# DEVELOPMENT OF AN IN-VITRO ASSAY FOR NEUROTROPHIN-INDUCED SEGREGATION OF NOCICEPTIVE AND PROPRIOCEPTIVE SENSORY AXONS

by

EBRAHIM SHABBIR BENGALI

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT ARLINGTON

DECEMBER 2006

ACKNOWLEDGEMENTS

The work described in the thesis could not have been completed without the guidance and collaboration of a number of people. I would like to take this opportunity to thank each one of them for their valuable contributions.

I would like to start out by expressing my deepest appreciation to my thesis advisor, Dr. Mario Romero-Ortega, for his tireless support and encouragement. I am extremely grateful to him for allowing me to be a part of his visionary research group. His knowledge and talent as a researcher earned him my respect, and his accessibility and support earned him my friendship. I would also like to express my deepest gratitude to Russ Daniels for his valuable assistance and insight during my entire lab tenure. Moreover, I would also like to thank Pedro Galvan-Garcia for his imparting his technical insights during the manufacture of the several in-vitro devices.

I would also like thank the other student members of the Regenerative Neurobiology Laboratory including Vishal Shah, Amit Chouhan, and GowriShankar Raman for the precious friendships I have acquired during my time in the lab.

I would like to dedicate this work to my mother for her continued patience and support during my education process. Last but not the least, I express my deepest appreciation to my fiancé, Sara Merhaba for being there for me and providing emotional support at times when I required it the most.

August 4, 2006

# ABSTRACT

## DEVELOPMENT OF AN IN-VITRO ASSAY FOR NEUROTROPHIN-INDUCED SEGREGATION OF NOCICEPTIVE AND PROPRIOCEPTIVE SENSORY AXONS

Publication No. _____

Ebrahim Shabbir Bengali, M.S.

The University of Texas at Arlington, 2006

Supervising Professor: Dr. Mario Romero-Ortega, Ph.D.

Peripheral nerves have the capacity for regeneration and if properly repaired, axon extensions can regenerate even across gaps caused by peripheral nerve loss. Complete functional recovery after gap injury repair remains suboptimal due to the erroneous pathways taken by the regenerating axons leading to aberrant target innervation. A segregation of axonal types can facilitate efficient target direction and innervation leading to improved functional recovery. Novel two-dimensional and three-dimensional Y-shaped assays were developed to test whether the nociceptive and proprioceptive sensory axons from the dorsal root ganglia could be segregated through

specific neurotrophin inducement; namely NGF and NT-3 respectively. A large number of the compartmentalized CGRP-positive axons growing toward NGF were long with little to no branches; a characteristic trait prevalent among nociceptive sensory axons. Similarly, the NT-3 attracted CGRP-negative axons that were short and highly arborized are characteristic of proprioceptive neurons. These findings provide evidence of segregation of sensory axonal subtypes using a novel assay and an appropriate delivery system for the release of specific neurotrophins.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

# CHAPTER 1

# PERIPHERAL NERVE PHYSIOLOGY

The nervous system can be divided structurally into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS comprises of the brain and the spinal cord and the PNS consists of peripheral nerves extending to a wide array of targets and the ganglia of these nerves. The peripheral nervous system includes both sensory neurons relaying sensory information from the external and internal environment to the brain and spinal cord, while the motor neurons connect the brain and spinal cord to the muscles and glands.

## 1.1 Anatomical Structure of the Peripheral Nerve

The two major anatomical components of the peripheral nervous system are the peripheral nerve consisting of bundles of axons and their supporting cells and ganglia, which are clusters of nerve cell bodies and supporting cells (Fig 1.1).

Peripheral nerve is an intricate and specialized structure composed of axons, schwann cells, fibroblasts, matrix and blood vessels. Schwann cells, the most abundant cell type in the peripheral nerve, envelop a single axon with multiple layers of membrane to form the insulating myelin sheath. On the outer surface of this Schwann cell layer is the neurilemma, a basement membrane similar to that found in epithelial layer. Endoneurium surrounds individual axons and their Schwann cell sheaths and is composed predominantly of oriented collagen fibers. The perineurium, formed from

many layers of flattened cells (i.e. fibroblasts) and collagen, surrounds groups of axons to form fascicles. The epineurium, an outer sheath of loose fibrocllagenous tissue, binds individual nerve fascicles into a nerve trunk. Peripheral nerves are well vascularized by capillaries within the support tissue of the nerve trunk or by vessels that penetrate the nerve from surrounding arteries and veins. Peripheral nerves may consist of only motor, sensory, or a mixed fiber composition. A large peripheral nerve like the sciatic nerve has a mixed fiber composition (Schmidt and Leach 2003).

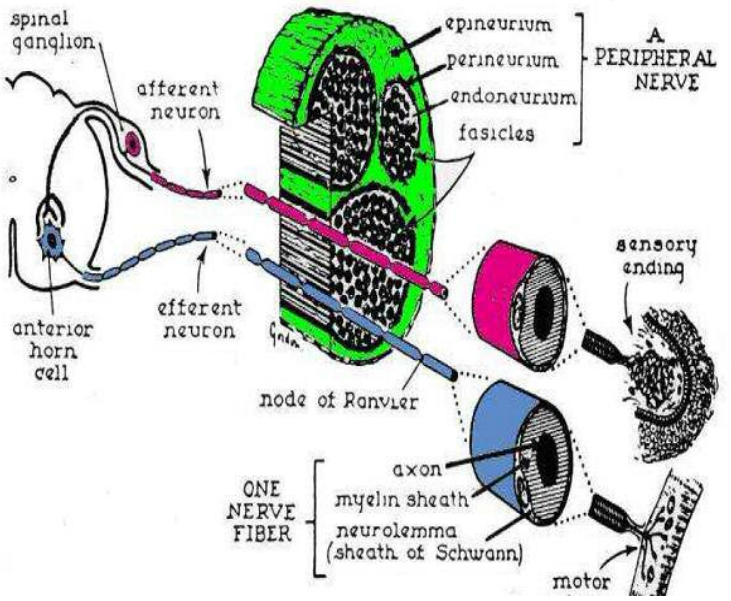


Figure 1.1 Structure of Peripheral Nerve: Diagram depicting a cross section through a peripheral nerve. Axons running parallel are grouped into tight bundles or fascicles. The protective sheaths surrounding the peripheral nerve are the epineurium (shown in green), perineurium, and the endoneurium. Sensory fibers (shown in red) relay information to the central nervous system (CNS) and motor fibers (shown in blue) carry signals to the muscles. Both myelinated and unmyelinated fibers traverse through large peripheral nerves like the sciatic nerve. Adapated from Ham's Histology.

## 1.2 Myelin Function and Composition

Myelin is a number of layers of Schwann cell membranes packed around a single axon. A single Schwann cell supplies myelin for a length of about 1mm around the axon. Myelin is a lipid rich membrane composed of about 80% lipid and 20% protein in which cholesterol is one of the major lipids. The main protein constituents include myelin associated glycoprotein (MAG), protein zero ($P_0$), peripheral myelin protein-22 (PMP-22) and myelin basic protein. Myelin acts as an electrical insulator and speeds up action potential conduction. Unmyelinated axon conduction velocities are in the range of 0.5 to 10 m/s, whereas myelinated axons can conduct at velocities up to 150 m/s. The node of Ranvier is the gap of 1-2 μm between neighboring myelinated regions. The current tends to flow down the fiber to the next node instead of leaking back across the membrane due to the high resistance of the nerve membrane and the low capacitance of the myelin wrapping. The impulse thus jumps from node to node in a mode of propagation called saltatory conduction.

## 1.3 Dorsal Root Ganglion

Ganglia are neuronal relay centers in the peripheral nervous system comprising of neuron cell bodies, satellite cells (glia) and loose fibrocollagenous support tissue .The sensory ganglia form swellings or nodules along the dorsal root at each level of the spinal cord (dorsal root ganglia) or adjacent to the brainstem (cranial nerve ganglia) (Stevens 1997). The Dorsal root ganglia (DRG) contain the cell bodies of peripheral somatosensory neurons. These sensory neurons are pseudo-unipolar; their one major process dividing into two branches, one gathering information from the sensory

receptors in the body and the other entering the spinal cord to relay the information to the central nervous system. They are classified either by their size and conduction velocity or their modalities. The proprioceptive neurons project large, myelinated, and fast-conducting axons that innervate muscle spindle and Golgi tendon organs. In contrast, the nociceptive neurons have axons that are small, unmyelinated, and slow-conducting, generally classified as the pain and temperature fibers (Inoue, K et al 2002).

Depending on their sensory modality, DRG neurons send afferent axons to the gray matter of the spinal cord and to different types of sensory receptors in the periphery. Specifically, the central axons of DRG neurons that transmit nociception project to the deep layers of the dorsal horn. Three types of proprioceptive DRG neurons provide information about muscle length and tension to the spinal cord. Group Ia and II afferent axons innervate muscle spindles, and group Ib afferent axons innervate Golgi tendon organs in the periphery. Group Ia afferent axons centrally ramify in the intermediate zone of the spinal cord and terminate in the motor nucleus of the ventral horn, whereas group Ib and II afferent axons project to the intermediate zone (Inoue, K et al 2002).

# CHAPTER 2

## PERIPHERAL NERVE INJURY AND REPAIR STRATEGIES

Approximately 50,000 cases (Evans 2001) of peripheral nerve lesions occur annually, with a consequent cost of millions of dollars in medical care. Nerve injuries pose challenging and difficult surgical reconstructive problems because mature neurons tend to innervate inappropriate targets while spontaneously regenerating after injury. Transection of a nerve represents a very complex injury and has implications all the way from the nerve cell body at the spinal cord or dorsal root ganglion level to distal muscle and skin targets, thus necessitating repair process of neurites over very long distances (Lundborg 2000). However, in contrast to the lack of spontaneous regeneration in the mature central nervous system, peripheral neurons have the capacity for regeneration. Thus, under the right condition, axon extensions can regenerate even across gaps caused by peripheral nerve loss, reconnecting with the distal stump and eventually reestablishing functional contacts with their original targets. Although a number of repair techniques have been developed, aberrant target innervation continues to be unaddressed and remains the main cause of failure for complete functional recovery after gap injury repair in the peripheral nervous system. A segregation of different types of neurons with the purpose of directing their growth to their appropriate targets could potentially reduce the innervation of incorrect targets and improve functional recovery.

## 2.1 Peripheral Nerve Injury

In moderate nerve compression injuries, axonal continuity is usually preserved, thus allowing functional recovery within weeks or months. In more severe compression, tract, or crush injuries, axonal continuity is usually interrupted, resulting in degeneration of the distal axonal segment, however, leaving the continuity of the endoneurial tubes intact. This allows the regenerating axons from the proximal segment to follow the intrinsic guidelines provided by the endoneurial tubes and reinnervate the correct peripheral targets. With more severe trauma to the nerve trunk, the endoneurial tubes as well as all connective-tissue components of the nerve trunk may be damaged leaving no tissue structure to guide the growing axon across the lesion site thus creating a gap.

The healing of nerve injuries is unique among tissues since cellular repair, rather than tissue repair, is of prime importance. A great number of amputated neurons have to regain their original axoplasmic volume by extending new processes to compensate for their amputated parts (Dahlin, Nordborg et al. 1987). While the number of neurons does not increase, regeneration of their axons takes place in an environment of intense cellular proliferation, reflecting a wound-healing process (Lundborg 2004).

Injury to a peripheral nerve is followed by a stereotyped sequence of events which can be grouped into three major categories: i) the cell body reaction, ii) wallerian degeneration, and iii) axonal regrowth. These events as well as their area of occurrence are described in detail below.

*2.1.1 Neuron cell body response after peripheral nerve injury*

When an axon is severed, the corresponding nerve cell body undergoes characteristic structural and functional changes including an increase in cell body volume, displacement of the nucleus to the periphery and a structural reorganization of the rough endoplasmic reticulum, aiming at increasing the regeneration potential of the neuron (Fu and Gordon 1997). Chromatolysis is accompanied by an increase in RNA and protein synthesis thus, serving as a means for the cell to reprogram its synthetic machinery to produce substances required for axonal reconstruction (Lundborg 2004). Expression of Jun, GAP-43, peripherin, and β-tubulin are upregulated while expression of peptides is downregulated (Lindwall, Dahlin et al. 2004). In sensory neurons, axonal injury leads to additional changes in neuropeptide and cytokine expression whereas in motor neurons, there are changes in neurotrophin receptor, glutamate receptor, and calcitonin gene-related peptide expression (Stoll and Muller 1999). A prerequisite for axonal regeneration is survival of the corresponding nerve cell bodies. A nerve injury close to the cell soma causes an amputation of a large amount of the cellular axoplasm from each neuron and thus, creating an enormous impact on cell body viability with as much as 40% to 50% of the cell body population dying as a consequence (Fu and Gordon 1997).

*2.1.2 Axonal Response to injury in the Peripheral Nervous System*

In the distal nerve stump, Wallerian degeneration (Fig 2.1) takes place during the first few days post-injury and prepares the local environment distal to injury for regeneration, removing potentially inhibitory myelin debris, increasing synthesis of

growth factors, and creating a matrix to support axonal growth. In the initial phase, the axons degenerate and the myelin sheaths detach from the degenerating axons resulting in the degradation of the myelin by the resident as well as the infiltrating blood-borne macrophages that invade the site of lesion within a week subsequent to injury (Terenghi 1999). The myelin degradation products together with macrophage secretion stimulate the Schwann cells within the distal stump to proliferate within their basal lamina tubes and this proliferation continues for approximately two weeks (Son and Thompson 1995). During this initial proliferation phase, the Schwann cells align to form Bands of Bungner which act as conduits to guide the regenerating axons to their target. The contact with regenerating axons is vital for a second phase of Schwann cell proliferation, which is mediated by a neuronally derived glial growth factor because a delayed axonal regeneration causes a progressive decrease in Schwann cells as well as reduced responsiveness to axonal regeneration (Terenghi, Calder et al. 1998).

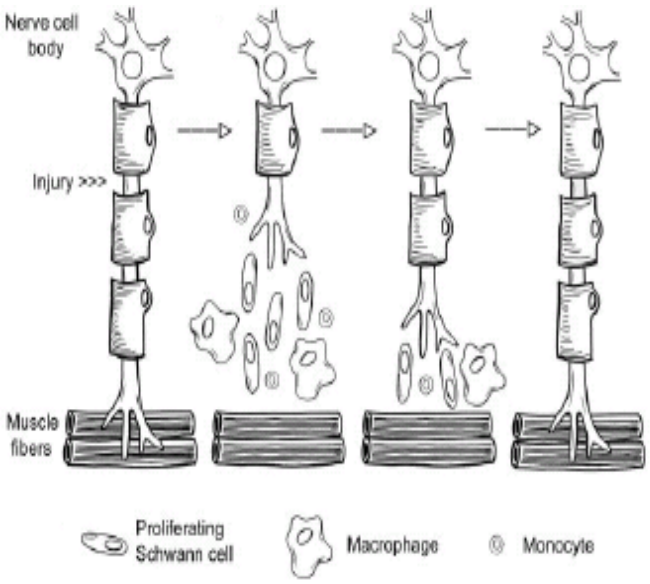


Figure 2.1 Schematic Representation of the process of Wallerian Degeneration in the PNS: In the PNS, neuronal regeneration is aided by the proliferating Schwann cells, macrophages, and monocytes, which work together to remove myelin debris, release neurotrophins, and lead axons toward their synaptic targets, resulting in restored neuronal function. Figure adapted from Schmidt and Leach, 2003.

In the proximal stump, the axons degenerate retrogradely as far as to the first node of Ranvier, creating a small area of Wallerian degeneration. Within a few hours, the injured axons give rise to several neuronal sprouts that are normally in excess of the original number of axons, presumably to maximize the chances of each neuronal cell reaching its target organ (Terenghi 1999). Some of these sprouts will successfully reinnervate target organs while the rest will 'die back' through axonal pruning (Brushart 1993). The growth cone of the regenerating axon responds to contact guidance cues and actively searches for a suitable matrix and environment to support its growth (Ming, Wong et al. 2002). During regeneration, axons while responding selectively to tropic and trophic cues attempt to regrow preferentially towards the target organ which they

initially innervated (Gu, Thomas et al. 1995). However, in gap nerve injuries, axonal misdirection during regeneration remains a key problem. Axon sprouts regenerating within incorrect Schwann cell tubes, end in an aberrant target organ which then compromises functional outcome of the spontaneous repair. The mismatch that occurs between sensory-motor fibers and sensory-motor pathways results in dysfunctional coupling between the neuron type and the innervated target in such a way that a sensory pain fiber might innervate a motor muscle fiber (Nguyen, Sanes et al. 2002).

To reach the distal segment, the regenerating axons have to pass a critical area between the proximal and distal stumps of the cut nerve: the interstump zone, the events occurring in which greatly determines the success of nerve regeneration. The interstump zone is characterized by exudation, cell proliferation, and collagen synthesis. Within a day post-injury, the gap is filled with an exudate containing blood cells, macrophages as well as proteins exhibiting neurotrophic or neurite-supporting activity. A week later, a fibrin bridge forms between the proximal and distal nerve stumps with consequent ingrowth of capillaries and fibroblasts from the nerve stumps as well as from the surrounding tissues (Lundborg 2004). By the second week, Schwann cells migrate into the gap from the proximal as well as distal nerve stump, forming a continuous tubular structure through which axons regenerate across the interstump gap and enter the distal nerve stump before proceeding towards their final targets (Terenghi 1999). Finally, over a period of two to eight weeks, Schwann cells myelinate the axons, resulting in a new nerve structure bridging the former gap between the nerve stumps.

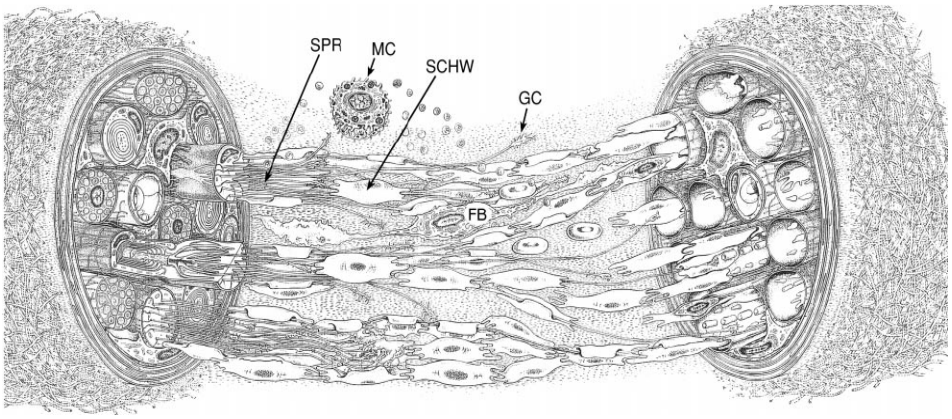


Figure 2.2 Local cellular response to nerve transection: Sprouting occurs at the cut axonal ends in the proximal nerve segment (left). Sprouts (SPR) arising from one myelinated axon form a regenerating unit surrounded by a common basal lamina. At the tip of each sprout there is a growth cone (GC). Sprouts advance over the zone of injury in immediate association with Schwann cells (SCHW). In the injury zone there are macrophages, fibroblasts (FB), mast cells (MC), and blood cells. In the distal segment sprouts attach to the band of Bungner and become enclosed in the Schwann cell cytoplasm. Axonal misdirection is frequent. Figure adapted from (Lundborg 2000).

### *2.1.3 Cellular and molecular basis for nerve regeneration*

Our understanding of biological mechanisms regulating outgrowth and orientation of nerve fibers has increased substantially during the last two to three decades. The importance of Schwann cells and target-derived neurotrophic factors during the regeneration process has been widely documented.

#### 2.1.3.1 Importance of Schwann cells in peripheral nerve regeneration

Schwann cells play an indispensable role in promoting axonal regeneration. After peripheral nerve injury, Schwann cells in the distal stump undergo proliferation and prepare the local environment for axonal regeneration by aiding in the clearance of myelin and axonal debris (Terenghi 1999). Schwann cells also migrate from the two

nerve ends and eventually connecting them through cell cords (bands of Bungner) within the basal lamina tubes. The regenerating axons grow in close contact with these Schwann cell cords as they secrete extracellular molecules, including laminin, collagen, and heparin sulfate proteoglycan, which injured axons recognize as permissive substrates for growth and regeneration (Frostick, Yin et al. 1998). Schwann cells also present cell-adhesion molecules (CAMs) such as N-CAM, Ng-CAM/L1, N-cadherin, and L2/HNK-1 for recognition of proper neuron types. In addition, these cells also secrete neurotrophic factors to aid in the neuronal regenerative process (Hadlock, Sundback et al. 2001).

2.1.3.2 Neurotrophic support in peripheral nerve regeneration

Neurotropism or chemotaxis is meant to illustrate the directional movement of an axon toward a distant source of chemical release. It was discovered by Cajal (Cajal 1928) that subsequent to the cutting of a peroneal nerve of a cat, there was an abundant outgrowth of regenerating axons from the proximal segment which found their way into the distal nerve stump even if the nerve segment was placed out of alignment. Cajal attributed this phenomenon to the chemotactic or neurotropic effect exerted by the neurolemmal cells in the degenerating distal nerve segment. Politis et al (Politis, Ederle et al. 1982) proved in consistent with Cajal's hypothesis that the distal stumps of severed nerves can attract regenerating peripheral nerve axons and that this effect is mediated over distances of several millimeters by diffusible neurotrophic factors. Moreover, it was also proved that the regenerating nerve fibers from a proximal stump of a transected nerve 'preferentially' regenerate toward the original (native) rather than

a different (foreign) distal nerve stump (Politis 1985). A nearly exclusive native preference is observed if the interfaces of the proximal and distal nerve stumps are exposed to each other while if the distal stump tissue is inverted, the extent and frequency of native preference is diminished (Politis 1985). This phenomenon is also explained by tropic guidance from the target organs.

