will enable us to determine critical design characteristics and sensitivity of the electrodes themselves.

The second important limitation is the acute time point – while the 4-week time point was selected in order to understand and solve the short-term design challenges, a thorough understanding of the benefits of this approach can only be reached after several months of implantation. It is vital to determine whether the axons can be maintained in proximity to the array for months to years, as well as if they demonstrate signs of continued development, such as fasciculation and myelination, which are essential for robust electrophysiological function. Based

on our results, we believe that a comprehensive histological assessment of the neural interface at 6 months and 1 year *in vivo* is merited.

## Chapter 6

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### Synthesis and Future Work

## **Chapter 6: Synthesis and Future Work**

### **6.1 Introduction**

Peripheral nerve damage following trauma can often be a debilitating condition resulting in pain and disability for prolonged periods. In its most severe form, the entire nerve trunk is transected resulting in complete discontinuity and a total loss of function without surgical intervention. If the distal stump is still present, bridging techniques using nerve guidance channels can be employed; however, if the injury is too extensive or too proximally located, the prognosis is invariably poor. If a distal pathway is absent, as in the case of a limb amputation, the nerve will either aberrantly innervate surrounding tissue or form a neuroma at the site. Without a suitable means to chronically record intent-related activity from the nerve, it essentially becomes extraneous.

The purpose of this study was to evaluate the ability of transplantable nervous tissue constructs to augment natural regeneration of transected peripheral nerves in order to (1) enable repair of a short lesion at both the proximal and distal ends, and (2) facilitate extensive and maintenance of the axons in the absence of a distal target, with the ultimate goal of creating a bi-directional neural interface with the PNS.

## 6.2 Summary and Synthesis of Major Findings

The first major goal in this project was to create a stable and reproducible encapsulation methodology for the axonal constructs. Previous collagen gel-based encapsulation schemes provided minimal physical support for the axonal constructs, resulting in frequent disruption of the tracts during removal from the culture environment. The stretch-growth protocol was modified to incorporate agarose hydrogel as the base layer upon which cell bodies were plated, and the optimal parameters for neurite outgrowth and tissue encapsulation were determined. Finally, salmon fibrin was incorporated as an end sealant at the time of implantation, in order to attenuate macrophage and glial infiltration as well as provide additional stability at the site of the suture. The salmon fibrin appeared to have contributed to the slow disintegration of the agarose over the initial 4 weeks, although this disintegration was complete within 8 weeks. Since allograft tissue is employed in these studies, the benefit provided by the fibrin in preventing macrophage infiltration outweighs the short-term drawbacks, and as such it will continue to be incorporated into the transplantation design.

Stretch-grown axonal constructs appeared to offer a clear benefit over static neural populations in repairing short PNS lesions. This benefit was most apparent during electrophysiological evaluation, where a statistically significant difference in the conduction velocity ratios was observed. This appeared to correlate with the extent of regeneration seen within the graft tubes; the axonal

construct group appeared to demonstrate robust neural and vascular growth through the tube, whereas growth was limited to along the walls in the static group. This suggests that regenerating neurites, and their accompanying vasculature, preferentially proceed along aligned axonal pathways, in a process approximating naturally occurring axon-mediated axon growth. The neural cell bodies themselves, which are entirely absent in the periphery, may simply be indiscernible to the host axons and thus provide no therapeutic benefit.

On the other hand, these cell bodies – and, more specifically, the neurites emanating from them – seem to play a significant role in maintaining the distal pathway far longer than naturally possible. The morphology of the distal stump at 4 months resembled that of control nerves at 2 weeks, thus potentially increasing the time window within which successful reinnervation may occur. The absence of any surviving neurites and cell bodies suggests, however, that this protective effect is transient. In the vast majority of cases, reinnervation of the distal pathway will have occurred within this timeframe. However, for very extensive lesions this delay may itself not be sufficient. The use of immune suppressants may be able to prolong the eventual atrophy of the grafted cells, and would be an important future area of study.

Stretch-grown axons were also shown to enable host axons regeneration and maintenance for 4 weeks without the presentation of any distal target. This runs contrary to conventional dogma, wherein regenerating neurites will retract and attempt collateral branching in the continued pursuit of a target. Excessive

collateral branching often leads to neuroma formation – indeed, this was uniformly observed in static culture group. The mechanisms by which the axonal constructs enable this axonal maintenance is unclear, as is the temporal limits if any. However, we did observe that incorporating an electrode substrate into the design enabled host axonal growth into its vicinity. The extent of host infiltration as well as the long-term behavior of these axons will need to be quantified in order for this neural-interface platform to be feasible. However, the short-term results obtained thus far merit a more comprehensive investigation of this architecture.

### **6.3 Future Work**

The preceding studies described a novel method of repairing nerve lesion, and potentially interfacing with the peripheral nervous system, using engineered living nervous tissue. However, several areas warrant further study which should be considered for future investigations.

#### *Developing transplantable axon tracts*

Currently the axonal tracts generated are planar in nature – this is particularly disadvantageous during removal from culture environment for nerve repair applications, where the tracts need to be compressed into a narrow tube in

order to achieve a reasonable axon density. An important solution to this would be to create 3-dimensional axonal tracts that conform to the dimensions of the nerve guidance channels. We are evaluating the use of various hydrogels as the embedding matrix, and will modify the stretch-growth chambers to incorporate these structures.

### *Repair of PNS lesions*

A more thorough understanding of the precise benefit offered by the stretch-grown axons is needed. While methods such as electrophysiology are immensely informative, it would be useful to correlate this information with a more quantitative assessment of the regeneration, including axonal density, myelination, etc. In addition, earlier (less than 2 weeks) and later (greater than 4 months) time points would be useful in determining the rate of regeneration and then extent and nature of reinnervation (motor versus sensory). A clearer understanding is also needed of the mechanisms by which axonal constructs augment host axonal regeneration. It would be important to isolate those components (chemotactic and/or haptotactic) that especially influence regeneration. By doing so, we may be able to create artificial conduits incorporating these elements, thus eliminating the need for living tissue transplantation.



For distal degeneration, it is important to determine the mechanism by which the constructs provided pathway maintenance, as well as the reasons for their eventual degradation. Evaluation at an earlier time point would be useful in determining the lifespan of the constructs. Finally, the use of immune suppressants or other agents as a means of prolonging their survival would be important to determine.

#### *Development of a neural interface*

A clearer quantitative understanding of the extent and nature of the regenerating axons is critical in determining the viability of this neural interface platform. While axon retraction and neuroma formation were not observed in the acute phase, this must be verified over the long-term. An evaluation of the host axon density and myelination must also be performed in order to determine the appropriate electrical characteristics of the electrodes. Finally, an improved integrated electrode design needs to be developed. Although we are merely evaluating the feasibility of this approach using a variety of commercially available components, none of them have been developed for this application. A more appropriate neural-interface design would incorporate a multi-channel electrode substrate encapsulating a 3-dimensional nervous tissue construct. Such a design would allow for more robust host innervation, and potentially enable selective stimulation and recording from individual fascicles.

## **6.4 Conclusion**

In summary, this thesis demonstrates the feasibility of using engineered nervous tissue constructs in the repair of peripheral nerve lesions and amputations. While this is only a preliminary analysis, further development of these techniques may result in clinically applicable therapeutic strategies for extensive nervous system repair.

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