The neurotrophic factors represent a group of soluble molecules with strong influence on survival of neurons and outgrowth of their axons thus, playing a pivotal role in the regeneration of the injured peripheral nerves. Neurotrophic factors are synthesized by the target tissues, and delivered to the neuronal soma via retrograde transport where they exert a trophic and survival effect (Terenghi 1999). The neurotrophin family; nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurtotrophic factor (BDNF), and neurotrophin-4/5 (NT-4/5) have been shown to be required for nerve development and injury repair (Schmidt and Leach 2003). Additionally, other factors such as the ciliary neurotrophic factor (CNTF), glial cell line-derived growth factor (GDNF), and acidic and basic fibroblast growth factor (aFGF, bFGF) have been demonstrated to be beneficial in nerve regeneration (Blesch, Lu et al. 2002). The neurotrophins mediate their effects by binding to high-affinity receptor kinases of the Trk family (Trk A, Trk B, Trk C) or the low affinity receptor p75 (). While the p75 receptor can bind all neurotrophins, NGF binds preferentially to Trk A, BDNF and NT-4 bind to Trk B, and NT-3 binds to Trk C (Tuszynski, Conner et al. 2002).

Nerve growth factor (NGF) is the first and best-characterized nerve-derived factor and considered to be crucial during the regeneration process of pain sensory fibers in the peripheral nerve. NGF is a dimeric 26-kDa neurotrophic factor that supports the survival of embryonic Trk A positive dorsal ganglion neurons and increases the survival of sympathetic and sensory, but not parasympathetic neurons during the critical stage of development (Jones, Oudega et al. 2001). NGF mRNA is normally present at low concentrations in healthy nerves but is upregulated in the distal stump upon injury (Terenghi 1999) where it has been postulated to play an important role in survival of sensory neurons after injury as well as for the outgrowth of regenerating sensory axons (Yin, Kemp et al. 1998). Only small, unmyelinated (pain) nociceptive neurons within the sensory ganglia that bear NGF receptor, Trk A, are affected by NGF. It was proved that injection of adenov-viruses encoding NGF into the dorsal horn of the adult rat spinal cord promoted an extensive sprouting of nociceptive sensory axons throughout the dorsal horn (Romero, Rangappa et al. 2000). In addition to being a neurotrophic factor, NGF also possesses a chemotactic effect on the extension and orientation of growing sensory neuritis (Jones, Oudega et al. 2001). Indeed, a diffusion gradient of NGF can promote directed neurite extension in a two-dimensional assay containing cultured embryonic dorsal root ganglia (DRGs) (Lundborg 2004).

Neurotrophin-3 (NT-3) mRNA is present in significant amounts in adult skeletal muscle, exerting a trophic role for proprioceptive sensory neurons innervating muscle spindles and for motor neurons. The strong trophic effect on muscle sensory afferents is

evident following exogenous administration of NT-3, even in the absence of the target organ (Jones, Oudega et al. 2001). *In-vitro* and *in-vivo* experiments have shown that NT-3 exerts a trophic effect on the Trk C containing proprioceptive and mechanoreceptive sensory neurons (Inoue, Ozaki et al. 2002). *In-vitro* NT-3 administration promotes neurite outgrowth from DRGs which innervate limb muscles (Sterne, Coulton et al. 1997). An enhanced neurite outgrowth from spinal cord explants as well as the increased number of motor end-plates formed in cocultures of neurons and muscles corroborates the trophic action of NT-3 on motor neurons (Braun, Croizat et al. 1996).

Romero et al (unpublished data; Fig 2.3) has demonstrated that an axonal morphological distinction exists between the nociceptive and proprioceptive sensory axons. In a culture medium containing dissociated dorsal root ganglion neurons, an exogenous administration of NGF promoted the growth of long axons with minimal to no branching. Since NGF specifically binds to the Trk A receptors which are exclusively present on the nociceptive sensory axons, it was determined that NGF promoted the growth of the long nociceptive sensory axons showing very few branches. Similarly, an exogenous administration of NT-3 promoted the growth of only short and highly arborized axons in a dissociated dorsal root ganglion neuronal culture. NT-3 binds with a high affinity to the Trk C receptors that are exclusively present on the proprioceptive sensory axons. Thus, it was determined that this highly arborized morphological feature is characteristic of the proprioceptive sensory axons.

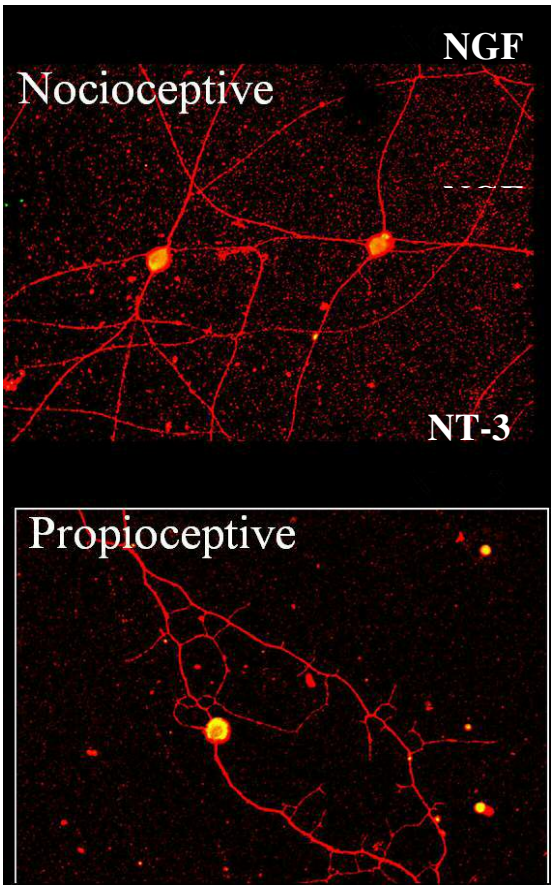


Figure 2.3 Axonal morphological distinctions between the nociceptive and proprioceptive sensory axons: It was demonstrated that an administration of NGF promotes the growth of the long nociceptive sensory axons possessing few branches. In contrast, the administration of NT-3 promotes the growth of proprioceptive sensory axons that possess a highly arborized morphological feature. Adapted from Romero et al, unpublished data.

In addition to NGF and NT-3, NT-4/5, GDNF, and BDNF support the sensory neuron survival and outgrowth. CNTF, on the other hand, promotes motor neuron survival and enhances their regeneration. aFGF and bFGF are strong promoters of angiogenesis and therefore, directly and indirectly, aid in the healing of injured nerves. Similar to NGF, NT-3, and BDNF, bFGF has been associated with increased outgrowth of sensory neurons from the dorsal root ganglion (Schmidt and Leach 2003).

## 2.2 Peripheral Nerve Repair Strategies

Nerve injuries resulting in extensive defects in nerve continuity pose a reconstructive challenge. The current clinical repair method of choice for nerve transaction is the direct end-to-end surgical reconnection, which involves the suturing of individual fascicles and/or the epineurium of the injured nerve. However, this method can only be performed if the nerve stumps are directly adjacent and thus, is pertinent only for small defects or gaps in the nerve (Schmidt and Leach 2003). For longer nerve gaps (greater than or equal to 2 mm), this approach is not desired because any tension introduced into the nerve would inhibit nerve regeneration.

### *2.2.1 Nerve Autografts: The Gold standard Repair method for gap nerve injuries*

For larger nerve defects, an autologous nerve graft harvested from another site in the body is used to span the injury site (Rutkowski and Heath 2002). This currently accepted method has remained the "gold standard" for nerve gap repair during the past three decades and despite creating new possibilities in reconstructive nerve surgery, disadvantages of this include the need for multiple surgeries, donor site morbidity, as well as limited availability. There are a few FDA approved devices only for small nerve defects (several millimeters) including Integra Neurosciences Type I collagen tube (NeuraGen Nerve Guide) (Archibald, Shefner et al. 1995) and SaluMedica's SaluBridge Nerve Cuff (Lundborg, Dahlin et al. 1997).

Allografts and xenografts have also been used since they possess the advantages of large supplies and their use does not require harvest from the patient (Schmidt and Leach 2003). However, these tissues possess the risk of disease transmission and must

be either used in conjunction with immunosuppressants or must be processed to remove immunogenic components.

*2.2.2 Artificial Nerve Grafts used in peripheral nerve repair*

A promising alternative for extending the length over which nerves can successfully regenerate is the artificial nerve graft (also known as the nerve guidance channel). The artificial graft is a synthetic conduit that bridges the gap between the nerve stumps, directing and supporting nerve regeneration by concentrating neurotrophic factors, reducing cellular invasion and scarring of the nerve, and providing directional guidance to prevent neuroma formation or excessive branching (Rutkowski and Heath 2002). An ideal nerve graft or conduit would consist of a scaffold/matrix that can offer contact guidance and a pathway for the regenerating axons. The scaffold could be constructed of a natural or synthetic material and preferentially bioresorbable. In addition, it could be of various topographic natures such as tubes, fibrils, gels, and fibers (Yannas and Hill 2004). The biosynthetic nerve might also contain cells capable of producing growth factors that would provide a permissive physiologic local environment.

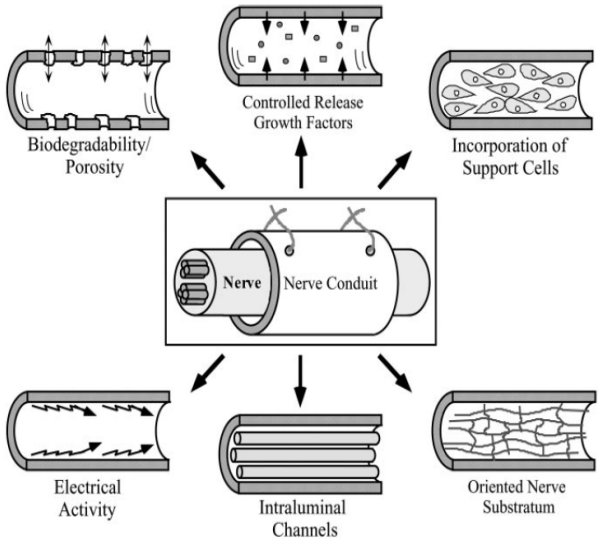


Figure 2.4 Properties of the ideal nerve guidance channel: The desired physical properties of a nerve conduit include: a biodegradable and porous channel wall; the ability to deliver bioactive factors, such as growth factors; the incorporation of support cells; an internal oriented matrix to support cell migration; intraluminal channels to mimic structure of nerve fascicles; and electrical activity. Figure adapted from (Schmidt and Leach 2003).

Synthetic, non-degradable grafts including silicone tubing have been widely used to study neural regeneration. Early studies involving empty or saline-filled silicone tubes showed that nerve repair using these tubings was possible for up to 10mm (in rats) (Lundborg, Dahlin et al. 1982) defects. For larger gaps, little or no regeneration was observed underlying the importance of support cells such as Schwann cells (Lundborg, Dahlin et al. 1982). In later experiments, silicone tubes filled with laminin (Labrador, Buti et al. 1998), fibronectin (Rodriguez, Verdu et al. 2000), and collagen (Verdu, Labrador et al. 2002) showed improved regenerative capacity as compared to empty silicone controls with respect to longer nerve gaps. Nerve graft made of expanded poly (tetrafluoroethylene) or ePTFE (Gore-Tex) have also been successfully employed to

bridge nerve gaps (Miloro, Halkias et al. 2002). Poly (ethylene glycol) (PEG) is another synthetic, non-resorbable material that has been applied to nerve regeneration applications. In a study, PEG (Lore, Hubbell et al. 1999) was used to fuse the severed ends of the rat sciatic nerve while re-establishing functional and morphological continuity. However, this technique could be applied only if the severed nerve ends are directly adjacent rendering it ineffective for larger nerve defects. A major disadvantage of these non-degradable grafts is that they remain *in situ* as foreign objects and this leads to long-term complications including fibrosis and chronic nerve compression. Thus, research is now focused more on degradable nerve grafts for improved nerve regenerative capacity over longer nerve gaps.

Nerve grafts made of bioresorbable materials have demonstrated excellent biocompatibility and biodegradability (Sundback, Hadlock et al. 2003). In addition to providing a structural support for the regenerating nerve fibers, they also degrade over time providing space for the growing axons. These degradable nerve conduits also preclude the detrimental effects of the synthetic, non-degradable grafts mentioned previously. While several studies have shown good acceptance of these implants by the body, a few have reported mild inflammatory reactions (Hadlock, Sundback et al. 2000) attributed to the increased acidity caused by the degradation process. Poly (esters) such as poly (glycolic acid) (PGA), poly (lactic acid) (PLA), and poly (lactic-co-glycolic acid) (PLGA) were some of the first synthetic biodegradable polymers researched in nerve regeneration studies (Schmidt and Leach 2003). Their advantages included ease of availability and processing, biodegradation characteristics, and FDA approval. To

date, extensive studies involving these materials have been performed to enhance their potential as nerve conduits with additional modifications including processing them into foams (Evans, Brandt et al. 2000) and seeding them with Schwann cells (Evans, Brandt et al. 2002). Tubes manufactured from other biodegradable poly (esters), such as poly (caprolactones) have also demonstrated promising results in peripheral nerve regeneration models. Poly (DL-lactide-ε-caprolactone) nerve guides were able to bridge 15mm defects in the rat sciatic nerve with partial recovery of motor function (Meek, van der Werff et al. 2003). Poly (urethane) (Soldani, Varelli et al. 1998) and poly (organo phosphazene) (Nicoli Aldini, Fini et al. 2000)are example of other resorbable materials that have been successfully used to manufacture synthetic nerve grafts for peripheral nerve repair. Several limitations of the bioresorbable nerve grafts include poor functional recovery due to the persistent innervation of aberrant targets and their expensive and difficult manufacturing procedures.

*2.2.3 Neurotrophin-assisted peripheral nerve regeneration*

The neurotrophic factors specifically influence neuronal activity by promoting development and maturation during embryonic life, and by sustaining maintenance during adult life and regeneration after injury (Terenghi 1999). Following peripheral nerve injury and axotomy, there is a disruption in the supply of retrogradely transported neurotrophic factors, leading to neuronal cell death and lack of regeneration. This process can be reversed if the neurons regenerate back to peripheral targets, indicating the dependence on target-derived neurotrophic factors. Hence, it seems feasible that the

addition of exogenous trophic factors to the micro-environment of the injured nerve might produce results similar to those of target innervation (Terenghi 1999).

A variety of techniques to deliver neurotrophic factors to the injured peripheral nervous system have been established, including mini-osmotic pumps. The mini-osmotic pumps have proven useful for the continuous delivery of neurotrophins with controlled concentration over a well-defined time period. These pumps operate due to an osmotic pressure difference and a variety of neurotrophins including NGF (Karchewski, Gratto et al. 2002), NT-3 (Young, Miller et al. 2001), GDNF (Gordon, Sulaiman et al. 2003) and BDNF (Boyd and Gordon 2003) have been used with the mini-osmotic pumps to examine the exogenous effects of these proteins in the peripheral nerve injuries.

In order to avoid the implant of pumps and second surgery associated with their removal, release of neurotrophic factors have been designed to occur as polymer degradation or by diffusion through pores in the polymer matrix. Microspheres, with diameters in the range of 1μm to 1mm, have been used as a less invasive technique (through injections) for controlled neurotrophin delivery in the peripheral nerve repair. Moreover, neurotrophin-loaded biodegradable microspheres have also been loaded in nerve grafts to allow for a prolonged, site-specific delivery. Growth factors encapsulated in chitosan, alginate, poly (lactic acid), poly (glycolic acid), poly (lactic-co-glycolic acid), and poly (caprolactone) have been widely used to enhance regeneration across a nerve gap (Schmidt and Leach 2003). However, a major disadvantage of the degradable microspheres is that the release profile of the

neurotrophins is characterized by a marked initial burst followed by a slow, continuous release and thus, the subsequent release of the growth factor could be low in relation to initial burst (Xu, Yee et al. 2003). This is in contrast to the desired slow, continuous release profile for a prolonged period of time.

Despite a controlled release by polymer matrices and polymer-based microspheres, only a finite reservoir of active neurotrophic agents is possible with this approach. Thus, gene therapy is being explored as a favorable alternative for the long-term expression of neurotrophic or regenerative factors within the peripheral nervous system subsequent to an injury. A number of viral vectors including herpes simplex virus, adenovirus and adeno-associated virus have been employed in the neuroregenerative field since they are capable of inducing high levels of gene expression from the vector (Smith and Romero 1999). However, the viral gene delivery techniques pose risks such as inflammation and severe immune response. Since the controlled delivery of active neurotrophic factors is difficult for the period of time that spans the entire regeneration process, transplanted genetically modified cells overexpressing one or more neurotrophins is a feasible methodology allowing their continuous supply. It has been reported that the continued expression of neurotrophic factors by the transduced Schwann cells do not allow the axons to leave the permissive milieu provided by these growth factors consequently, resulting in a failure to exit the graft and re-innervate the intended targets (Blesch, Conner et al. 2001). An inducible gene expression in these transfected cells would allow a significant control over neuronal survival and axonal growth (Blesch, Lu et al. 2002) by facilitating the axonal

exit from the grafts due to the cascade of neurotrophin expression. Genetically modified fibroblasts overexpressing NGF, NT-3, BDNF, CNTF, and GDNF have been widely used either in cell grafts or incorporated in nerve grafts, biodegradable or otherwise for enhancing the repair process (Schmidt and Leach 2003). To control the genetically modified expression of neurotrophins in fibroblasts, methods employed include Muristerone A- inducible expression (Patrick, Zheng et al. 2001) and tetracycline-responsive promoters (Blesch, Conner et al. 2001). Similarly, Schwann cells have also been genetically transduced to over-secrete particular neurotrophic factors including NGF, NT-3, and BDNF and these modified cells have also been utilized either in Schwann cell grafts or placed inside nerve guidance conduits (Schmidt and Leach 2003). Although a majority of studies involving neurotrophin-expressing genetically modified Schwann cells, fibroblasts or other cells have been only used in the spinal cord repair model; the utility of this delivery system for augmenting the regenerative process in the peripheral nerve cannot be underestimated.

However, despite all the recent advances in the neuroregenerative field and the intrinsic capacity of the peripheral nerves to demonstrate spontaneous regeneration, the functional recovery after simple tubularization nerve repair methods remain suboptimal as opposed to the autologous nerve grafts widely accepted as the gold standard in nerve repair. This vital limitation has been attributed to the inappropriate regeneration pathways taken by the regenerating axons through and distal to the nerve graft leading to a compromised functional recovery when the regenerating axons innervate aberrant targets and make dysfunctional connections.

## 2.3 Axonal guidance by transparent multiluminal hydrogels

Axonal regeneration and functional recovery might be enhanced if the axonal growth inside the guidance tube could be directed in a desired longitudinally organized direction. Lundborg et al. (Lundborg 2004) pioneered this concept when successful axonal regeneration was reported across 10 to 15mm gaps by using bridging filaments made from bioresorbable sutures. Since then, several *in-vitro* studies have been conducted using this theory, including using laminin-coated PLLA filaments to induce directional orientation to the neurite outgrowth from the dorsal root ganglia (DRG) explant cultures (Rangappa, Romero et al. 2000). In addition, magnetic fields have also been employed in *in-vitro* experiments to align fibrin and collagen matrices in order to promote and direct axonal growth in a specified direction (Dubey, Letourneau et al. 2001). Novel bioresorbable polymer scaffolds containing multiple longitudinally aligned internal channels have been designed to better replicate the natural microanatomy of the peripheral nerves (Sundback, Hadlock et al. 2003).However, the major shortcomings of the current methods of multiluminal nerve repair are the advanced and complicated fabrication techniques in addition to the demonstrated incompleteness or absence of functional reinnervation.

### *2.3.1 The TSRH Biosynthetic Nerve Implant*

In order to linearly direct axonal and cell growth within an *in-vitro* three-dimensional, multi-conduit matrix, a transparent multi-luminal hydrogel matrix (TMM) gel was developed Romero and colleagues (Abstract, Galvan-Garcia, Romero et al.). This system can be customized to specifically arrange conduits in multiple geometric

configurations, with variable lengths and internal diameters. A novel casting device was also fabricated to reproducibly prepare these multiluminal hydrogel matrices wherein the hydrogel micro-channel casting and the loading of intraluminal material such as collagen alone or collagen/cells mixture is simultaneously achieved. In addition to the directed axonal growth, multiple advantages of this TMM three-dimensional matrix casting system include sequential seeding of multiple cell types as well as cell compartmentalization and cell co-culture in a three-dimensional matrix.

To translate the successful directed nerve growth within the TMM micro-channels into an *in-vivo* model, a cylindrical multiluminal matrix was developed by Romero and colleagues (Abstract, Galvan-Garcia, Romero et al.) for peripheral nerve regeneration studies aimed at facilitating axonal regeneration and resembling the fascicular structure of a normal nerve. Experiments have been conducted using these hydrogel-based biosynthetic nerve implants (BNIs) encased in micro-Renathane tubings and containing either 7 or 14 250μm channels in a rat sciatic nerve injury model. For a reproducible preparation of the BNIs, a novel casting device was developed allowing controlled deployment of collagen and/or desired cells in suspension within the micro-channels. Results of the *in-vivo* experiments exhibited high axonal density and fascicular-like nerve growth throughout the length of the BNI that included multiple nerve cables occupying all the available area within each of the 7 or 14 available micro-channels. This finding is in sharp contrast to the singular nerve cable normally associated with the conventional nerve grafts. Moreover, substantial vascularization of

the nerve cables was also evident with only mild fibrosis observed, thus proving the biocompatibility of the implant.

## 2.4 Hypothesis

Despite the intrinsic capacity of peripheral nerves for spontaneous regeneration and advances made in peripheral nerve gap repair, the functional outcome after repair remains disappointing. This is due to the fact that the regenerating axons take erroneous pathways leading to the innervation of aberrant targets and development of dysfunctional connections. A critical factor contributing to these erroneous pathways taken by the regenerating axons is the presence of mixed nerve fibers consisting of both motor and sensory axons, within and distal to the nerve grafts. We hypothesize that segregation of axonal subtypes can vastly improve axonal regeneration and functional recovery after neurotomy.

In this study, while enticing segregation of sensory axonal subtypes, four different aspects of axonal growth and direction were analyzed including: i) a three-dimensional, linear axonal growth was examined using the existing TMM assay, ii) novel, two-dimensional and three-dimensional physical guidance models were developed to direct axonal growth in specified directions to aid in the segregation studies, iii) specific neurotrophic factors including NGF and NT-3 were employed to attract specific axonal types, and iv) multiple neurotrophin delivery methods were examined including passive diffusion, neurotrophin-coated latex beads, genetically modified Schwann cells, and mini-osmotic pumps. We demonstrate that using a novel three-dimensional assay and an appropriate delivery system for the controlled release of

neurotrophins; namely NGF and NT-3, it is possible to entice the *in-vitro* segregation of nociceptive and prpriocetpive sensory axons, respectively.

# CHAPTER 3

# TWO-DIMENSIONAL IN-VITRO ASSAYS FOR TESTING AXONAL GUIDANCE AND SEGREGATION OF SENSORY AXONAL SUBTYPES

## 3.1 In-vitro two-dimensional drop assay

*In-vitro* cultures of neurons, whether in a two-dimensional or three-dimensional assay, exhibit a radial axonal growth. A two-dimensional assay was designed to test if specific neurotrophic gradients could guide the growth of axons in a specific direction with the aim of segregating the sensory axonal subtypes. This assay consisted of a couple of small gelfoam pieces soaked in a desired neurotrophin solution, the release of which would present a gradient to the sensory axons growing from a dorsal root ganglion explant embedded in an ECM- drop (Fig 3.1A).

A couple of small gelfoam pieces (15 mm X 15 mm) were soaked in the desired neurotrophin solutions (mNGF, 100ng/ml; Sigma, St. Louis, MO; hNT-3, 5ng/ml; Sigma, St. Louis, MO) and embedded in a drop of growth factor reduced Matrigel Matrix (ECM-; BD Biosciences, San Jose, CA) in a 35-mm cell culture dish. This ECM- drop was allowed to polymerize in the 5% CO2 incubator for 5 minutes. Neonate mice (P2) DRGs were isolated as per described protocols and placed adjacent to the neurotrophin-soaked ECM- drops (Fig 3.1A). After polymerization, Neurobasal culture medium (Invitrogen, Carlsband, CA) supplemented with L-glutamine (Gibco Invitrogen, Carlsband, CA), B-27 supplement (Invitrogen, Carlsband, CA), and Penicillin/Streptomycin (Gibco Invitrogen, Carlsband, CA) was added. The cultures

were then cultured for 3 days, subsequent to which, fixing and staining protocols were performed.

A distinction in axonal morphology was considered during examination of axons growing towards the neurotrophin-soaked gelfoams. As shown in figure 3.1C, there is a significant axonal outgrowth from the DRG towards the gelfoam pieces soaked in NGF and NT-3 after 3 days in culture. Axons growing toward the NGF soaked gelfoam were longer and showed few to no branches (arrows in Fig 3.1D). This is consistent with the morphology of the nociceptive sensory axons. In contrast, axons growing toward the NT-3 soaked gelfoam were short and showed an increased number of branches per axon (arrows in Fig 3.1C). The short and arborized morphological feature of the NT-3 attracted axons is consistent with the morphology of the proprioceptive sensory axons. Although a clear distinction could be made based on axonal morpohology, a number of morphologically mixed axons were also observed.

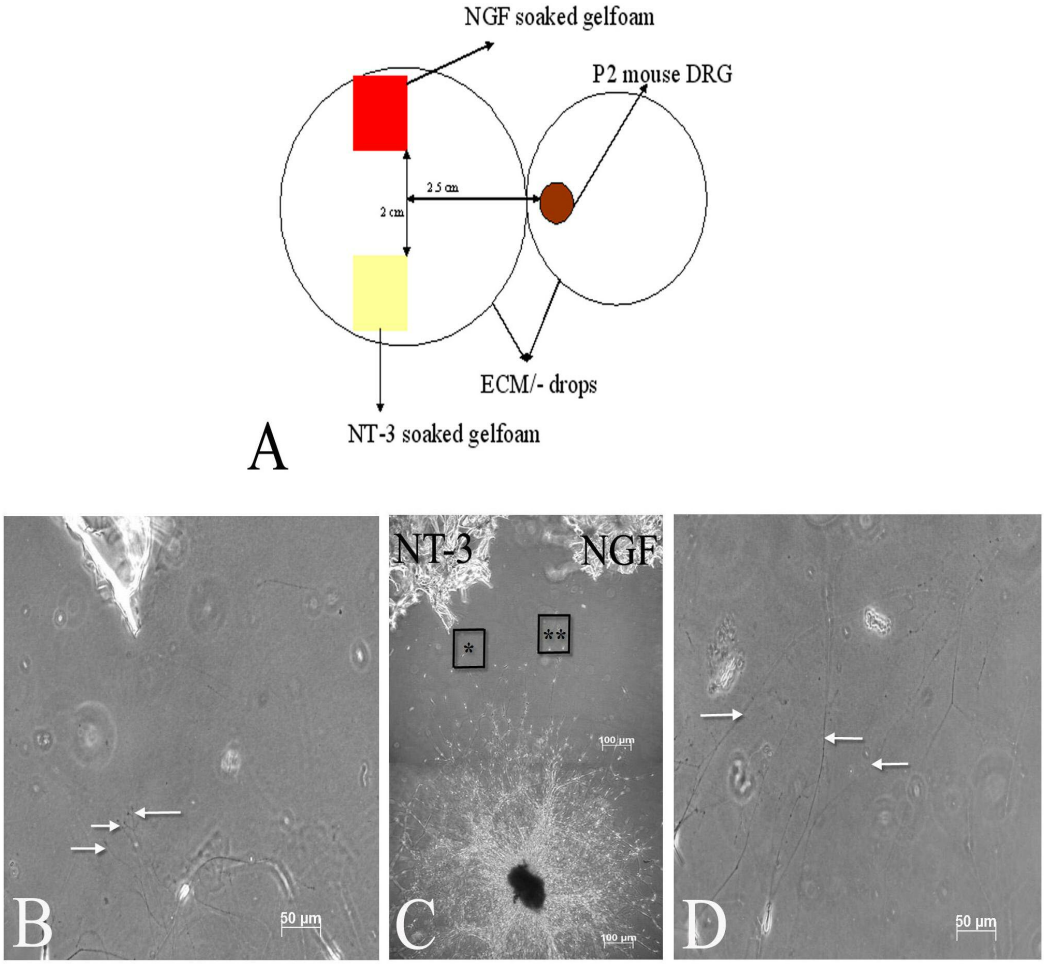


Figure 3.1 In-vitro two-dimensional drop assay for testing axonal guidance and segregation: A. Schematic representation of the assay. C. Light micrograph depicting the axonal outgrowth towards the NGF and NT-3 soaked gelfoams after 3 days in culture. B & D. Higher magnification pictures of the axonal growth in areas depicted by * and ** respectively in Fig 3.1C. A distinct morphological difference was observed between some axons growing toward the NGF and NT-3 soaked gelfoams. Magification: 5X (C), 20X (A, B).

## 3.2 In-vitro two-dimensional Y- PDMS assay

A novel two-dimensional Y-shaped assay was developed to prove the principle that mixed sensory fibers from a dorsal root ganglion explant can be segregated into distinct channels using appropriate neurotrophic factors. This assay would further allow

a more potent formation of specific neurotrophic gradients within the two distinct channels of the Y-assay while simultaneously minimizing any cross-talk between the gradients. This assay was fabricated from a poly dimethylsiloxane (PDMS) mold and Fig 3.2A illustrates a schematic representation of this Y-shaped assay that incorporates areas for placing gelfoam pieces soaked with particular neurotrophic factors. The DRG explant would be placed close to the bifurcation point to allow the axons to respond to the neurotrophins diffused from the gelfoams and enter the respective arm of the Y according to the preferred neurotrophins. This assay was created using curing agent (Sylgard 184® Silicone Elastomer Base, Dow Corning Corporation, Midland, MI) and elastomer base (Sylgard 184® Silicone Elastomer Curing agent, Dow Corning Corporation, Midland, MI) at a ratio of 1:10 to form the PDMS mold. The entire mold contained three Y-shaped assays to allow the experiments to be performed in triplicates.

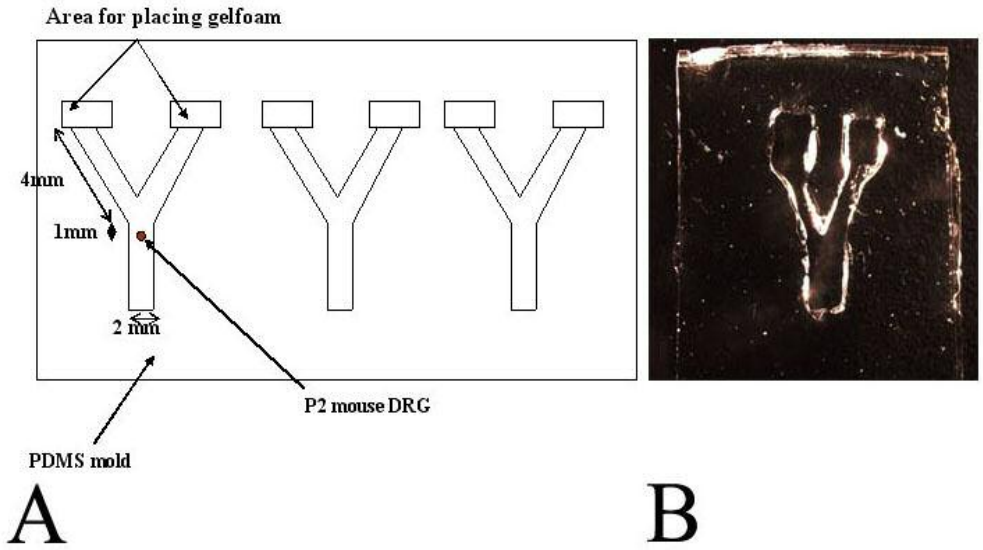


Figure 3.2 Two-dimensional Y-shaped PDMS assay for axonal segregation: A. Schematic representation of the two-dimensional Y-shaped assay fabricated from PDMS mold. B. Light micrograph of the Y-shaped PDMS mold.

*3.2.1 Demonstration of the gradient formation using the Y-PDMS assay*

Fluorescin solution (Molecular Probes, Eugene, OR) was used to test the gradient formation within the PDMS Y-shaped assay in addition to determining the amount of time for the solution to spill over and dilute in the media. A mold containing a single Y-shaped assay was placed in a 35-mm culture dish and sterilized by washing in 70% ethanol and distilled water before drying under UV-light. A couple of small gelfoam pieces (15 mm X 15 mm ) were carefully cut with the aid of a scalpel and soaked in 4µl of the fluorescin solution (in methanol) or 1X PBS and placed in the gelfoam placement areas in the mold. Subsequently, 3 ml of 1X PBS was added to the culture dish and the gradient formation of the fluorescin solution was analyzed 30 minutes later. Fluorescent micrograph pictures were taken at 5 minute intervals for the initial 30 minutes and the gradient preservation was analyzed again after 24 hours.

Fig 3.3B is a light micrograph of the Y-shaped PDMS mold used for the purpose of this experiment. Fig 3.3A is a fluorescent micrograph reconstruction of the fluorescin gradient formation in the boxed area indicated by * in Fig 3.3B 30 minutes subsequent to the media addition. This is the arm of the Y-assay corresponding to the placement area containing the fluorescin-soaked gelfoam. Fig 3.3C is a fluorescent micrograph reconstruction of the boxed area depicted by ** in Fig 3.3B 30 minutes subsequent to the media addition. This is the arm of the Y-assay corresponding to the placement area containing the gelfoam soaked in 1X PBS serving as the control. It is evident that a distinct fluorescin gradient had begun to form by passive diffusion up till the bifurcation point merely 30 minutes after the media addition and there was no spill-

over of the fluorescin solution to the control arm containing 1X PBS. Fig 3.3D is a graph plotted using the optical densitometry measurements at 30 minutes after the media addition. It recorded the intensity of the fluorescin in the boxed area indicated by * in Fig 3.3B. There is an evident progressive gradient formation of the fluorescin within the confines of the arm with no spill-over into the control arm of the Y-assay as is indicated by the fluorescin intensity graph in Fig 3.3E. A dilution of the gradient in the media was observed after 24 hours indicating a distinct gradient is preserved in this assay for this time period.

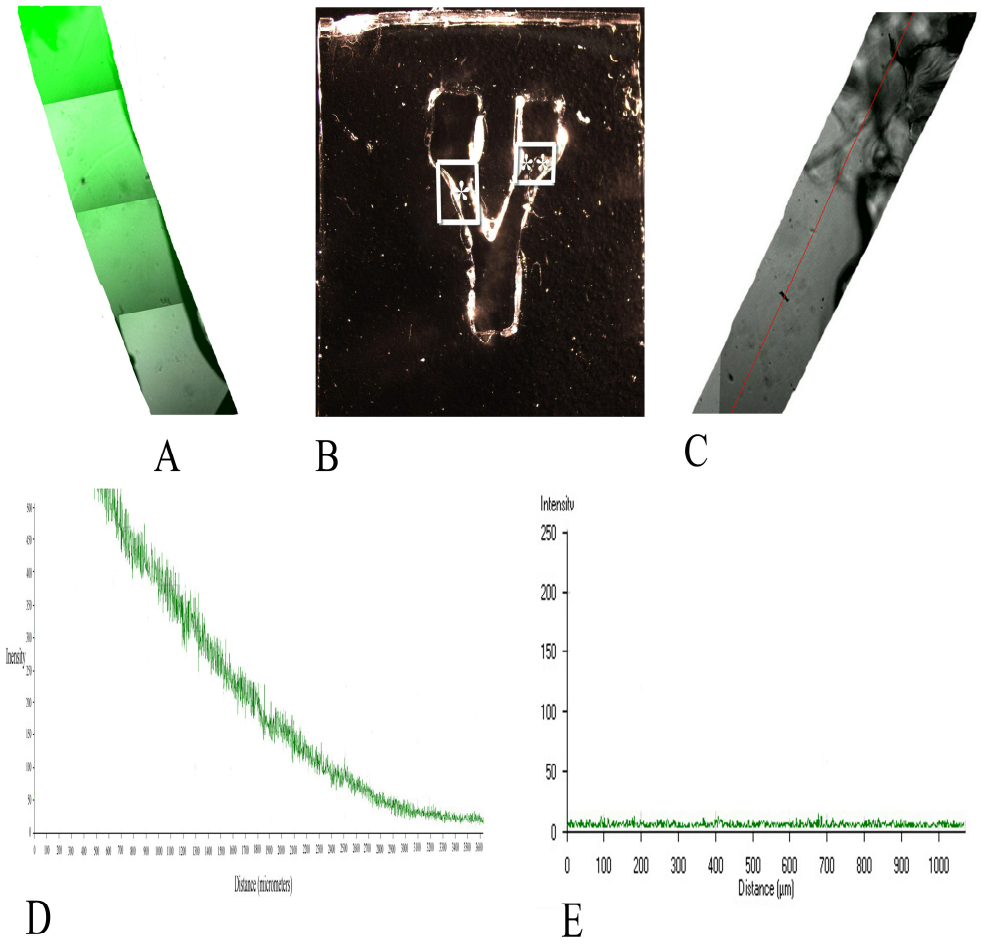


Fig 3.3 Gradient formation and preservation in the Y-shaped PDMS assay: B. Light micrograph of the Y-shaped PDMS mold used for the experiment. A. Fluorescent micrograph reconstruction of the fluorescin gradient formation in the boxed area depicted by * in Fig 3.3B at 30 minutes after media addition. This arm corresponds to the placement area containing the gelfoam soaked in fluorescin solution. C. Fluorescent micrograph reconstruction of the boxed area depicted by ** in Fig 3.3B at 30 minutes after media addition. This arm corresponds to the placement area containing 1X PBS-soaked gelfoam (Control). An evident gradient formation of the fluorescin solution within the confines of the arm with no spill-over into the control arm is demonstrated. D. Fluorescin intensity graph using optical densitometry measurements of the boxed area depicted by * in Fig 3.3B at 30 minutes after media addition. An evident gradient formation of the fluorescin is further demonstrated wit the aid of this graph. E. Fluorescin intensity graph of the boxed area depicted by ** in Fig 3.3B at 30 minutes after media addition. Negligible intensity evident in the graph suggests no spillover of the fluorescin into the Control arm.

*3.2.2 Axonal growth in Y-PDMS assay using neurotrophin-soaked gelfoams*

The molds containing the three Y-shaped assays were placed in 100 mm cell culture dishes and sterilized by washing in 70% ethanol and distilled water before drying under UV-light. The base of the Y-areas in each mold was coated with a thin film of ECM- to facilitate the axonal outgrowth from the DRG explants. Neonate mice (P2) DRGs were isolated as per the described protocols and placed at 1 mm from the bifurcation point in each assay. The minimal media method was employed to eliminate the need of anchoring the DRG using an ECM- drop. This method included the addition of small media volume, sufficient for the DRG to survive for 24 hours. The 24 hour time period would be sufficient for the migration of Schwann cells out of the DRG explant and extending processes in the interim, thus, anchoring the DRG without the need for any external substrate. Additional media was added after that. Also, neurotrophin-soaked gelfoam pieces were added in the assay 24 hours subsequent to the placement of the DRG explant. According to the results obtained with the fluorescin gradient formation, we believed that if the neurotrophin-soaked gelfoams are added at least a day after the DRG explant placement, a distinct neurotrophic gradient would be available for the axonal fibers to respond to. Small gelfoam pieces (15 mm X 15 mm) were carefully cut with the aid of a scalpel and soaked in 4µl of desired neurotrophin solutions, (mNGF, 100ng/ml; Sigma,St. Louis, MO, hNT-3 5ng/ml; Sigma, St. Louis, MO). For the control treatment, the gelfoam piece was soaked in Saline. Table 3.1 lists the different treatment groups employed for this assay. In each assay, as per the treatment group, the gelfoam pieces soaked in a particular neurotrophin treatment were

placed in the gelfoam placement areas and encapsulated in ECM- drops to slow the neurotrophin release subsequent to the addition of the media. The culture medium utilized is described in Chapter 7. Established fixing and staining protocols were performed on all the cultures.

Table 3.1 List of the different treatment groups used in the Y-shaped PDMS assay

| **Group Number** | **Treatments used in Group** | |
|---|---|---|
| Group 1 | Saline (Control) – Saline (Control) | (n = 3) |
| Group 2 | Saline (Control) – NGF | ( n = 3) |
| Group 3 | Saline (Control) – NT-3 | ( n = 3) |
| Group 4 | NGF – NT-3 | ( n = 3) |

In all the groups tested, axons were able to enter the two arms of the Y proving that the axons in an *in-vitro* two-dimensional culture can be guided into distinct chambers. Evaluation of all the control groups was performed with the PDMS Y-assay to enable a greater understanding of the morphological differences amongst the sensory axonal subtypes attracted towards NGF or NT-3. In Group 1 containing only Saline-soaked gelfoams, a radial outgrowth of axons was observed with no detectable distinction in axonal morphology amongst the axons that grew towards both the gelfoam pieces (Figs 3.4A and B). While examining the axonal outgrowth in Group 2, it was evident that a large number of axons growing attracted toward NGF were long and contained only few to no branches; a characteristic trait prevalent among the nociceptive sensory axons (Figs 3.4C and D). In contrast, a large number of axons

growing toward the NT-3 soaked gelfoam in Group 3 were short and highly arborized signifying the morphological feature of the proprioceptive sensory axons (Figs 3.4E and F). Having determined that a separate delivery of NGF or NT-3 can indeed promote the growth of either nociceptive or proprioceptive sensory fibers, axonal growth in Group 4 was evaluated to test whether the simultaneous presentation of both the neurotrophins would segregate these axons from the same ganglia in a two-dimensional assay. Fig 3.5A is a light micrograph of the axonal outgrowth from the DRG towards the NGF and NT-3 soaked gelfoams. It was indeed evident that a majority of the axons growing toward the NGF containing gelfoam were long and possessed minimal branching (Fig 3.5a), if any, whereas the NT-3 attracted axons were highly arborized with a comparatively shorter length (Fig 3.5b).

To further corroborate that these NGF and NT-3 attracted sensory axons are functionally different, dual immunocytochemistry labeling was performed. The culture was incubated with monoclonal mouse Beta-tubulin as the primary antibody and green fluorescently labeled Cy2 Donkey anti-mouse secondary antibody. The beta-tubulin is a neuronal marker and labels all the axons, irrespective of their type and the staining result is visualized in Figure 3.5B**.** The culture was also stained for CGRP-positive nociceptive sensory axons using rabbit CGRP as the primary antibody and red fluorescently labeled Cy3 Donkey anti-rabbit secondary antibody. As is evident from Figure 3.5C**,** the red CGRP-positive pain/nociceptive fibers are predominantly attracted towards NGF. Figure 3.5D is a merged image of the green beta-tubulin positive mixed sensory axons and the red CGRP-positive nociceptive sensory axons. While a co-

localization of the red and green axons is detected in the area populated by the nociceptive axons attracted towards NGF, co-localization is non-existent in the area populated by the CGRP-negative proprioceptive axons attracted towards NT-3 thus; conclusively proving a segregation of the functionally different sensory axonal subtypes is attainable in a two-dimensional system.

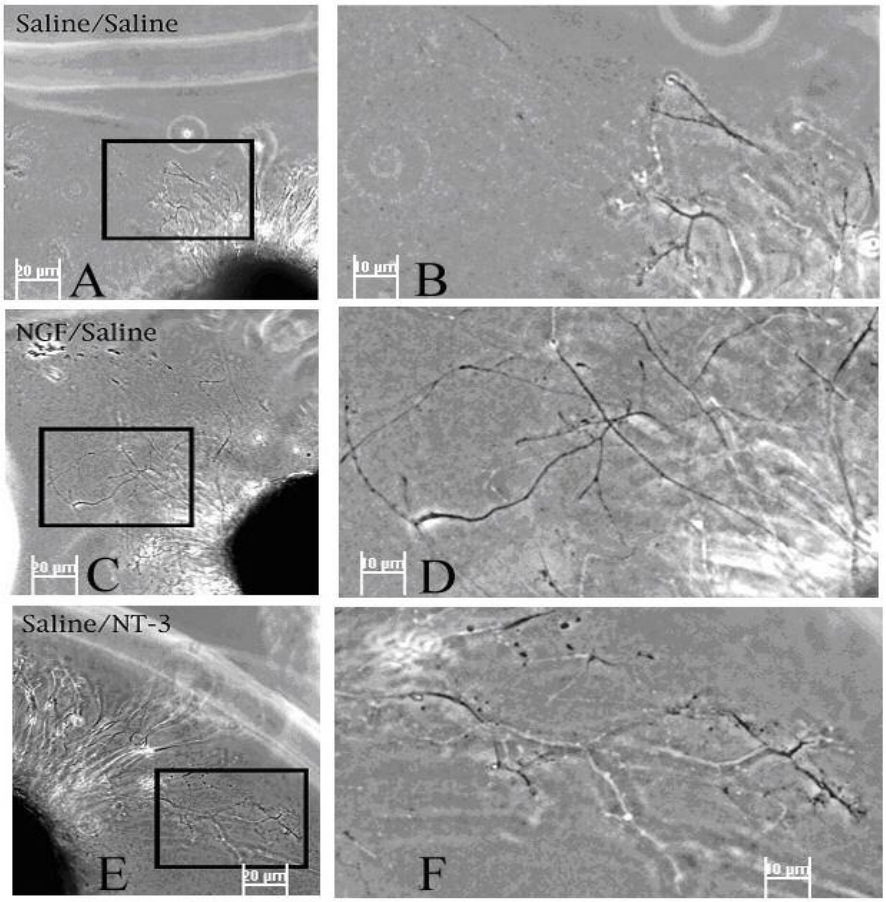


Figure 3.4 Axonal growth in the different control groups using the PDMS Y-assay: A. Light micrograph of the axonal outgrowth from the DRG in Group 1 where each of the gelfoam pieces was soaked in Saline. B. Higher magnification picture of the highlighted area in Fig 3.4A. No distinction in the axonal morphology could be concluded amongst the axons that grew towards both the gelfoam pieces. C. Light micrograph of the axonal outgrowth from the DRG in the PDMS Y-assay in Group 2 where one gelfoam was soaked in NGF (100ng/ml concentration) and the other with Saline. D. Higher magnification picture of the highlighted area in Fig 3.4C. While examining the axonal outgrowth, it was evident that the axons growing towards the NGF-soaked gelfoam were long and contained very little to no branches; a characteristic trait prevalent amongst the nociceptive sensory axons. E. Light micrograph of the DRG axonal outgrowth in the PDMS Y-assay in Group 3 where one gelfoam was soaked in NT-3 (5ng/ml) and the other with Saline. F. Higher magnification picture of the highlighted area in Fig 3.4E. While the axons growing towards the Saline-soaked gelfoam were mixed morphologically, the axons attracted towards NT-3 were short and highly arborized exhibiting an anatomical feature of the proprioceptive sensory axons. Magnification: 5X (A, C, E), 20X (B, D, F).

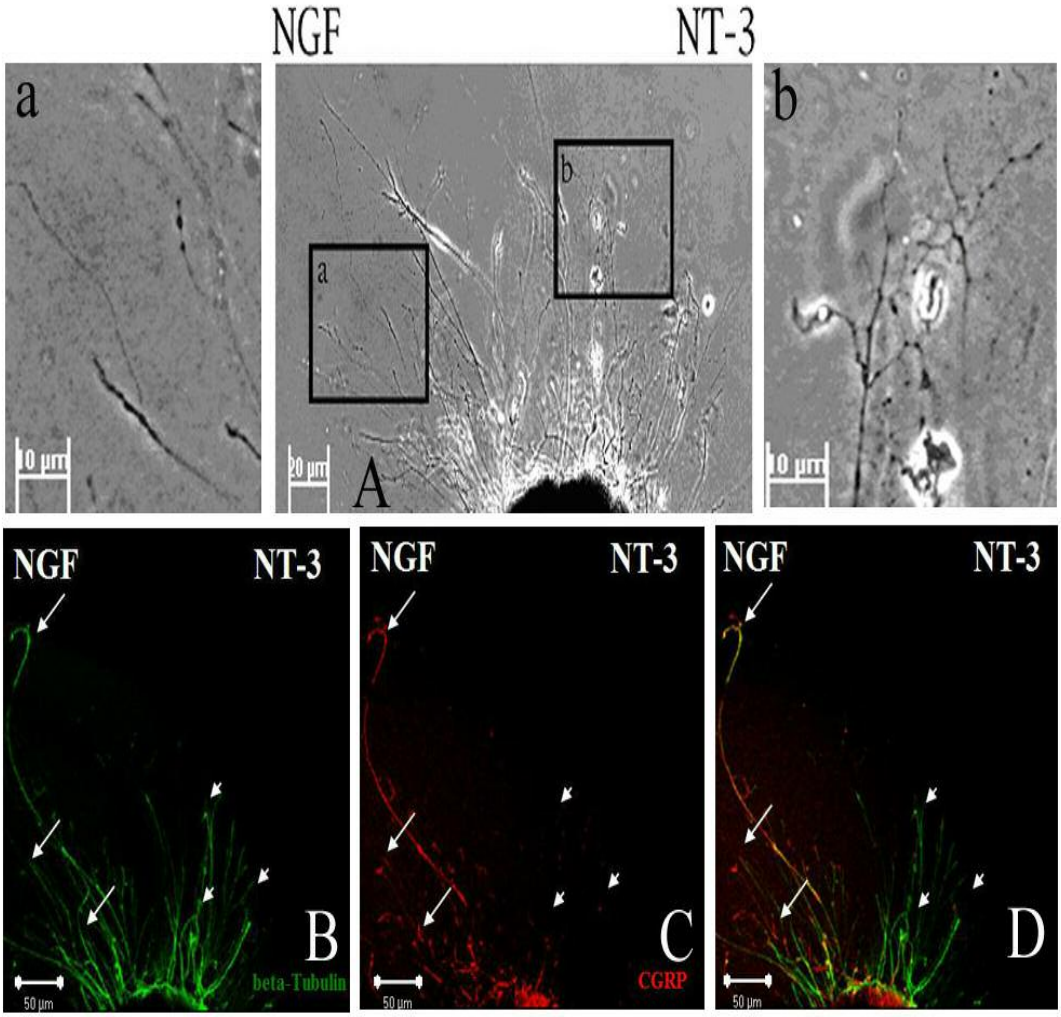


Figure 3.5 Axonal segregation in the PDMS Y-assay using NGF and NT-3: The same PDMS Y-shaped assay included both NGF and NT-3 soaked gelfoams and axonal outgrowth was evaluated three days subsequent to the placement of the DRG. A. Light micrograph of the axons growing towards the NGF and NT-3 soaked gelfoams. a and b depict areas at higher magnification represented in Fig 3.5A indicating a distinct morphological difference between the NGF- and NT-3-attracted sensory axons emanating from the DRG. B. Immunocytochemical visualization of all the axons using beta-tubulin (green) as an axonal marker. C. Immunocytochemical visualization of only the CGRP positive (red) nociceptive sensory axons growing only toward NGF. D. Merged immunoctyochemical visualization of the beta-tubulin positive mixed sensory axons and CGRP-positive nociceptive sensory axons. A co-localization of the red and green axons is detected in the area populated by the nociceptive axons attracted towards NGF and co-localization is non-existent in the area populated by the CGRP-negative proprioceptive axons attracted towards NT-3. Magnification: 5X (A), 20X (a, b).

# CHAPTER 4

# IN-VITRO THREE DIMENSIONAL ASSAYS FOR DIRECTING AXONAL GROWTH

## 4.1 Transparent Multi-luminal Matrix (TMM) assay

Having achieved axonal segregation of sensory neurons in a two-dimensional assay, it was decided to direct axonal growth in a three-dimensional to better mimic the nerve growth in the nervous system. To that end, it was decided to use the existing transparent multi-luminal hydrogel matrix (TMM) developed by Romero and colleagues (Abstract, Romero et al). Genetically modified Schwann cells over-expressing a particular neurotrophin (provided by the University of Miami) were decided to be the neurotrophin delivery system to be tested to direct and segregate the sensory axonal subtypes.

### *4.1.1 TMM hydrogel preparation and cell loading within micro-channels*

The efficacy of the cell loading within the micro-channels of the transparent multichannel hydrogel matrix (TMM) gels was tested by using the GFP-expressing Schwann cells (provided by the University of Miami). Figure 4.1 depicts a schematic representation of the method employed to load the hydrogel micro-channel casting and the loading of the intra-luminal material using the TMM casting device.

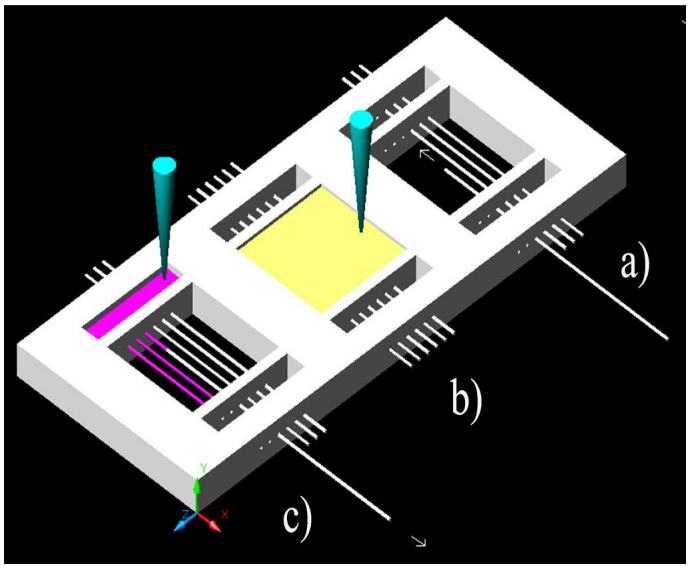


Figure 4.1 Schematic representation of the TMM gel casting including cell seeding method: a) alignment of the fibers in casting device, b) agarose polymerization c) cell loading in the micro-conduits.

A rectangular casting frame manufactured from dental cement (12.5 mm X 36 mm) for casting gels in triplicate was employed to guide multiple plastic polymer fibers (0.25 mm X 17 mm) through perforations (0.26 mm diameter) at both ends of the device (Fig 4.1). The sterilization procedure included washing the device in 70% ethanol (15 minutes), rinsing in distilled three times and allowing to air dry initially followed by UV light drying. After placing the TMM casting device containing the inserted polymer fibers in a cell culture plate, 1.5% ultrapure agarose (Invitrogen, Carlsbad, CA) in 1X phosphate buffered saline (Fisher Scientific, Fairlawn, NJ) was allowed to polymerize over the fibers in the central matrix casting well. A suspension of Matrigel Matrix (ECM, BD Biosciences, San Jose, CA), at a 1:1 ratio with P-3 GFP-expressing Schwann cells (4 X $10^6$cells/ml) was added into the cell suspension well and

subsequently loaded into the lumen of the multi-channel hydrogel due to the negative pressure created when the plastic polymer fibers were extracted from the polymerized agarose gel. Immediately after cell loading, the device was placed in the incubator (5% CO2) for 5 minutes to allow the suspension to polymerize within the conduits before its placement in D-10 mitogen culture media. The assay was cultured for 7 days with periodic media change and fluorescent as well as light micrograph pictures were taken after 24 hours in culture to determine the efficacy of the cell survival within the micro-channels.

Each of the six micro-channels of the TMM hydrogel was homogenously seeded with the GFP-expressing Schwann cells/ECM mix. The cells in each micro-channel proliferated within the matrix, and formed three-dimensional cellular cables within 24 hours. A majority of the seeded Schwann cells survived for the evaluation period consisting of 1 week. Figure 4.2 shows the fluorescent (A, D, G), phase contrast (B, E, H) and overlay micrographs (C, F, I) of the seeded GFP-expressing Schwann cells within the TMM micro-channels at various magnifications 24 hours after seeding. Figures 4.2 G-I illustrate the three-dimensional cellular cables formed only 24 hours after cell loading within micro-channels.

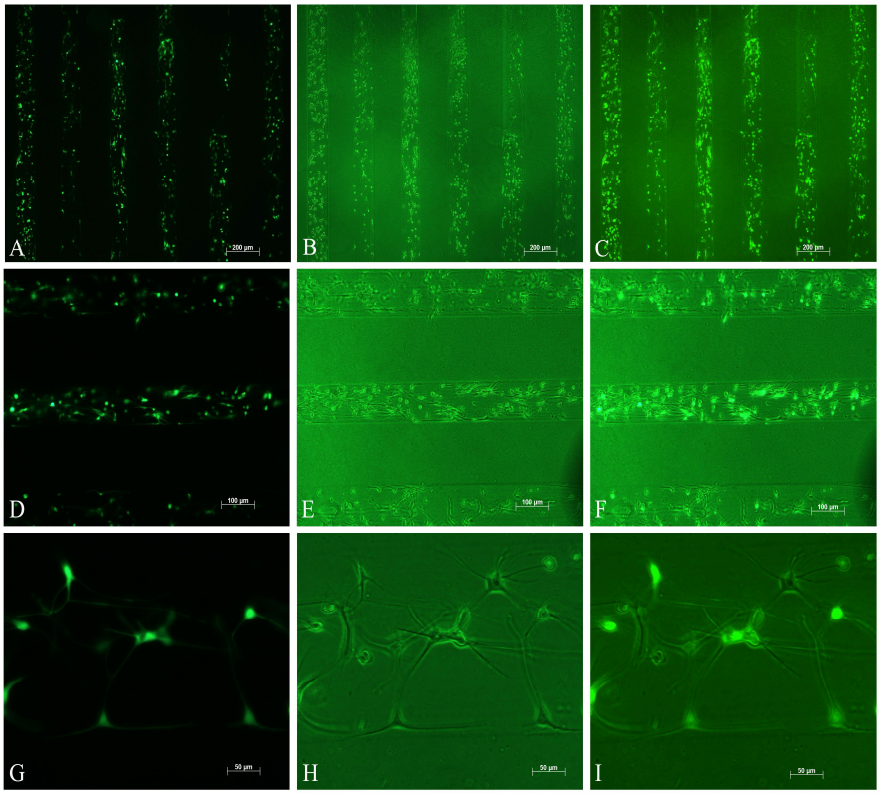


Figure 4.2 GFP Schwann cells loaded in TMM micro-channels 24 hours after seeding: Fluorescent micrograph of GFP-Schwann cells loaded in TMM micro-channels (A, D, G). Phase contrast of GFP-Schwann cells in TMM micro-channels (B, E, H). Overlay of fluorescence and phase contrast (C, F, I). Three-dimensional cellular cables formed by the Schwann cell within 24 hours of seeding (G, H, I). Magnification: 5X (A-C), 10X (D-F), 20X (G-I).

*4.1.2 Fabrication of a modified TMM casting device with a DRG placement region*

The existing TMM casting device did not accommodate the need for placing DRG explants, thus, necessitating a modification in the design that would incorporate a placement region for the explant tissue. A detachable 2 mm wide comb device containing perforations (0.260 mm diameter) aligned with the rest of the TMM casting device was manufactured from dental cement. The conduit-casting fibers are guided through the perforations in the detachable comb and casting device thus, holding the

comb. Subsequent to the polymerization of agarose and extraction of the fibers, the removal of the comb results in the formation of an agarose well that could be utilized for placing the DRG explants in a three-dimensional matrix. After the initial manufacture and testing of these devices made from dental cement, a three-well TMM casting device including the detachable comb was manufactured by Zyvex Corporation with modifications that included the diameter of the perforations (0.275 mm), fabrication material (plastic) as well as the inclusion of dual cell seeding wells to accommodate multiple cell type loading within micro-channels (Fig 4.3A and B).

Employment of this device would accommodate the placement of the DRG explants in an agarose well filled with a three-dimensional matrix. This agarose well would be created subsequent to the polymerization of agarose, extraction of the conduit-casting fibers and the detachment of the comb apparatus. Figure 4.3A shows a top view of the modified TMM casting device including the comb apparatus. A part of the comb device (denoted by *) is also shown in Fig 4.3B to illustrate the fact that the comb device rests 0.2 mm from the bottom to facilitate the formation of the agarose well.

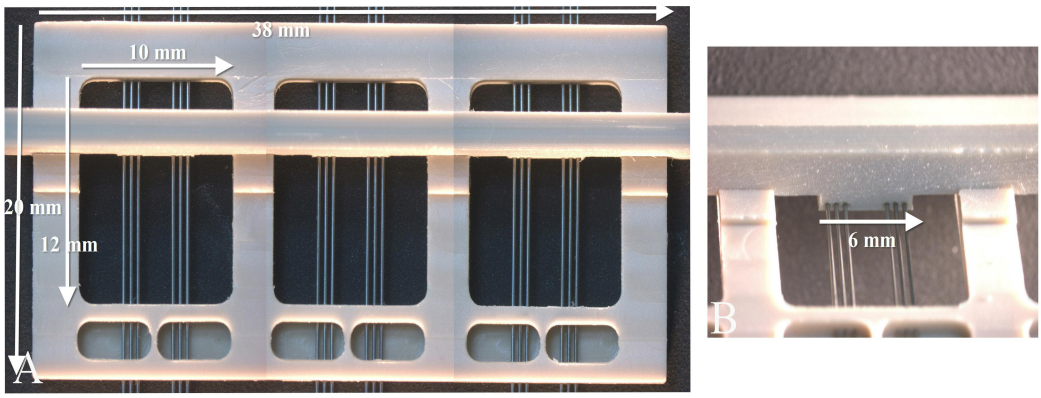


Figure 4.3 Modified TMM casting device with the comb apparatus: A. Top view of the TMM casting device including the conduit casting titanium fibers and the comb apparatus. B. Area of the comb apparatus (denoted by * in Fig 4.3A) illustrating the fact that the comb rests above the surface to facilitate the formation of agarose well.

*4.1.3 Analyzing axonal entry into micro-channels*

Fig 4.3A depicts a schematic representation of the modified TMM assay to be employed for the segregation of the sensory axonal subtypes. It illustrates the TMM micro-channels loaded with different neurotrophin expressing cells (as depicted by yellow and black) and a central agarose well (as indicated by the rectangular region) to be used for the placement of the DRG explants in a three-dimensional matrix provided by growth factor reduced Matrigel Matrix (BD Biosciences, San Jose, CA) or ECM-. Loading the TMM micro-channels with genetically modified Schwann cells that constitutively express a particular neurotrophin, either NGF or NT-3, will provide attractive cues for nociceptive (pain fibers) and proprioceptive sensory axons, respectively. These DRG fibers would respond to the neurotrophic gradient from the micro-channels and segregate based on appropriate pathfinding cues.

This experiment was conducted to determine the effectiveness of the TMM assay with regards to the axonal entry inside the micro-channels. For the purpose of this experiment, P-3 wild-type and GFP/NT-3-overexpressing Schwann cells provided by the University of Miami) were employed to entice the axonal outgrowth from the DRG explants placed in the central agarose well. The Zyvex-manufactured three-well TMM casting device was sterilized according to the previously mentioned protocol and multiple titanium wires (0.25 mm X 17 mm, Small Parts, Inc., Miami Lakes, FL) were guided through the perforations at both ends of the device including the detachable comb apparatus. After allowing the 1.5% agarose (in 1X PBS) to be polymerized over the fibers in each of the three central wells of the TMM casting device, a suspension of ECM- at 1:1 with either wild-type or GFP/NT-3 over-expressing Schwann cells (4 X $10^6$ cells/ml) were loaded in alternate cell seeding wells. Subsequent to the extraction of the fibers, multiple adjacent micro-channels were seeded with either wild-type or GFP/NT-3 expressing Schwann cells thus, potentially creating a neuotrophin gradient within the lumen of the channels. The comb apparatus was detached from the casting device creating an agarose well in each of the three hydrogels that was then filled with growth factor reduced Matrigel (ECM-) to provide a three-dimensional matrix for the DRG explants. Neonate mice DRGs (P2) were isolated following described protocol and placed in the ECM- filled central well in each of the three hydrogels. The device was placed in the culture medium (described in Chapter 7) and the assay was cultured for 5 days with periodic media change after which the culture was fixed as per the described protocol.

The axonal outgrowth from the P2 mouse DRG placed in the central agarose well filled with ECM- was assessed 7 days after its placement. Both the cell types survived within the micro-channels during the entire assessment period of 7 days (Figs 4.4B and C). Although a robust outgrowth towards the Schwann cells-filled micro-channels can be observed (Fig 4.4D), there was very little to no axonal entry into the channels. Figures 4.4E and F illustrating a higher magnification of axonal growth in areas denoted by * and ** respectively in Figure 4.4D further demonstrate the observation that axons are unable to cross the interface between the well and the channel entry point leading to no entry within the channels.

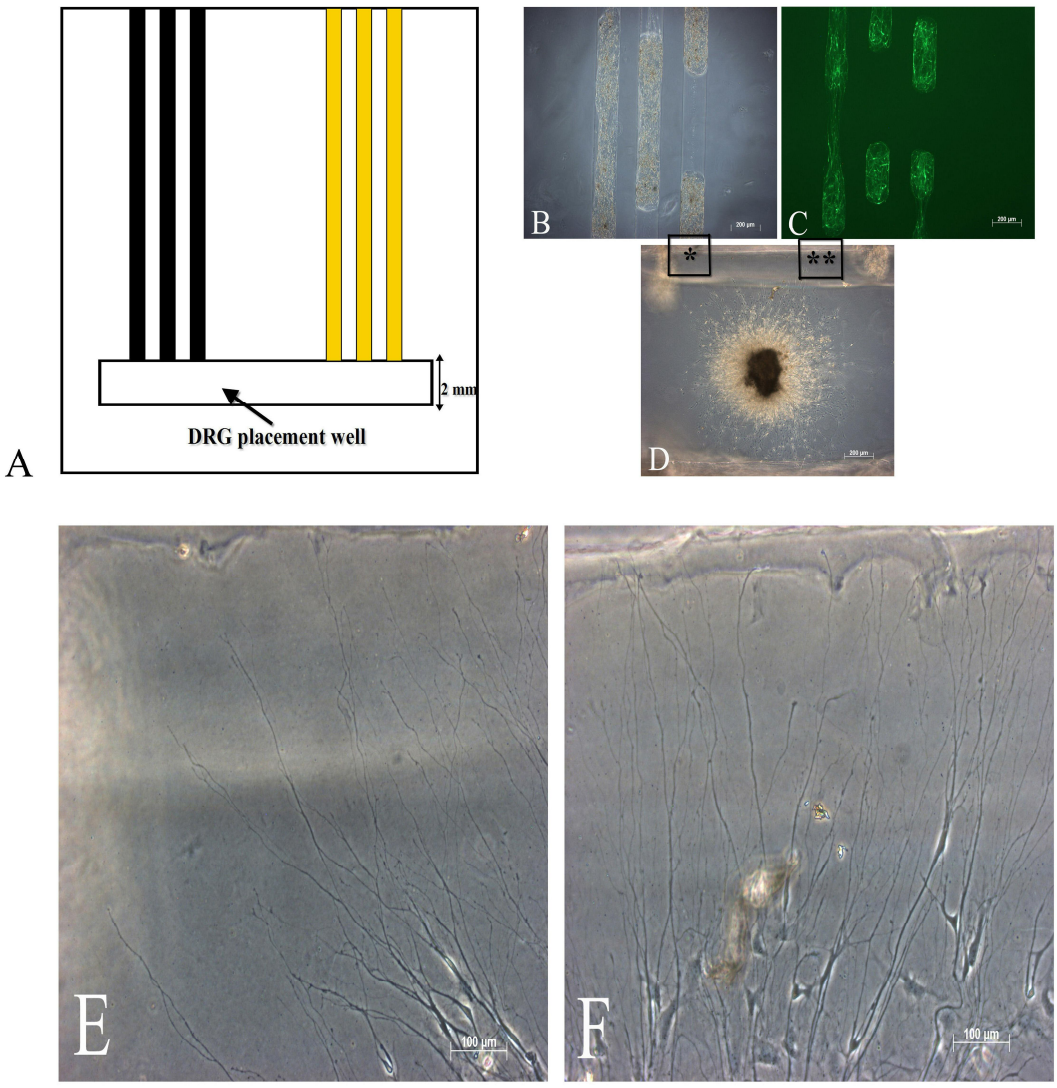


Figure 4.4 In-vitro axonal outgrowth using the TMM assay: A. Schematic representation of the TMM illustrating channels loaded with different neurotrophin expressing cells (as depicted by yellow and black). Note that a central agarose well (as indicated by the rectangular region) was used for the placement of the DRG explants in reduced growth factor Matrigel (ECM-). Light (Fig 4.4B) and fluorescent (Fig 4.4C) micrographs of TMM micro-channels loaded with wild type Schwann cells and GFP/NT-3 expressing Schwann cells respectively. A mouse neonate DRG (P2) was placed in an ECM- filled central well and axonal fibers can be observed growing towards the Schwann cell filled micro-channels (Fig 4.4D). Figures E and F illustrate areas at higher magnification of axonal outgrowth represented by * and ** respectively in Fig 4.4D. Magnification: 5X (B-D), 20X (E, F).

## 4.2 Three-dimensional Y-TMM assay

A novel assay was designed and fabricated employing the TMM hydrogels to mimic the three-dimensional axonal growth in the nervous system and translate the results obtained in the PDMS Y- assay in a three-dimensional setting.

### *4.2.1 Fabrication of the three-dimensional Y-assay*

The TMM casting devices that were employed for the fabrication of this assay were manufactured by Zyvex Corporation with modifications that included incorporating a perforation (0.500 mm diameter) at the center of one end of the device and smaller, singular perforations (0.275 mm diameter) in each of the cell suspension wells on the opposite end of the device (Fig 4.4A). The length of the central matrix casting well was also shorter, i.e. 6 mm. A novel, brush system was also designed to aid in the formation of the Y-micro-channel within the TMM hydrogel. This Y-brush was fabricated using a couple of plastic polymer fibers (0.25 mm X 17 mm) that were glued within hypodermic tubing (OD 0.508 mm ID 0.356 mm; Small Parts, Inc., Miami Lakes, FL).

The Zyvex-manufactured three-well TMM casting device was sterilized as per the standard protocol described previously after the Y-brush was guided through the perforations at both ends of the device (Fig 4.4A). After casting 1.5% agarose (in 1X PBS) in each of the three central matrix casting wells of the TMM device and allowing them to be polymerized, collagen (Chemicon International, Temecula, CA) was added into each of the cell suspension wells, and effectively into the lumen of the micro-channel, by utilizing the negative pressure created when the Y- brush is extracted in

each of the three polymerized gels. The collagen within the Y-micro-channel is allowed to polymerize for 1.5 hours in the 5% CO2 incubator before its placement in D-10 culture medium.

Figure 4.5A is a light micrograph of the Y-TMM casting device with the inserted brush and Figure 4.5B is a three-dimensional reconstruction of the Y-micro-channel loaded homogenously with collagen while depicting the area of the placement of the DRG explant at the channel entry point. The common arm of the Y-shaped micro-channel would act as a spacer region within which all the DRG axons would grow together and nearing the bifurcation point, the specific axonal types would respond to the particular neurotrophic gradients within each arm of the micro-channel.

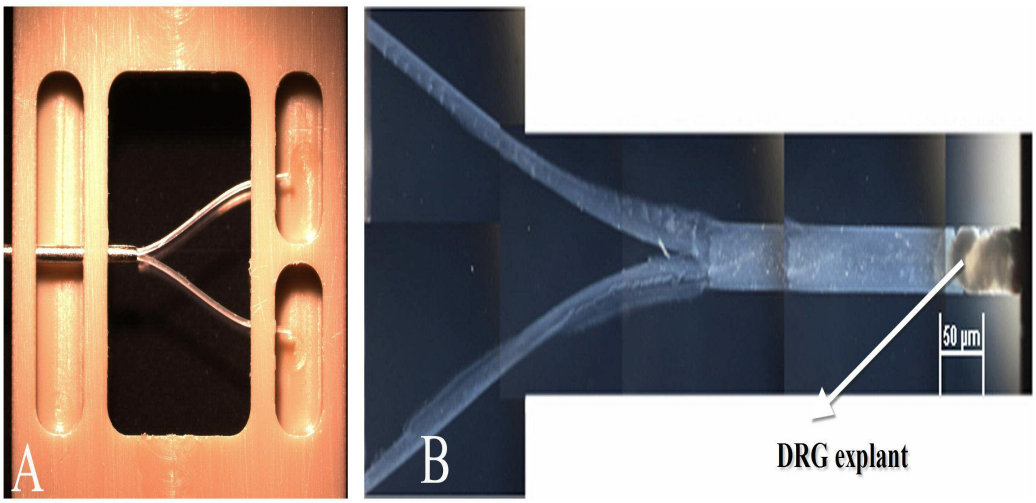


Figure 4.5 A three-dimensional Y-assay for in-vitro axonal segregation: A. TMM casting device with dual cell seeding wells and containing a Y-shaped brush (two 250µm plastic fibers glued within 500µm hypodermic tubing). Subsequent to the addition of agarose in the central well and collagen in both the cell-seeding wells, the brush is extracted thus, forming a collagen-filled Y-shaped channel. B. Reconstruction of the Y-micro-channel homogenously filled with collagen and denoting the placement of the DRG explant at the channel entry point.

*4.2.2 Testing the efficacy of the different neurotrophin delivery methods*

Multiple neurotrophin delivery systems were utilized to test their efficiency within the three-dimensional Y-assay. These systems included i) Schwann cells, ii) neurotrophin-coated latex beads iii) Passive diffusion of neurotrophins, and iv) Alzet® manufactured mini-osmotic pumps.

4.2.2.1 Testing Schwann cell seeding within the three-dimensional Y-assay

This experiment was conducted to test the effectiveness of the Schwann cell loading within the Y-micro-channels. The Y-TMM casting device manufactured by Zyvex Corporation was used to prepare the transparent 1.5% agarose (in 1X PBS) hydrogels in triplicate. A suspension of collagen (Chemicon International, Temecula, CA) with P-3 wild type Schwann cells (3 X $10^6$ cell/ml) at a 1:1 ratio was added into the cell suspension wells. Subsequent to the extraction of the Y-brushes, the collagen suspension containing the Schwann cells were loaded into the lumen of the formed Y-micro-channel. The culture medium utilized was D-10 mitogen and the assay was cultured for 3 days to examine the cell survival. Figure 4.6 is a three-dimensional reconstruction of the Y-micro-channel loaded homogeneously with the wild-type Schwann cells immediately after seeding.

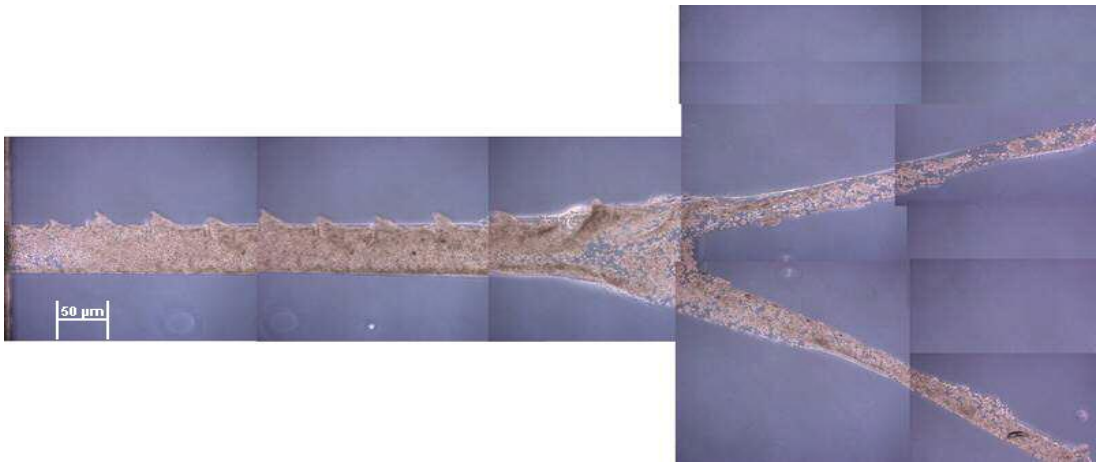


Figure 4.6 Reconstruction of the Y-micro-channel loaded with Schwann cells immediately after seeding

4.2.2.2 Employment of the neurotrophin-coated latex beads as a delivery system

This experiment was conducted to test the effectiveness of loading of the protein-coated beads within the Y-micro-channels and the efficacy of this neurotrophin delivery system within the assay. 10µm latex beads (Coulter Corporation, Miami, FL) were used for neurotrophin coating and seeding within the Y-micro-channel. 100µl of the beads suspension was centrifuged using a table-top centrifuge and washed three times with 70% ethanol before allowing the suspension to dry using different suspension drops thus, exposing only the beads concentrate. To better visualize the different neurotrophin-coated beads, Cy2- and Cy3-conjugated secondary antibodies in 1X PBS were used (1:400, Jackson Labs, West Grove, PA). While some batches of the beads concentrate were resuspended in Cy2- and Cy3- secondary antibodies solution, the rest were resuspended in the mNGF (100ng/ml; Sigma,St. Louis, MO) and hNT-3 (5ng/ml; Sigma, St. Louis, MO) solutions. After allowing these resuspensions to dry, all

of them were washed in 1X PBS to remove the uncoated proteins. Subsequent to the drying process, all the beads concentrate batches were resuspended in collagen (Chemicon International, Temecula, CA). Collagen solutions containing the Cy2-coated beads and the NGF-coated beads were mixed. Similarly, the collagen solutions containing the Cy3- and NT-3- coated beads were also mixed. The modified three-well Zyvex-manufactured TMM casting device including the Y-brushes was sterilized and loaded with 1.5% agarose gels according to the protocols described previously. The collagen solutions containing NGF/Cy2- coated beads and NT-3/Cy3- coated beads were each added into alternate cell suspension wells and effectively loaded into the lumen of the Y-micro-channels when the Y-brushes are removed. The collagen solution within the micro-channels is allowed to polymerize in the incubator (5% CO2) for 1.5 hours before its placement in D-10 culture medium. Through the employment of this technique, the two arms of the Y-micro-channel would be loaded with different neurotrophin-coated beads, thus establishing distinct neurotrophic gradients within the two arms. Although a uniform loading of the beads within the Y-micro-channel was accomplished (Fig 4.7), the auto-fluorescence property of the beads rendered it difficult to examine the effectiveness of the neurotrophin or the antibody coating.

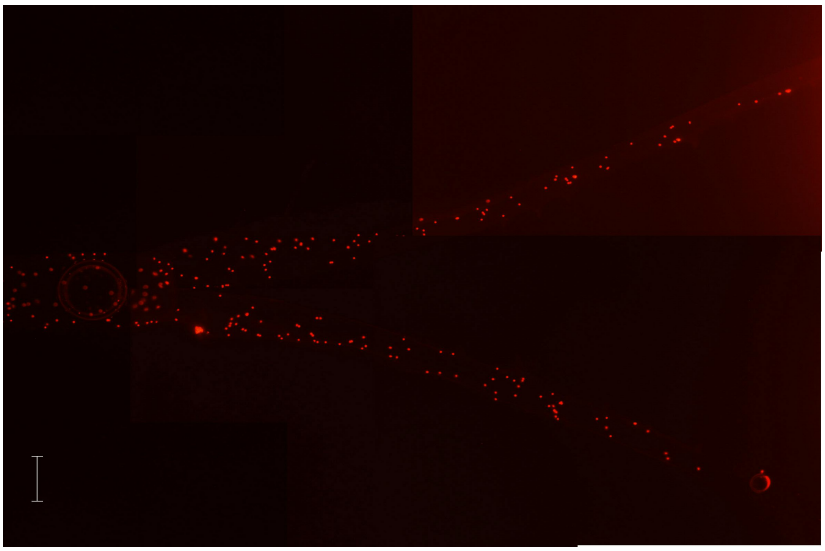

Figure 4.7 Reconstruction of the Y-micro-channel loaded with neurotrophin-coated latex beads

4.2.2.3 Employment of passively diffused neurotrophins as a delivery system

The three-well Y-TMM casting device manufactured by Zyvex Corporation was used. The sterilization and the gel casting in the device were performed according to the protocols described previously. Collagen (Chemicon International, Temecula, CA) was added in each of the cell suspension wells and the Y-brush was removed in each of the polymerized gels to allow the homogenous loading of collagen within the Y-micro-channels. The collagen within the micro-channels was allowed to polymerize for 1.5 hours in the incubator (5% CO2). Subsequently, 4µl of mNGF (100ng/ml; Sigma,St. Louis, MO) and hNT-3 (5ng/ml; Sigma, St. Louis, MO) solutions were added to the appropriate cell suspension wells. The culture(s) was placed in the appropriate culture medium (described in Chapter 7). However, for the initial 24 hours after DRG placement, the cultures were not entirely submerged in media. This would allow enough

time for the neurotrophins in the cell suspension wells to be diffused within the Y-micro-channel through capillary action without dilution in the media.

4.2.2.4 Testing the dilution of Trypan blue dye using Alzet® mini-pumps

The Alzet® manufactured mini-osmotic pumps were employed as a delivery system for allowing a controlled and pre-determined release of the desired neurotrophic proteins within the three-dimensional Y-assay. The mini-pumps operate through an osmotic pressure difference principle ensuring that the loaded proteins to be delivered at a controlled rate independent of their physical and chemical properties.

This experiment was conducted to analyze the release of the Trypan blue dye (Sigma, St. Louis, MO) within the arm of the Y-micro-channel using the Alzet® manufactured mini-osmotic pumps (Model 1007D, Alzet® Corporation, Cupertino, CA). Moreover, an examination of the diffusion of the dye within and around the micro-channel as well as its progressive dilution would provide valuable insight into the appropriate neurotrophin concentrations to be used. This pump model has a reservoir volume of 100µl and a release rate of 0.5µl /hr for a period of 7 days. Following an established protocol, the pump was loaded with 100µl of the blue dye using a 1.0ml syringe (BD Biosciences, San Jose, CA) and the provided 27 gauge filling tube. To ensure a localized delivery of the blue dye only within the lumen of the micro-channel, a novel system was designed. This included connecting a vinyl catheter tubing (ID 0.685 mm, OD 1.14 mm; Durect Corporation, Cupertino, CA) to a hypodermic needle (30G ½ ; BD Biosciences, San Jose, CA) with the aid of Krazy Glue (Elmer's Products Inc., Bainbridge, NY). The distal end of the catheter tubing was connected to the

provided flow moderator (i.e. stainless steel tube) which was, in turn, connected to an additional vinyl catheter tubing that was primed with blue dye until the hypodermic needle is flush with the dye. This system was essential to ensure the elimination of any air pockets within the catheter which would impede the flow of the loaded solution. Subsequent to this step, the additional vinyl catheter tubing is removed and the flow moderator is inserted all the way into the loaded pump until the attached catheter and hypodermic needle is flush with the surface of the pump.

The Zyvex-manufactured Y-TMM casting device was sterilized, casted with 1.5% agarose gel and loaded with collagen within the lumen of the Y-micro-channel following the established protocol. To ascertain a localized delivery, the loaded pump was connected to a catheter and a hypodermic needle (as described above) with the needle tip placed at the entry point of one arm of the Y-micro-channel with the aid of Krazy Glue (Elmer's Products Inc., Bainbridge, NY). The assay was cultured for 7 days and the culture medium utilized was Neurobasal medium (Invitrogen, Carlsband, CA).

To calculate the extent of dilution of the blue dye at the bifurcation point within the Y-micro-channel after 7 days, serial dilutions of the dye of the order of 1:4 was performed to generate a standard calibration curve of its absorbance using a spectrophotometer. The absorbance of the various dye dilutions were measured and recorded. The hydrogel employed in this experiment was cut in several 1mm square zones, placed in 750µl of 1X PBS and heated at 80°C for 10 minutes to allow the gel to dissolve and release the dye. The absorbance of the dye released from these different gel sections were measured and compared against the standard calibration curve to

calculate the extent of dilution of the dye at the bifurcation point of the Y-micro-channel after 7 days.

Fig 4.8A is a three-dimensional reconstruction of the Y-micro-channel 7 days subsequent to the implantation of the blue-dye loaded pump. The boxed area in Fig 4.8A demonstrates the extent of the diffusion of the blue dye within and around the micro-channel 7 days subsequent to the pump implantation. Fig 4.8B is a light micrograph of the agarose gel 7 days after the pump implantation showing the diffusion of the dye with the arrows indicating the extent of diffusion within the micro-channel. It is evident from Figs 4.8A and B that the blue dye gets diffused around the micro-channel in addition to its diffusion within it. Moreover, the dye was progressively diluted as it was injected further into the micro-channel with substantial dilution at the bifurcation point.

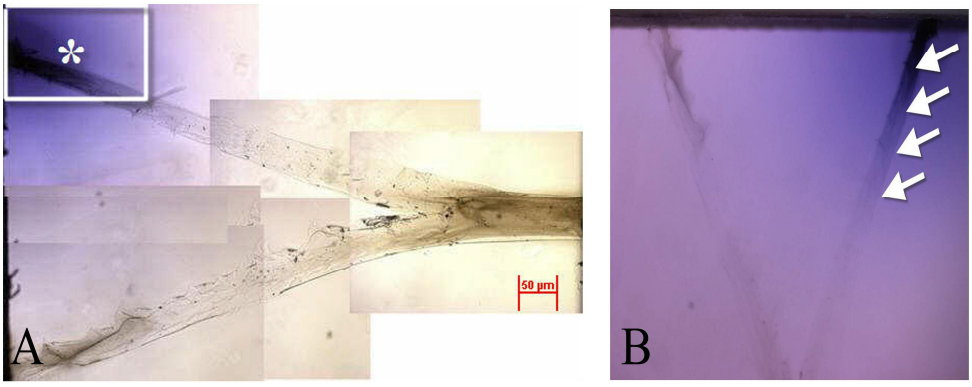


Figure 4.8 Alzet® manufactured mini-osmotic pumps releasing Trypan blue dye: A. Three-dimensional reconstruction of the Y-micro-channel 7 days after the implantation of the blue-dye loaded mini-pump. The diffusion of the dye within and around the micro-channel is maximum in the boxed area. B. Light micrograph picture of the agarose gel demonstrating the diffusion and dilution of the dye in the gel. The arrows indicate the extent of diffusion of the dye within the micro-channel.

To gain a further insight into the appropriate neurotrophin concentrations to be used with this assay, a calculation of the extent of the dilution of the dye at the bifurcation point after 7 days was performed using serial dilutions of the blue dye of the order of 1:4. The absorbance readings of the dye dilutions were generated with the aid of a spectrophotometer. Table 4.1 lists the absorbance readings of the various dye dilutions. The hydrogel employed in this experiment was cut in several 1 mm square zones (Fig 4.9) to measure the absorbance of the dye released from these sections. Table 4.2 lists the absorbance readings of the dye released from these different gel sections.

Table 4.1 Absorbance readings of the blue dye dilutions

| **Dilution** | **Absorbance** | **Dilution w/ respect to original** |
|---|---|---|
| Blank (Undiluted) | 0.000 | |
| ***** 1:16 | 2.015 | |
| 1:4 | 0.511 | 1:64 |
| 1:4 | 0.147 | 1:256 |
| 1:4 | 0.040 | 1:1024 |
| 1:4 | 0.028 | 1:4096 |
| 1:4 | 0.002 | 1:16384 |
| 1:4 | -0.003 | 1:65536 |

Note. * The absorbance of the initial four 1:4 dilutions were not detected so they were combined.

Since the absorbance reading of zone A falls in the 1:4096 range in the standard calibration readings of the serial dye dilutions, it can be assumed that the dye when being injected into the micro-channel entry point is already diluted 1:4096 times. When the dye reaches the bifurcation point (i.e. zone D), it gets diluted 1:3.67 times (0.011/0.003). Thus, it was calculated that the total dilution of the dye at the bifurcation point (zone D) is 1:(4096 *3.67) or 1:15032.

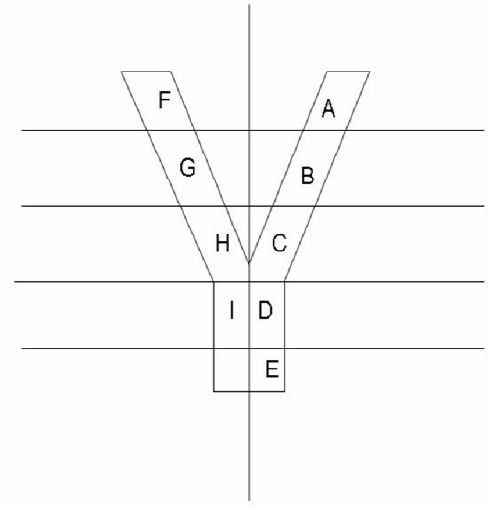


Figure 4.9 A representation of the several gel sections (1 mm X 1 mm) cut to measure the absorbance of the dye released from them

Table 4.2 Absorbance readings of the dye released from the different gel sections

| **Gel section** | **Absorbance** |
|---|---|
| A | 0.011 |
| B | 0.009 |
| C | 0.003 |
| D | 0.003 |
| E | -0.002 |
| F | 0.003 |
| G | 0.000 |
| H | 0.000 |
| I | -0.001 |
| J | -0.003 |
| K | -0.003 |
| L | -0.004 |
| M | -0.003 |

Note. Zones J-M were sections cut from a hydrogel with no pump thus, serving as control absorbance readings.

# CHAPTER 5

# PILOT STUDY USING PASSIVELY DIFFUSED NEUROTROPHINS WITH THREE-DIMENSIONAL Y-ASSAY

## 5.1 Employment of passively diffused neurotrophins to entice segregation of sensory axonal subtypes within the three-dimensional Y-assay

The Y-TMM casting device manufactured by Zyvex Corporation (described previously) was used in this experiment and the sterilization and the gel casting in each of the casting devices was performed according to the protocols described previously. Table 5.1 lists the different treatments groups used in the experiment. The same casting device was used for the similar treatment groups. Collagen (Chemicon International, Temecula, CA) was added in each of the cell suspension wells and the Y-brush was removed in each of the polymerized gels to allow the homogenous loading of collagen within the Y-micro-channels. The collagen within the micro-channels was allowed to polymerize for 1.5 hours in the incubator (5% CO2). Subsequently, 4µl of mNGF (100ng/ml; Sigma,St. Louis, MO) or hNT-3 (5ng/ml; Sigma, St. Louis, MO) solutions were added to the appropriate cell suspension wells as per the treatment group. For the control treatment, merely additional collagen was added to the cell suspension wells. Neonate mouse (P2) DRGs were extracted as per the established protocols and a DRG explant was placed at the channel entry in each of the gels. The culture(s) was placed in the appropriate culture medium (described in detail in Chapter 7). However, for the initial 24 hours after DRG placement, the cultures were not entirely submerged in media. This would allow enough time for the neurotrophins in the cell suspension wells

to be diffused within the Y-micro-channel through capillary action without dilution in the media. The assay was cultured for 5 days with periodic media change prior to fixing. Immunocytochemistry staining was conducted on these cultures using the Oregon Green® phalloidin 514 probe (Molecular Probes, Eugene, OR) to visualize the axons within the micro-channels. The staining protocol is described in detail in the General Materials and Methods chapter.

Table 5.1 List of the treatment groups with the passively diffused neurotrophins as a delivery system

| **Group Number** | **Treatments used in Group** A | **B** | A B |
|---|---|---|---|
| Group 1 | Saline (Control) | Saline (Control) | (n = 1) |
| Group 2 | Saline (Control) | NGF | (n = 1) |
| Group 3 | Saline (Control) | NT-3 | (n = 1) |
| Group 4 | NGF | NT-3 | (n = 5) |

In Group 1 that did not include any neurotrophin treatments, no axonal growth from the DRG explant was observed. This could be attributed to the damage incurred to the DRG explant while pushing it into the entry point of the micro-channel. Due to the three-dimensionality of the assay, multiple fluorescent micrographs of the axons at particular focal planes were taken and collapsed into a single image as visualized in Figs 5.1A-C. Fig 5.1A is a fluorescent micrograph of the phalloidin green-positive axons within the collagen-filled Y-micro-channel in Group 2 (Control - NGF). Fig 5.1D is a phase contrast (bright field) micro-photograph of Fig 5.1A indicating the Y-micro-channel and the axons growing within it. It is evident from Figs 5.1A and D that the

axons from the DRG explant are able to enter the two distinct arms of the Y-micro-channel. Fig 5.1B is a fluorescent micrograph representing the reconstruction of the phalloidin-green positive axons within the Y-micro-channel in Group 3 (Control – NT-3) and Fig 5.1E is a phase contrast (bright field) micro-photograph of Fig 5.1B. Similar to the results obtained from Group 2, axons were able to grow into the two arms of the collagen-filled Y-micro-channel. Fig 5.1C is a fluorescent micrograph of the reconstruction of the phalloidin-green positive axons within the Y-micro-channel in Group 4 (NGF – NT-3) and Fig 5.1F is a phase-contrast (bright field) micro-photograph of Fig 5.1C. In addition to the significant number of axons that had entered the two distinct arms of the collagen-filled Y-micro-channel, it was evident that the axons within the NGF-treatment arm had grown further into the arm as compared to the NT-3 treatment arm. This phenomenon was consistent across all the cultures belonging to Group 4. Optical density measurements were performed at particular areas (indicated by the boxed areas in Fig 5.1C) in the two arms of the Y-micro-channel, 300μm from the bifurcation point. The optical density in the boxed area indicated by * is 193.68 ± 29 while that in the boxed area indicated by ** is 137.71 ± 32. Fig 5.2 is a comparison of the maximum axonal length calculated in all the arms of the Y-micro-channel of the treatment groups. The maximum axonal length is the length of the longest axon in each arm of the Y-micro-channel and its length was calculated from the DRG explant. The maximum axonal length in the NGF-treatment arm of the Y-micro-channel is significantly greater than the NT-3 treatment and the Control (no neuotrophin treatment) arms.

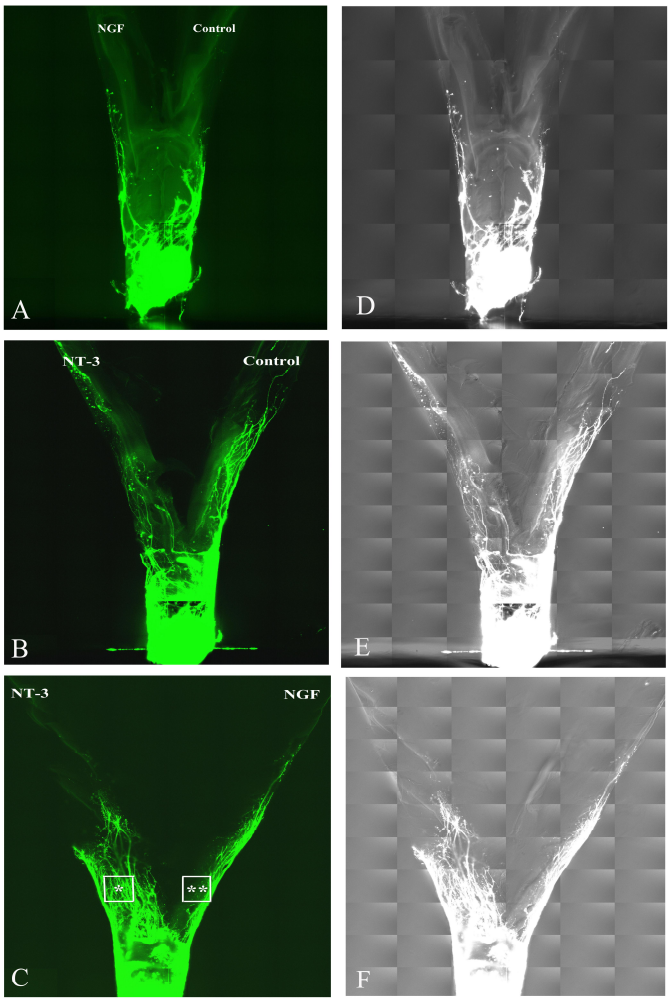


Fig 5.1 Axonal growth within three-dimensional Y-micro-channel with passively diffused neurotrophins (at 5 days): A-C. Fluorescent micrographs of the reconstruction of the phalloidin green-positive axons within the Y-micro-channel in Groups 2, 3, and 4 respectively. D–F. Phase contrast (bright field) micro-photograph of the Y-micro-channel in Groups 2, 3, and 4 respectively and the axons growing within it. It is evident from Figs 4.1A-F that the axons from the DRG explant placed at the channel entry-point had grown into the two arms of the collagen-filled micro-channel. In Group 4 (Figs 5.1C and F), axons in the NGF containing arm of the Y-micro-channel grew further into the arm as compared to the NT-3 treatment arm. Optical density measurements in the boxed areas indicated by * and ** (Fig 5.1C) were recorded.

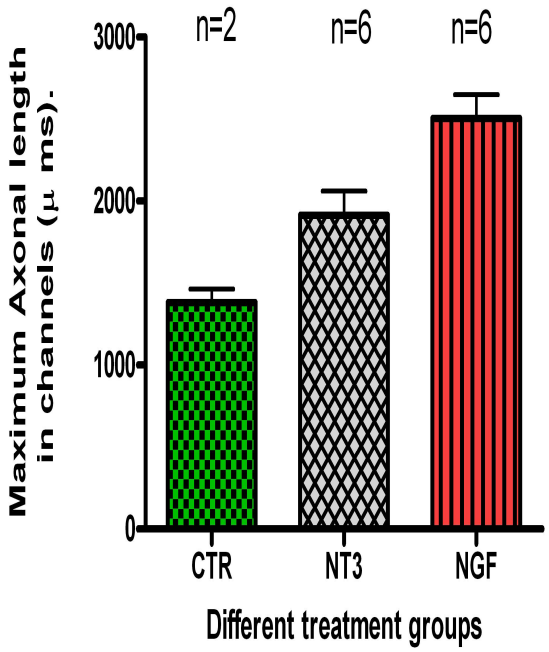


Fig 5.2 Maximum axonal length of the different treatment arms of the Y-micro-channel using passively diffused neurotrophins: The maximum axonal length was determined by calculating the length of the longest axon in each arm of the Y-micro-channel across all the treatment groups. The maximum axonal length in the NGF-treatment arms of all the groups was significantly greater than the NT-3 and the Control treatment arms. One-way ANOVA test revealed $P < 0.05$ ($P = 0.0040$).

# CHAPTER 6

# PILOT STUDY USING ALZET® MINI-PUMPS WITH THREE-DIMENSIONAL Y-ASSAY

## 6.1 Employment of neurotrophin-loaded Alzet® mini-pumps to entice segregation of sensory axonal subtypes within the three-dimensional Y-assay

To test the principle of axonal segregation using the three-dimensional Y-assay, the Alzet® manufactured mini-osmotic pumps were utilized to ensure a pre-determined and controlled release of the desired neurotrophins; namely NGF and NT-3. Following the protocols described in detail previously, the Y-TMM casting devices manufactured by Zyvex Corporation were used to prepare transparent hydrogels loaded with collagen within a three-dimensional Y-shaped micro-channel. The Alzet® mini-osmotic pumps were loaded with 100µl of the mNGF (5µg/ml; Sigma,St. Louis, MO) or hNT-3 (5µg/ml; Sigma, St. Louis, MO) solutions. Taking into account the extensive dilution of the blue dye observed within the Y-micro-channel (experiment results described in detail in Section 4.2.2.4), it was decided to use a higher concentration of the neurotrophins. As per the treatment group, the loaded pump was connected to a catheter and a hypodermic needle (as described previously) with the needle tip placed at the entry point of one arm of the Y-micro-channel within the polymerized gel. For the control treatment, pumps were not utilized. Neonate mice (P2) DRGs were isolated as per the described protocols and a DRG explant was placed at the channel entry in each of the gels. The assays were cultured for 7 days and allowed to continue with no media

change for their entire duration of 7 days. This was done to preserve the neurotrophic gradient within the micro-channel for the duration of the culture.

Table 6.1 List of the treatment groups with the Alzet® mini-pumps

| **Group Number** | **Treatments used in Group** A | **B** | A B |
|---|---|---|---|
| Group 1 | Saline (Control) | Saline (Control) | (n = 2) |
| Group 2 | Saline (Control) | NGF | (n = 2) |
| Group 3 | Saline (Control) | NT-3 | (n = 1) |
| Group 4 | NGF | NT-3 | (n = 2) |

Minimum to no axonal growth was observed after 7 days in all the treatment groups. Fig 6.1 is a fluorescent micrograph of the phalloidin green-positive axons in the Y-micro-channel in Group 2 after 7 days demonstrating limited axonal growth in the collagen-filled Y-micro-channel.

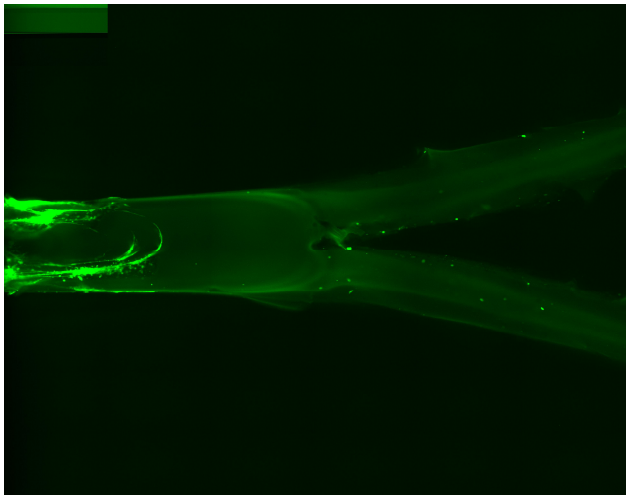

Figure 6.1 Axonal growth within the Y-micro-channel with Alzet® mini-pumps (at 7 days): Limited axonal growth in the Y-micro-channel in Group 2 at 7 days after the implantation of the neurotrophin-loaded Alzet® mini-pump. This observation was consistent amongst the cultures in all the groups.

Assuming that the higher concentration of the neurotrophins and no media change might have been detrimental to the survival of the axons, the pumps were removed at 7 days, and media was changed with an additional media change at the 10$^{th}$ day and the assays cultured for a total of 14 days. Immunocytochemistrya labeling with phalloidin-green probes was performed. No axonal growth was observed in all the groups except for one culture in Group 4. Fig 6.2A is a reconstruction of the fluorescent micrograph of the phalloidin green-positive axons within the Y-micro-channel in Group 4 at 14 days and Fig 6.2B is its phase contrast (bright field) micro-photograph. Significant axonal growth was observed within the two arms of the Y-micro-channel.

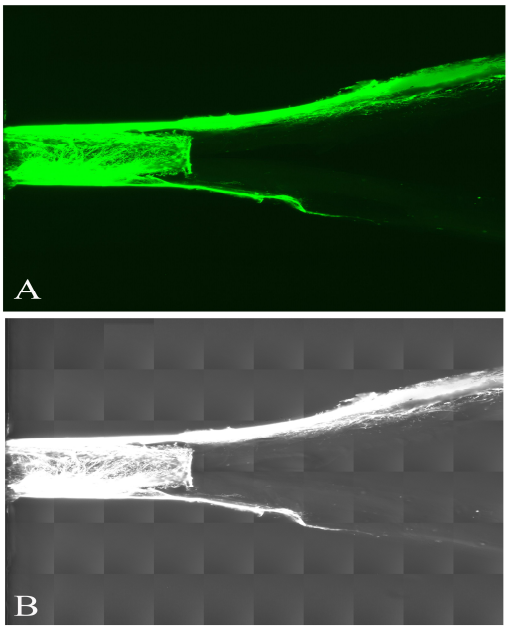


Figure 6.2 Axonal growth within the three-dimensional Y-assay in Group 4 with Alzet® mini-pumps (at 14 days): A. Reconstruction of the fluorescent micrograph of the phalloidin-green positive axons within the three-dimensional Y-micro-channel in Group 4. B. Phase contrast (bright field) micro-photograph of Fig 6.2A indicating the Y-micro-channel and the axons growing within it. Significant axonal growth was observed within the two arms of the Y-micro-channel.

Fig 6.3B is a reconstruction of the fluorescent micrograph of the phalloidin green-positive axons in the Y-micro-channel in Group 4 at 14 days. Fig 6.3A is a higher magnification three-dimensional z-stack reconstruction of the axonal growth in the boxed area indicated by * in Fig 6.3B demonstrating the highly arborized feature of these NT-3 attracted axons, a morphological characteristic of the proprioceptive sensory axons. Fig 6.3C is a three-dimensional z-stack reconstruction of the axonal growth in the boxed area indicated by ** in Fig 6.3B. These NGF attracted axons are long with minimal branching, a morphological characteristic prevalent among the nociceptive sensory axons. These morphological distinctions amongst the axonal types growing within the two arms of the three-dimensional Y-micro-channel provided promising evidence for the segregation of the sensory axonal subtypes with the aid of specific neurotrophin delivery.

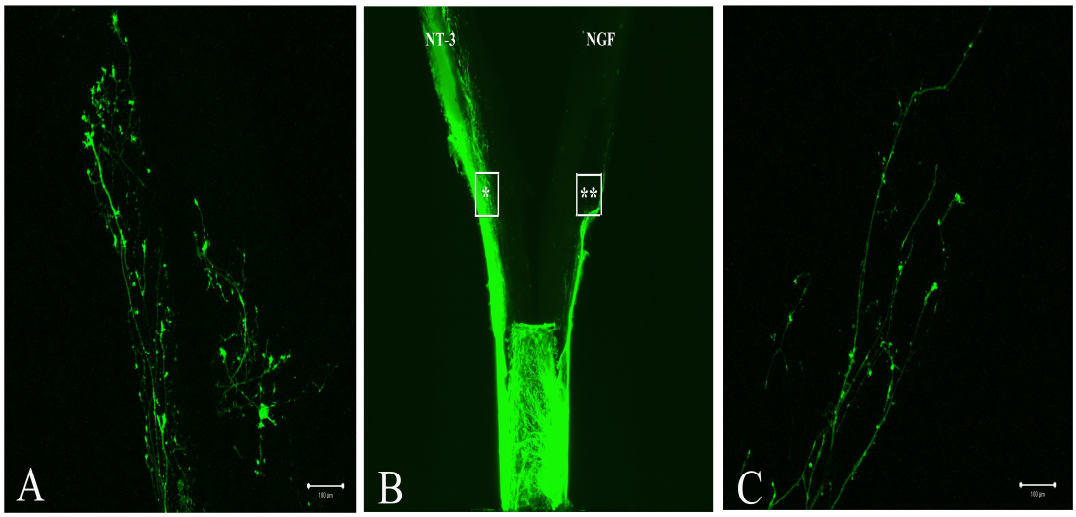


Figure 6.3 Distinction in the axonal morphologies amongst the NGF and NT-3 attracted sensory axons growing within the three-dimensional Y-micro-channel (at 14 days): B. Reconstruction of the fluorescent micrograph of the phalloidin green-positive axons within the Y-micro-channel in Group 4. A. z-stack reconstruction of the axonal growth in the boxed area indicated by * in Fig 6.3B. The highly arborized morphological feature of the NT-3 attracted sensory axons growing within this arm of the Y-micro-channel is evident. C. z-stack reconstruction of the axonal growth in the boxed area indicated by ** in Fig 6.3B. The NGF attracted sensory axons growing within this arm of the Y-micro-channel are long with minimal branching. Scale bars = (A, C) 100µm.

# CHAPTER 7

# GENERAL MATERIALS AND METHODS

## 7.1 Fixing and immunocytochemistry

For the fixing of the cultured cells, media was removed and 4.0% paraformaldehyde (PFA) added and washed briefly with 1X PBS. Incubation was then achieved at room temperature for 30 minutes with gentle agitation on the lab rotator with the aid of magnetic stir bars. The PFA was subsequently removed and the culture washed 4 times with 1X PBS for 5 minutes each at room temperature.

For immunostaining of the DRG axons in the two-dimensional cultures, the tissue was permeabilized in 2.5% Triton X (Sigma, St. Louis, MO) in 1X PBS solution for 30 minutes at room temperature using a 35-mm cell culture dish. The tissue culture was then incubated in a blocking solution consisting of 1% Normal Donkey Serum (Jackson Labs, West Grove, PA) in 2.5% Triton X (in 1X PBS) for 30 minutes. The culture was subsequently incubated with a combination of primary antibodies against monoclonal beta-tubulin produced in mouse (1:500; Sigma, St. Louis, MO) and calcitonin gene-related peptide produced in rabbit (1:2500; Sigma, St. Louis, MO). The beta-tubulin is a neuronal marker and labels all the axons, irrespective of their type and the calcitonin gene-related peptide is a positive marker for nociceptive sensory axons. After overnight incubation in the primary antibodies, the culture was rinsed 4 times in 1X PBS for 5 minutes each. Visualization was achieved by incubation of the culture in

Cy2-donkey anti-mouse (1:250; Jackson Labs, West Grove, PA) and Cy3-donkey anti-rabbit (1:250; Jackson Labs, West Grove, PA) and rinsing 4 times in 1X PBS for 5 minutes each subsequently.

For immunostaining of the DRG axons within the three-dimensional Y-TMM assay, the tissue was permeabilized in 2.5% Triton X (Sigma, St. Louis, MO) in 1X PBS solution for 30 minutes at room temperature using a 35-mm cell culture dish. The tissue culture was then incubated in a blocking solution consisting of 1% Normal Donkey Serum (Jackson Labs, West Grove, PA) in 2.5% Triton X (in 1X PBS) for 30 minutes. The culture was incubated overnight in the Oregon Green® 514 phalloidin probe (1:40; Molecular Probes, Eugene, OR). Subsequently, the culture was rinsed 4 times in 1X PBS for 5 minutes each.

The staining was evaluated using a Zeiss Pascal confocal microscope. Z-stack reconstructions were also performed in select cultures to visualize the three-dimensional outgrowth from the DRGs. Phase contrast and light micrograph pictures were taken using Zeiss inverted microscope.

### 7.2 Neonate mouse Dorsal Root Ganglion isolation

The neonate (P0-2) mouse pups were sacrificed by decapitation. The mouse pup was decapitated and the skin removed from the back. A transverse cut in the spinal cord above the thoracic region was made to implement a lateral cut down the spinal cord on both the sides. Subsequently, the spinal cord was removed and placed in Hank's Buffered Salt Solution medium (Gibco; Carlsbad, CA). With the aid of surgical scissors, the tissue was removed on the ventral side of the spinal cord until the bony

structure became visible. A V-shape (10° from the vertical plane) was cut in the transverse section of the spinal cord and continued in the ventral portion until it has a trough from the distal to the proximal end. After cutting the spinal cord in half, nerve fibers in the interior of the spinal cord were removed exposing the dorsal root ganglia (DRG). Inserting the forceps under a DRG, the underlying nerve fascicle was cut thus, pulling out the DRG. For explant cultures, all but a small protrusion of the nerve fascicles were removed from the DRGs. The DRG explants were cultured in Neurobasal medium (Invitrogen, Carlsband, CA) supplemented with L-glutamine (Gibco Invitrogen, Carlsband, CA), B-27 supplement (Invitrogen, Carlsband, CA), and Penicillin/Streptomycin (Gibco Invitrogen, Carlsband, CA).

### 7.3 Schwann cell cultures

Schwann cells were obtained from adult rat sciatic nerves, and expanded in vitro according to established protocols. The cells were cultured in DMEM/10% FBS, supplemented with forskolin, and pituitary gland extract. The genetically modified Schwann cells were kindly provided by the University of Miami. In all the experiments requiring the use of the Schwann cells, P-3 cells with 70-80% confluency were utilized.

### 7.4 Culture medium for DRG explant cultures

The culture medium that was utilized for all the DRG explant cultures was Neurobasal medium (Invitrogen, Carlsband, CA) supplemented with L-glutamine (Gibco Invitrogen, Carlsband, CA), B-27 supplement (Invitrogen, Carlsband, CA), and Penicillin/Streptomycin (Gibco Invitrogen, Carlsband, CA)

# CHAPTER 8

# DISCUSSION

The major determinant of functional recovery after nerve lesions in the peripheral nervous system is the accurate regeneration of axons to their original target end organs. The definitive measure of such accuracy is physiological proof of functional reinnervation of the distal target end organ. Regenerating axons tend to grow along Schwann cell basal lamina tubes in the distal nerve stump; also termed the bands of Bungner. Myelinating Schwann cells are associated with just a single axon and form continuous, un-branched basal lamina tubes all the way from the nerve ransection site to the distal end organ target (Madison, Archibald et al. 1999). The intactness of these basal lamina tubes would forcibly direct regenerating axons into the original terminal nerve branches thus, determining the ultimate destination of these axons.

Despite the advent of a number of repair techniques, the inadequacy of the repair concept is frequently emphasized by the resulting poor sensory and motor function thus, compromising overall functional recovery. The critical factor contributing to this limitation is the regeneration of axons into inappropriate distal pathways. Motor axons enter sensory Schwann cell tubes and are directed to skin, while sensory axons enter motor Schwann cell tubes and are directed to muscle (Brushart 1993). These misdirected axons cause a neuron-target mismatch leading to failed functional connections and also excluding appropriate axons from the pathways they occupy. The misdirection may occur between motor and sensory pathways (Schwwann

cell tubes) during mixed-axon regeneration, between different muscles during motor axon regeneration, between different sensory corpuscles, and between different sensory areas during sensory axon regeneration (Fig 6.1) (Maki 2002). Identification of factors contributing to the specificity in innervation could reduce the resulting functional compromise.

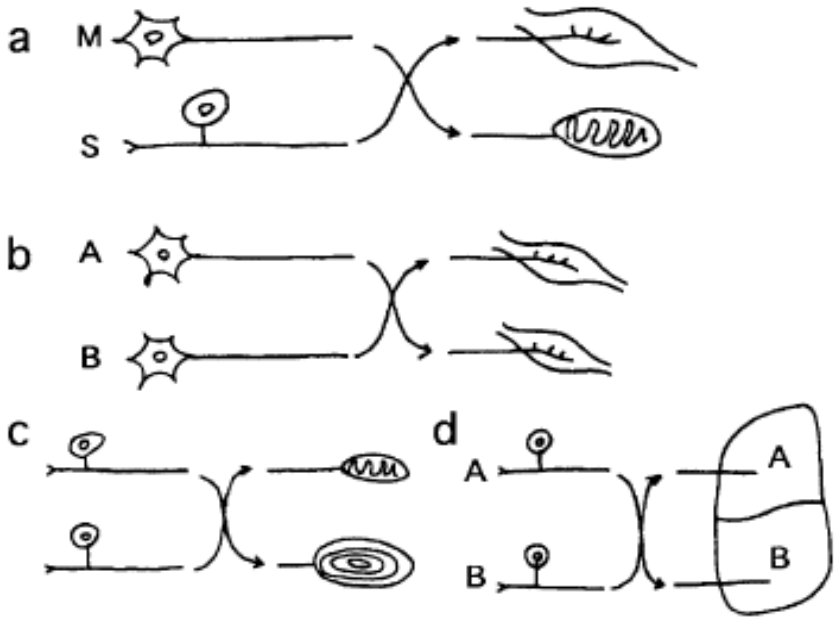


Figure 8.1 Types of misdirected axonal regeneration: a. misdirection between a motor pathway (M) and a sensory pathway (S); b. misdirection between different muscles; c. misdirection between different sensory corpuscles; d. misdirection between different sensory areas.

The term specificity can be defined as the ultimate extent of accuracy in reinnervation of distal targets after various mechanisms for axonal guidance have had a possibility to operate at various time points and various levels during the regeneration process (Lundborg 2004). Specificity can be categorized into different types: i) tissue

specificity, meaning that regenerating axons preferentially grow toward distal neuronal tissue, as compared to non-neuronal tissue signifying a role of neurotropism in directing the axons (Lundborg, Dahlin et al. 1994); ii) motor versus sensory specificity (end-organ specificity), indicating preferential motor innervation by motor axons (Brushart 1993) or preferential sensory innervation of sensory targets (Maki 2002); iii) topographic specificity, signifying preferential reinnervation of targets for e.g. belonging to tibial pathways by tibial nerve fibers and targets belonging to peroneal pathways by peroneal nerve fibers (Lundborg 2004).

The search for evidence of sensory/motor specificity has produced contradictory results. Brushart (Brushart 1988; Brushart 1993) coined the term preferential motor reinnervation after he demonstrated the ability of the regenerating motor axons in rat femoral nerve to preferentially, albeit incompletely, reinnervate muscle versus cutaneous distal nerve branches. Provided equal access, regenerating motor axons initially grow into both nerve branches but over time are pruned from the cutaneous branch, preferentially innervating the terminal muscle branch (Madison, Archibald et al. 1996). On the other hand, similar experiments performed with the rat femoral nerve by Maki (Maki, Yoshizu et al. 1996; Maki 2002) demonstrated that specificity is found in sensory regeneration and not motor regeneration. Moreover, any evidence of pruning of the misdirected axons could not be validated.

Achieving directed axonal growth with any peripheral nerve gap repair strategy has been recognized to be essential to guide the regenerating axons to their original distal target and improve functional recovery. Bridging filaments made from

bioresorbable sutures of either polyglactin or polyamid were used to successfully bridge 10 to 15 mm gaps while allowing a longitudinal arrangement of the regenerating axons (Lundborg 2004). *In-vitro* (Rangappa, Romero et al. 2000) and *in-vivo* (Ngo, Waggoner et al. 2003) studies with poly (L-lactide) microfilaments facilitated directional orientation to the axons in DRG explant cultures as well as across peripheral nerve gap lesions. Additional efforts to improve directed axonal growth include the deployment of collagen filaments (Yoshii, Oka et al. 2003) and the use of multiluminal conduits (Hadlock, Sundback et al. 2000; Yang, De Laporte et al. 2005). While more closely resembling the fascicular micro-architecture of the peripheral nerves, the multiple longitudinally aligned channels in a nerve graft would facilitate the regenerating axons to grow in a longitudinally organized manner.

In this study, we hypothesized that segregation of axonal types would greatly reduce axonal misdirection and facilitate their entry into appropriate distal pathways leading to correct target innervation and improved functional recovery. Sorting of regenerating axons has been partially investigated with a Y-tube model and target-derived molecules (Jerregard, Nyberg et al. 2001). The two distal branches of the Y-tube were filled with agarose primed with filtrate prepared from either skin or muscle homogenates and the tibial and sural nerves were inserted in the two outlet branches of the Y-tube distal to the position of the homogenates. The motor axons were found to have preferentially grew into the muscle filtrate branch but no demonstrated sorting of the sensory axons seemed to have occurred with both the branches of the Y-tube containing sensory axons albeit, the larger-sized, myelinated proprioceptive sensory

axons were enriched in the muscle-filtrate branch and the smaller, unmyelinated nociceptive sensory axons were larger in number in the skin-filtrate branch of the Y-tube. To accomplish an efficient *in-vitro* segregation of axonal subtypes, a three-dimensional system allowing a linear, directed axonal growth is essential to mimic the *in-vivo* conditions in the peripheral nerve. Moreover, this system must also act as a physical guidance model to direct axonal growth in a specified direction. A delivery system allowing a controlled of specific, target-derived neurotrophic factors must be employed to entice the compartmentalized growth of specific types of axons. The purpose of this study was to examine if using a novel *in-vitro* assay and an appropriate delivery system for the release of specific neurotrophins; namely NGF and NT-3, it would be possible to entice the segregation of nociceptive and proprioceptive sensory axons, respectively.

Considering that the *in-vitro* cultures of neurons results in a two-dimensional, random growth of axons, it was decided to develop an appropriate two-dimensional assay with specific neurotropic cues to guide the axons in a specific direction and effectively segregate sensory axonal subtypes. A two-dimensional drop assay was devised wherein a couple of small gelfoam pieces (15 mm X 15 mm) embedded in an ECM- drop were soaked in either NGF or NT-3, the release of which would present a potent neurotrophic gradient to the sensory axons growing from a dorsal root ganglion explant anchored in an adjacent ECM- drop (Fig 3.1). Due to the fact that this experiment was an initial study to identify the various parameters to be considered for the development of an appropriate two-dimensional segregation assay, it did not include

the different control groups. While examining the axonal outgrowth after 3 days in culture, it was determined that a number of NGF-attracted axons were long with few branches and numerous axons growing toward the NT-3 soaked gelfoam were short and highly arborized (Fig 3.1). These distinct axonal morphologies were characteristic of the nociceptive and proprioceptive sensory axons respectively. However, owing to the presence of a number of morphologically mixed axons as well, no conclusive evidence of segregation was obtained. The addition of media may have resulted in the immediate release of the neurotrophins from the gelfoams leading to immediate dilution thus, contributing to the lack of formation of a potent and distinct neurotrophic gradient. This phenomenon may have been further compounded by the absence of a physical barrier between the gelfoams facilitating the mixing of the different neurotrophins resulting in a diluted and ineffective gradient. To circumvent these shortcomings, a novel, two-dimensional PDMS Y-shaped assay including segregated gelfoam placement areas was developed to facilitate the production of an effective neurotrophic gradient within specific channels for enticing the segregation of nociceptive and proprioceptive sensory axons growing from a dorsal root ganglion explant placed close to the bifurcation point (Fig 3.2) In all the control groups tested, a distinction in axonal morphologies was observed amongst the axons attracted toward NGF or NT-3 (Fig 3.3). Having determined that the separate delivery of either NGF or NT-3 can indeed promote the growth of nociceptive (pain) or proprioceptive fibers, it was decided to test whether the simultaneous presentation of both neurotrophins could segregate these axons from he same ganglia. To that end, the subsequent experiments included NGF and NT-3 soaked

gelfoams in the same PDMS Y-assay and it was indeed evident that a majority of the axons growing toward the NGF and NT-3 soaked gelfoams possessed nociceptive and proprioceptive-like morphological characteristics (Fig 3.4). Dual immunocytochemistry labeling with beta-tubulin and CGRP was further carried out to examine whether the NGF and NT-3 attracted sensory axons are functionally different. While beta-tubulin is a known neuronal marker, the nociceptive sensory axons can be visualized by their content of CGRP, which serves as a specific marker for these fibers (Oku, Satoh et al. 1987; Romero, Rangappa et al. 2000; Zhang, Hoff et al. 2001). The immunocytochemistry results demonstrated that the CGRP-positive nociceptive sensory axons were attracted toward NGF while the CGRP-negative proprioceptive sensory axons were growing toward NT-3 (Fig 3.4). These results demonstrated that segregation of functionally different sensory axonal subtypes is attainable in a two-dimensional system. However, these findings needed to be translated in a three-dimensional assay to more accurately mimic the *in-vivo* situation. A further testing of appropriate neurotrophin-delivery methods needed to be accomplished with the three-dimensional assay for the segregation studies.

The transparent multiluminal hydrogel matrix developed by Romero and colleagues (Abstract, Romero et al) was used to direct the three-dimensional axonal growth (Fig 4.1). The genetically modified Schwann cells over-expressing a particular neurotrophin (provided by the University of Miami) were decided to be the neurotrophin delivery system that would be used with this particular assay. The micro-channels of the TMM hydrogels were homogeneously loaded with GFP-expressing

Schwann cells and they successfully survived within the micro-channels for the evaluation period of 7 days with a majority of them exhibiting three-dimensional cellular cables only 24 hours after seeding (Fig 4.2). To employ this assay for the segregation studies, the TMM casting device was modified to incorporate a DRG placement region with the aid of a detachable comb device. The NGF- or NT-3-secreting Schwann cells loaded within the lumen of the micro-channels would provide neurotrophic gradients for the nociceptive and proprioceptive sensory axons respectively which would then segregate based on appropriate path-finding cues. However, in the initial set of experiments, it was found that a majority of the axonal fibers growing from the dorsal root ganglion explant placed in an ECM- filled well were unable to cross the interface between the well and the channel entry point leading to no entry within the micro-channels (Fig 4.4). It may be possible that owing to the three-dimensionality of the ECM- filled well housing the DRG explant; the axons grew in multiple focal planes, thus curbing the micro-channel entry. To effectively translate the results obtained with the PDMS Y-shaped assay in a three-dimensional setting while simultaneously mimicking the nerve growth in the nervous system, a novel three-dimensional Y-shaped assay was devised with the TMM hydrogels. Employing a modified TMM casting device, a Y-shaped micro-channel was created within the TMM hydrogel. The lumen of this micro-channel was homogeneously loaded with collagen to provide a three-dimensional facilitatory substrate for the axons (Fig 4.5). The common arm of the Y-micro-channel would act as a spacer region within which all the axons from the DRG explant placed at the channel entry point would grow together. Nearing

the bifurcation point, the specific types of axons would respond to particular neurotrophic gradients within each arm of the Y-micro-channel leading to the segregation of the subtypes of sensory axons.

Multiple neurotrophin-delivery methods were tested with this three-dimensional Y-assay including i) Schwann cells, ii) neurotrophin-coated latex beads, iii) passive diffusion, and iv) Alzet®-manufactured mini-osmotic pumps. Although Schwann cells were successfully loaded within the Y-shaped micro-channel (Fig 4.6), it was decided not to employ them as a neurotrophin-delivery method in the segregation assay. This was attributed to the knowledge that Schwann cells would not be granted FDA approval for any future clinical studies. 10µm latex beads were coated with NGF or NT-3 and loaded within each arm of the Y-micro-channel. To effectively visualize the different neurotrophin-coated beads, they were also coated with Cy2 (green) or Cy3 (red) antibodies. However, the auto-fluorescence property of the beads rendered it difficult to analyze the effectiveness of the neurotrophin or the antibody coating (Fig 4.7). Moreover, it was further recognized that the employment of this neurotrophin-delivery system would make the quantification of the diffused neurotrophins difficult since potent antibodies against NGF and NT-3 are not available. However, in lieu of the fact that beads can be homogeneously loaded within the lumen of the Y-microchannel, an employment of micro-spheres as a delivery system with this three-dimensional Y-assay could be a further possibility. Passively diffused neurotrophins within the Y-micro-channels was another neurotrophin delivery method tested wherein 4µl of NGF and NT-3 were added to cell suspension wells of the casting device and allowed to diffuse into

the micro-channels through capillary action. The three-dimensional matrix of the polymerized collagen within the Y-micro-channel would bind the neurotrophins allowing only minimal flow out of the proteins subsequent to the addition of the media. The media was only added 24 hours after the placement of the neurotrophin solutions thus, allowing sufficient time for the neurotrophins to be passively diffused within the Y-micro-channels. The Alzet®-manufactured mini-osmotic pumps were also tested as a potential delivery system allowing a controlled and pre-determined release of the desired neurotrophic factors within the three-dimensional Y-assay. Owing to the osmotic pressure phenomenon governing the functioning of the pump, the loaded proteins are delivered at a controlled rate independent of their physical or chemical proteins. The osmotic pump employed for the purpose of the assay had a release rate of 0.5µl/hr for a period of 7 days. A novel system incorporating a catheter and a hypodermic needle was utilized to ensure a localized delivery of the desired solution within the micro-channel. To provide a valuable insight into the concentrations of the neurotrophins to be used, the initial testing of the pump included filling it with the Trypan blue dye and analyzing its diffusion within and around the micro-channel as well as its progressive dilution 7 days subsequent to its loading. In addition to the diffusion of the blue dye within the micro-channel, there was a certain amount of diffusion around the micro-channel as well which could be attributed to the porosity of the agarose hydrogel. Moreover, an evaluation of the dilution of the dye through the absorbance readings revealed that the dye was diluted approximately 15,000 times at the bifurcation point. The porosity of the hydrogel would render the lumen of the micro-

channel easily accessible to the media thus, contributing to the extensive dilution of the dye within the micro-channel. Owing to this expansive dilution, it was decided that when the pumps would be loaded with the neurotrophin solutions, a higher concentration of the proteins would offset the dilution problem. Of the four delivery methods tested with the three-dimensional Y-assay, passive diffusion and osmotic pumps were later employed to deliver neurotrophins to entice the segregation of nociceptive and proprioceptive sensory axons.

A pilot study was conducted with the three-dimensional Y-TMMs and the desired neurotrophins were allowed to be passively diffused within the micro-channels. After allowing the cultures to continue for 5 days, significant axonal growth was observed within each arm of the Y-micro-channels across all the groups, demonstrating the effectiveness of the assay with regards to the compartmentalized axonal growth. Dual immunocytochemistry labeling was attempted to corroborate the functional difference in the axonal types growing within the two arms of the Y-micro-channels. However, owing to the thickness of the agaorse hydrogel, non-specific binding of the staining antibodies (beta-tubulin and CGRP) resulted in ineffective visualization of the axons within the micro-channels. To circumvent this shortcoming, phalloidin green antibody was employed for effective axonal visualization. For determining an axonal morphological distinction between the axons growing within the two arms of the Y-micro-channel in Group 4 (NGF – NT-3), mean optical density measurements were recorded at a particular area in the two arms of the Y-micro-channel. Since the NT-3 attracted proprioceptive sensory axons are highly arborized with a large number of

branches per axon, the optical density measurement in the NT-3 treatment arm would be expected to greater than that of the NGF treatment arm containing long, un-branched nociceptive sensory axons. Indeed, the optical density measurement in the boxed area (indicated by * in Fig 5.1C) in the NT-3 treatment arm was significantly greater than that of the boxed area (indicated by ** in Fig 5.1C) in the NGF treatment arm. Thus, optical density measurements could be employed as a quantification tool for examining the axonal morphological distinction between the NGF and NT-3 attracted sensory axons in this three-dimensional assay or any other future segregation assay. However, it was recognized that although a greater optical density measurement in the NT-3 treatment arm could be construed as evidence for greater arborization, a significantly greater axonal growth in the arm could also generate a greater optical density recording. Maximum axonal length measurements in the different treatment arms across all the groups revealed that the NGF treatment arms in the Y-micro-channels contained significantly longer axons as compared to the NT-3 and the Control treatment arms. This finding could be attributed to the presence of the longer, nociceptive sensory axons within the NGF treatment arm of the Y-micro-channels. Thus, there was encouraging evidence of the segregation of the nociceptive and proprioceptive sensory axons within the three-dimensional Y-assay with the aid of passively diffused neurotrophins.

To enable a controlled release of neurotrophins within the three-dimensional Y-micro-channel, Alzet® mini-osmotic pumps were utilized in the pilot study. Minimum axonal growth observed in all the groups 7 days after the implantation of the pumps suggested that the higher concentration of the neurotrophins used could have proved

detrimental to the outgrowth of the axons. Moreover, the lack of media change to facilitate the preservation of the neurotrophic gradients could have added to the build-up of the neurotrophins, further compounding the excessive concentrations. After the pumps were removed and media changed at the $7^{th}$ day, the cultures were allowed to continue for an additional 7 days and significant axonal growth was observed in one of the cultures belonging to Group 4 (NGF – NT-3) further suggesting that the initial poor axonal growth could be attributed to the excessive neurotrophin build-up. A significant amount of the neurotrophins released by the pumps within the initial 7 days would have been embedded in the three-dimensional collagen matrix of the micro-channels allowing a potent neurotrophic gradient to be retained even after the removal of the pumps. A three-dimensional z-stack reconstruction of the axonal growth in a particular area of the two arms of the Y-micro-channel belonging to Group 4 revealed the highly arborized morphological feature of the axons in the NT-3 treatment arm (Fig 5.3A). In contrast, the axons in the NGF treatment arm were long with minimal to no branching (Fig 5.3C). Thus, promising evidence was obtained with regards to the segregation of the nociceptive and proprioceptive sensory axons within the three-dimensional Y-assay with the aid of a controlled release of neurotrophins by the Alzet® mini-osmotic pumps.

The extent of diffusion of the neurotrophins within the micro-channels can be estimated; however no quantitative data has been included in this study. Pore size of 1.5% agarose used in this study is in the range of 70-80 nm (Fatin-Rouge, Starchev et al. 2004). The neurotrophins within the micro-channels, released through the Alzet® mini-pumps or by passive diffusion, can be hypothesized to restrict themselves within

the micro-channels with minimal diffusion outward. The small pore size of the agarose hydrogel could pose as a barrier to restrict diffusion of these molecules. Moreover, the negative staining results obtained with the beta-tubulin and CGRP antibodies with the three-dimensional Y-assay could be attributed to the thickness of the agarose hydrogel as well as the small pore size of the hydrogel allowing minimal penetration of the antibodies within the micro-channels. This would further suggest that the neurotrophins would be restricted within their respective micro-channel with minimal cross-talk with the neurotrophins in the adjacent micro-channels. However imperfect casting or defects in the agarose scaffold could cause diffusion of these factors. A direct measurement by ELISA is needed to address this issue.

Although a promising evidence for the segregation of the sensory axonal subtypes was observed with the pilot studies conducted with the three-dimensional Y-assay using passively diffused neurotrophins and mini-pumps, additional experiments need to be conducted with a larger number of replicates for the control groups. This would enable a more conclusive evidence for segregation while allowing efficient quantification measurements. To efficiently corroborate that the axonal types in the segregated arms of the Y-micro-channels are functionally different, transgenic mice could be created containing specific sub-populations of neurons genetically labeled with different colors. This would circumvent the problems with the dual-immunocytochemistry labeling encountered with this three-dimensional Y-assay. Moreover, a reduced thickness of the agarose hydrogel, could potentially enhance the effectiveness of the penetration of the antibodies and reduce their non-specific binding

to enable an effective dual immunocytochemistry labeling. In lieu of the fact that high concentrations of neurotrophins can be detrimental to the survival and growth of the axons, an optimum concentration of neurotrophins can be determined using diffusion gradient calculations and ELISA tests.

The results obtained from this *in-vitro* segregation assay have allowed the conduction of *in-vivo* segregation studies wherein Y-shaped micro-Renathane tubing was employed as a physical guidance model in 10 mm rat peripheral nerve gap repair. In the first set of the *in-vivo* experiments, the Y-tubes were merely filled with collagen and the proximal portion of the sciatic nerve was inserted into the stem part of the Y-tube and one of the Y-tube branches was sutured into the tibial nerve and the other branch into the sural nerve. This experiment was conducted to examine whether the tropic cues from the tibial and sural nerves would be sufficient to allow the segregation of the sensory and motor axons regenerating from the proximal portion of the transected sciatic nerve. Furthermore, the next set of experiments has included collagen-mixed NGF and NT-3 in the two branches of the Y-tube to examine the direct effect of the specific neurotrophins on the segregation of the sensory and motor axonal fibers respectively. The future experiments involve using skin and muscle homogenates within the two branches of the Y-tube to determine whether the sensory and motor axon regenerating from the divided rat sciatic nerve can be segregated into separate bundles with the aid of a Y-tube and target-derived filtrates.

Besides enhancing functional recovery, a segregation of regenerating axons into bundles containing functionally different nerve fiber populations (e.g. sensory versus

motor) would greatly facilitate the development of hand prostheses with multiple degrees of freedom and direct neural control. A peripheral nerve graft containing specific cellular or molecular cues would be employed to entice the segregation of the sensory and motor axons within it. The segregated axonal subtypes would then make contact with appropriate neural interfaces allowing an enhanced distinction of sensory and motor neural signals. Neural interfaces include appropriate electrodes designed to make better interface with the regenerating axons allowing neuromuscular stimulation and neural signal recording (Navarro, Krueger et al. 2005). The enhanced distinction of neural signals would allow the motor neuronal signals to be transmitted to the prosthetic and the sensory neurons to be stimulated based on the external stimuli facilitating a neural-directed motor control and sensory feedback. Moreover, a further segregation of the sensory axonal subtypes would significantly enhance the cutaneous sensibility of the prosthetic including its dexterity and proprioceptive abilities.

In conclusion, the results of this study demonstrated a successful segregation of the nociceptive and proprioceptive sensory axons in a novel two-dimensional Y-shaped PDMS assay with the aid of specific neurotrophin inducement; namely NGF and NT-3 respectively. Furthermore, a Y-shaped hydrogel-based transparent matrix was developed as a three-dimensional substrate for directing and segregating sensory axonal subtypes. A couple of neurotrophin-delivery methods including passive diffusion and mini-osmotic pumps were used with this assay resulting in the growth of a large number of long, non-branchy, nociceptive axons toward NGF and the short, highly arborized, proprioceptive sensory axons toward NT-3. These results demonstrate the neurotrophin-

induced segregation of nociceptive and proprioceptive sensory axons with a novel assay and appropriate delivery system for the release of specific neurotrophins. A segregation of axonal types would facilitate efficient target direction and innervation leading to improved functional recovery after a peripheral nerve gap injury; a facet unaddressed in all the current nerve gap repair strategies.

## REFERENCES


Archibald, S. J., J. Shefner, et al. (1995). "Monkey median nerve repaired by nerve graft or collagen nerve guide tube." J Neurosci **15**(5 Pt 2): 4109-23.

Blesch, A., J. M. Conner, et al. (2001). "Modulation of neuronal survival and axonal growth in vivo by tetracycline-regulated neurotrophin expression." Gene Ther **8**(12): 954-60.

Blesch, A., P. Lu, et al. (2002). "Neurotrophic factors, gene therapy, and neural stem cells for spinal cord repair." Brain Res Bull **57**(6): 833-8.

Boyd, J. G. and T. Gordon (2003). "Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo." Exp Neurol **183**(2): 610-9.

Braun, S., B. Croizat, et al. (1996). "Neurotrophins increase motoneurons' ability to innervate skeletal muscle fibers in rat spinal cord--human muscle cocultures." J Neurol Sci **136**(1-2): 17-23.

Brushart, T. M. (1988). "Preferential reinnervation of motor nerves by regenerating motor axons." J Neurosci **8**(3): 1026-31.

Brushart, T. M. (1993). "Motor axons preferentially reinnervate motor pathways." J Neurosci **13**(6): 2730-8.

Cajal S.R. (1928). Degeneration and Regeneration of the Nervous System. London: Oxford University Press.

Dahlin, L. B., C. Nordborg, et al. (1987). "Morphologic changes in nerve cell bodies induced by experimental graded nerve compression." Exp Neurol **95**(3): 611-21.

Dubey, N., P. C. Letourneau, et al. (2001). "Neuronal contact guidance in magnetically aligned fibrin gels: effect of variation in gel mechano-structural properties." Biomaterials **22**(10): 1065-75.

Evans, G. R. (2001). "Peripheral nerve injury: a review and approach to tissue engineered constructs." Anat Rec **263**(4): 396-404.

Evans, G. R., K. Brandt, et al. (2002). "Bioactive poly(L-lactic acid) conduits seeded with Schwann cells for peripheral nerve regeneration." Biomaterials **23**(3): 841-8.

Evans, G. R., K. Brandt, et al. (2000). "Clinical long-term in vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration." J Biomater Sci Polym Ed **11**(8): 869-78.

Fatin-Rouge, N., K. Starchev, et al. (2004). "Size effects on diffusion processes within agarose gels." Biophys J **86**(5): 2710-9.

Frostick, S. P., Q. Yin, et al. (1998). "Schwann cells, neurotrophic factors, and peripheral nerve regeneration." Microsurgery **18**(7): 397-405.

Fu, S. Y. and T. Gordon (1997). "The cellular and molecular basis of peripheral nerve regeneration." Mol Neurobiol **14**(1-2): 67-116.

Gordon, T., O. Sulaiman, et al. (2003). "Experimental strategies to promote functional recovery after peripheral nerve injuries." J Peripher Nerv Syst **8**(4): 236-50.

Gu, X., P. K. Thomas, et al. (1995). "Chemotropism in nerve regeneration studied in tissue culture." J Anat **186 ( Pt 1)**: 153-63.

Hadlock, T., C. Sundback, et al. (2000). "A polymer foam conduit seeded with Schwann cells promotes guided peripheral nerve regeneration." Tissue Eng **6**(2): 119-27.

Hadlock, T. A., C. A. Sundback, et al. (2001). "A new artificial nerve graft containing rolled Schwann cell monolayers." Microsurgery **21**(3): 96-101.

Inoue, K., S. Ozaki, et al. (2002). "Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons." Nat Neurosci **5**(10): 946-54.

Jerregard, H., T. Nyberg, et al. (2001). "Sorting of regenerating rat sciatic nerve fibers with target-derived molecules." Exp Neurol **169**(2): 298-306.

Jones, L. L., M. Oudega, et al. (2001). "Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury." J Physiol **533**(Pt 1): 83-9.

Karchewski, L. A., K. A. Gratto, et al. (2002). "Dynamic patterns of BDNF expression in injured sensory neurons: differential modulation by NGF and NT-3." Eur J Neurosci **16**(8): 1449-62.

Labrador, R. O., M. Buti, et al. (1998). "Influence of collagen and laminin gels concentration on nerve regeneration after resection and tube repair." Exp Neurol **149**(1): 243-52.

Lindwall, C., L. Dahlin, et al. (2004). "Inhibition of c-Jun phosphorylation reduces axonal outgrowth of adult rat nodose ganglia and dorsal root ganglia sensory neurons." Mol Cell Neurosci **27**(3): 267-79.

Lore, A. B., J. A. Hubbell, et al. (1999). "Rapid induction of functional and morphological continuity between severed ends of mammalian or earthworm myelinated axons." J Neurosci **19**(7): 2442-54.

Lundborg, G. (2004). Nerve Injury and Repair. Regeneration, Reconstruction and Cortical Remodeling. Philadelphia: Elseiver Churchill Livingstone.

Lundborg, G. (2000). "A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance." J Hand Surg [Am] **25**(3): 391-414.

Lundborg, G., L. Dahlin, et al. (1994). "Trophism, tropism, and specificity in nerve regeneration." J Reconstr Microsurg **10**(5): 345-54.

Lundborg, G., L. Dahlin, et al. (1997). "A new type of "bioartificial" nerve graft for bridging extended defects in nerves." J Hand Surg [Br] **22**(3): 299-303.

Lundborg, G., L. B. Dahlin, et al. (1982). "Nerve regeneration in silicone chambers: influence of gap length and of distal stump components." Exp Neurol **76**(2): 361-75.

Lundborg, G., L. B. Dahlin, et al. (1982). "Nerve regeneration across an extended gap: a neurobiological view of nerve repair and the possible involvement of neuronotrophic factors." J Hand Surg [Am] **7**(6): 580-7.

Madison, R. D., S. J. Archibald, et al. (1996). "Reinnervation accuracy of the rat femoral nerve by motor and sensory neurons." J Neurosci **16**(18): 5698-703.

Madison, R. D., S. J. Archibald, et al. (1999). "Factors contributing to preferential motor reinnervation in the primate peripheral nervous system." J Neurosci **19**(24): 11007-16.

Maki, Y. (2002). "Specificity in peripheral nerve regeneration: a discussion of the issues and the research." J Orthop Sci **7**(5): 594-600.

Maki, Y., T. Yoshizu, et al. (1996). "The selectivity of regenerating motor and sensory axons." J Reconstr Microsurg **12**(8): 547-51.

Meek, M. F., J. F. van der Werff, et al. (2003). "Functional nerve recovery after bridging a 15 mm gap in rat sciatic nerve with a biodegradable nerve guide." Scand J Plast Reconstr Surg Hand Surg **37**(5): 258-65.

Miloro, M., L. E. Halkias, et al. (2002). "Low-level laser effect on neural regeneration in Gore-Tex tubes." Oral Surg Oral Med Oral Pathol Oral Radiol Endod **93**(1): 27-34.

Ming, G. L., S. T. Wong, et al. (2002). "Adaptation in the chemotactic guidance of nerve growth cones." Nature **417**(6887): 411-8.

Navarro, X., T. B. Krueger, et al. (2005). "A critical review of interfaces with the peripheral nervous system for the control of neuroprostheses and hybrid bionic systems." J Peripher Nerv Syst **10**(3): 229-58.

Ngo, T. T., P. J. Waggoner, et al. (2003). "Poly(L-Lactide) microfilaments enhance peripheral nerve regeneration across extended nerve lesions." J Neurosci Res **72**(2): 227-38.

Nguyen, Q. T., J. R. Sanes, et al. (2002). "Pre-existing pathways promote precise projection patterns." Nat Neurosci **5**(9): 861-7.

Nicoli Aldini, N., M. Fini, et al. (2000). "Guided regeneration with resorbable conduits in experimental peripheral nerve injuries." Int Orthop **24**(3): 121-5.

Oku, R., M. Satoh, et al. (1987). "Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats." Brain Res **403**(2): 350-4.

Patrick, C. W., Jr., B. Zheng, et al. (2001). "Muristerone A-induced nerve growth factor release from genetically engineered human dermal fibroblasts for peripheral nerve tissue engineering." Tissue Eng **7**(3): 303-11.

Politis, M. J. (1985). "Specificity in mammalian peripheral nerve regeneration at the level of the nerve trunk." Brain Res **328**(2): 271-6.

Politis, M. J., K. Ederle, et al. (1982). "Tropism in nerve regeneration in vivo. Attraction of regenerating axons by diffusible factors derived from cells in distal nerve stumps of transected peripheral nerves." Brain Res **253**(1-2): 1-12.

Rangappa, N., A. Romero, et al. (2000). "Laminin-coated poly(L-lactide) filaments induce robust neurite growth while providing directional orientation." J Biomed Mater Res **51**(4): 625-34.

Rodriguez, F. J., E. Verdu, et al. (2000). "Nerve guides seeded with autologous schwann cells improve nerve regeneration." Exp Neurol **161**(2): 571-84.

Romero, M.I., Galvan-Garcia, P., et al. (2006). *Abstract* "Directed axonal regeneration in peripheral nerve gap repair via a multiluminal biosynthetic implant." Exp Neurol.

Romero, M. I., N. Rangappa, et al. (2000). "Extensive sprouting of sensory afferents and hyperalgesia induced by conditional expression of nerve growth factor in the adult spinal cord." J Neurosci **20**(12): 4435-45.

Rutkowski, G. E. and C. A. Heath (2002). "Development of a bioartificial nerve graft. II. Nerve regeneration in vitro." Biotechnol Prog **18**(2): 373-9.

Schmidt, C. E. and J. B. Leach (2003). "Neural tissue engineering: strategies for repair and regeneration." Annu Rev Biomed Eng **5**: 293-347.

Smith, G. M. and M. I. Romero (1999). "Adenoviral-mediated gene transfer to enhance neuronal survival, growth, and regeneration." J Neurosci Res **55**(2): 147-57.

Soldani, G., G. Varelli, et al. (1998). "Manufacturing and microscopical characterisation of polyurethane nerve guidance channel featuring a highly smooth internal surface." Biomaterials **19**(21): 1919-24.

Son, Y. J. and W. J. Thompson (1995). "Schwann cell processes guide regeneration of peripheral axons." Neuron **14**(1): 125-32.

Sterne, G. D., G. R. Coulton, et al. (1997). "Neurotrophin-3-enhanced nerve regeneration selectively improves recovery of muscle fibers expressing myosin heavy chains 2b." J Cell Biol **139**(3): 709-15.

Stevens, A. and J. S. Lowe (1997). Nervous Tissue. In *Human Histology*. Second edition. Mosby, London. 77-98.

Stoll, G. and H. W. Muller (1999). "Nerve injury, axonal degeneration and neural regeneration: basic insights." Brain Pathol **9**(2): 313-25.

Sundback, C., T. Hadlock, et al. (2003). "Manufacture of porous polymer nerve conduits by a novel low-pressure injection molding process." Biomaterials **24**(5): 819-30.

Terenghi, G. (1999). "Peripheral nerve regeneration and neurotrophic factors." J Anat **194 ( Pt 1)**: 1-14.

Terenghi, G., J. S. Calder, et al. (1998). "A morphological study of Schwann cells and axonal regeneration in chronically transected human peripheral nerves." J Hand Surg [Br] **23**(5): 583-7.

Tuszynski, M. H., J. Conner, et al. (2002). "New strategies in neural repair." Prog Brain Res **138**: 401-9.

Verdu, E., R. O. Labrador, et al. (2002). "Alignment of collagen and laminin-containing gels improve nerve regeneration within silicone tubes." Restor Neurol Neurosci **20**(5): 169-79.

Xu, X., W. C. Yee, et al. (2003). "Peripheral nerve regeneration with sustained release of poly(phosphoester) microencapsulated nerve growth factor within nerve guide conduits." Biomaterials **24**(13): 2405-12.
Yang, Y., L. De Laporte, et al. (2005). "Neurotrophin releasing single and multiple lumen nerve conduits." J Control Release **104**(3): 433-46.

Yannas, I. V. and B. J. Hill (2004). "Selection of biomaterials for peripheral nerve regeneration using data from the nerve chamber model." Biomaterials **25**(9): 1593-600.

Yin, Q., G. J. Kemp, et al. (1998). "Neurotrophins, neurones and peripheral nerve regeneration." J Hand Surg [Br] **23**(4): 433-7.

Yoshii, S., M. Oka, et al. (2003). "Bridging a 30-mm nerve defect using collagen filaments." J Biomed Mater Res A **67**(2): 467-74.

Young, C., E. Miller, et al. (2001). "Nerve growth factor and neurotrophin-3 affect functional recovery following peripheral nerve injury differently." Restor Neurol Neurosci **18**(4): 167-75.

Zhang, L., A. O. Hoff, et al. (2001). "Arthritic calcitonin/alpha calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity." Pain **89**(2-3): 265-73.

# BIOGRAPHICAL INFORMATION

The author, Ebrahim Bengali, completed his Bachelor of Science in Computer Science from Georgia Institute of Technology, Atlanta, Georgia. He enrolled in the Master of Science, joint program of Biomedical Engineering at University of Texas at Arlington/ University of Texas Southwestern Medical center in January 2004. During the course of his education he has worked on various class projects. He worked as a Graduate Research assistant at the Southwestern Medical Center, Dallas/Texas Scottish Rite Hospital for Children, Dallas. This project provided an opportunity to work on an aspect of nerve repair that has been rarely attempted and involved the design and fabrication of novel *in-vitro* devices. The outcome of this study would assist in improving functional recovery after nerve injuries. In the future he looks forward to pursue a career in the Biotechnology field and eventually set up a company in the same.