Influence of Implant Parameters on the Mechanisms of Peripheral Nerve Regeneration

by

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S.M., Mechanical Engineering
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Doctor of Philosophy in Mechanical Engineering

at the
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Abstract

The regeneration of the rat sciatic nerve across a 10-mm gap was studied under a variety of experimental conditions. Large-pore collagen, small-pore collagen, and silicone tubes were implanted unfilled and filled with a specific collagen-glycosaminoglycan (CG) matrix. Sciatic nerve autograft and untreated groups served as controls. Following 6, 30, and 60 weeks, regenerated nerves were evaluated histologically for the number and diameter of axons and connective tissue composition. At 60 weeks, the nerves were evaluated electrophysiologically.

Implantation of a matrix-filled large-pore collagen tube (LC/M) resulted in regeneration of axon structure and electrophysiological function that were not significantly different from the autograft, suggesting that the LC/M implant can be used as an off-the-shelf replacement for autografting as a clinical treatment for peripheral nerve injuries. A significant amount of axon maturation was observed between 30 and 60 weeks, indicating that the regenerative process was ongoing at 1 year. Filling a tube with the CG matrix significantly increased the number of axons reaching the distal nerve branches and the amount of axon maturation compared to unfilled tubes. Based on these findings, it was hypothesized that the CG matrix enhances the rate of axon elongation across the nerve gap, which may lead to the observed long-term effects. In addition, it was observed that the silicone tube groups had significantly fewer mature axons than the large-pore collagen tube groups. In the silicone tube groups, the combination of necking of the nerve trunk at the center of the gap and the presence of a thick tissue capsule containing multiple layers of contractile cells suggested that the tissue capsule may be applying contractile forces on the nerve trunk. It was hypothesized that the restrictive forces caused by the contractile capsule prevented the growth of axons in diameter, leading to the significant effect of tube composition on axon maturation. It was also observed that a similar contractile capsule formed in the untreated group, hypothetically leading to the formation of a neuroma.

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For support with the experimentation, I have many people to thank. First, Dr. Hsu, thank you for performing the animal surgeries and sacrifices - I couldn’t have done it without you! Thanks also to Sandra Taylor and Martha Murray for advice and support on the α-smooth muscle actin immunohistochemical staining. Without your guidance, I might still be in the laboratory iterating the protocol.

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Chapter 1: Background

1.1 Clinical Significance

In the United States, approximately 200,000 patients are treated each year for peripheral nerve injuries that require surgical intervention (Madison, et al., 1992). These injuries are a result of trauma, disease, or surgical procedures that require transection of peripheral nerves to gain access to the surgical site. An example of a surgical trauma is the procedure used for oncological surgery to remove tumors from the cranial base, in which the facial nerve is severed to provide access to this region (Janecka, et al., 1990).

Regardless of the cause of peripheral nerve injury, the result is partial or total paralysis in the affected tissue if left untreated. Clinically, direct suturing or autografting of the gap between the two nerve stumps is commonly employed. Direct suture techniques are used when the two nerve stumps can be directly apposed without tension forming at the suture line (Madison, et al., 1992). With this type of treatment in the median nerve, only 25% of patients recovered full motor function and 3% recovered full sensory function (Mackinnon and Dellon, 1988). Autografting is the current clinical treatment for severed peripheral nerves where a significant gap (e.g., greater than 10 mm) exists between the nerve ends, preventing direct suturing. This procedure requires that an additional site be traumatized to remove an intact sensory nerve (e.g., sural nerve), which serves as the autograft tissue. With this type of treatment in the median nerve, only 20% of patients recovered full motor function and no patients recovered full sensory function (Mackinnon and Dellon, 1988). Since the results of such procedures have been very unsatisfactory, a need exists for new approaches to these injuries. Insertion of the two nerve stumps in a carefully designed tubular implant is a third approach which could conceivably improve the quality of regeneration and lead to a satisfactory clinical outcome.
The advantage of incorporating a tubular implant into the peripheral nerve treatment protocol, besides obviating the need for graft tissue, is that the tubular implant can be modified for improved performance. Parameters of the tube which have been varied include chemical composition (Fields, et al., 1989), permeability to molecules of different diameter (Aebischer, et al., 1988; Jenq, et al., 1987; Li, et al., 1990; Li, et al., 1992), degradability (Aldini, et al., 1996; den Dunnen, et al., 1993; Robinson, et al., 1991; Tountas, et al., 1993) and tube dimensions (Ducker and Hayes, 1968). Tubes can also be filled with substrate materials which may enhance the regeneration of axons across the gap. Substrates composed of extracellular matrix proteins (Bailey, et al., 1993; Bryan, et al., 1993; Chang, et al., 1990; Glasby, et al., 1986; Madison, et al., 1988; Ohbayashi, et al., 1996; Rosen, et al., 1990; Yannas, et al., 1987; Yoshii, et al., 1987) or Schwann cells (Guenard, et al., 1992; Kim, et al., 1994) have been implanted within tubes to study peripheral nerve regeneration. Several implant designs have been employed to date, however, few have performed as well as the autograft and none have improved the outcome over that of the autograft in cases where the gap between the two nerve stumps was 10 mm or more (Table 1.1).

1.2 Peripheral Nervous System Anatomy and Physiology

Peripheral nerves have a complex organization of conducting and non-conducting tissues which allow for the efficient transfer of electrical impulses to and from the central

<table>
<thead>
<tr>
<th>Equivalent to the Autograft</th>
<th>Inferior to the Autograft</th>
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<tbody>
<tr>
<td>Collagen tube filled with Schwann cells (Kim, et. al., 1994)</td>
<td>Unfilled silicone tube (Muller, et. al., 1987; Fields, et. al., 1989)</td>
</tr>
<tr>
<td></td>
<td>Collagen tube filled with collagen gel (Kim, et. al., 1994)</td>
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Table 1.1 Status of the tubular implants being employed today to bridge 10-mm gaps as compared with the current clinical standard, the autograft repair. Unfortunately, few studies directly compare the regeneration through the test device with an autograft control. However, in studies which incorporated both a tubular implant and the autograft, only one device was reported to be equivalent to the autograft repair.
nervous system (*i.e.*, brain and spinal cord). The conducting members of the peripheral nerve are nerve cells which can be broken down into three primary components: the neuron cell body, the axon (or nerve cell process), and the nerve terminal (or synapse). The non-conducting tissue protects the conducting tissue by surrounding it in three primary layers: epineurium, perineurium, and endoneurium.

### 1.2.1 Conducting Tissue

For motor neurons, the cell bodies reside within the ventral gray matter of the spinal cord and extend their processes, or axons, out of the spinal cord through the ventral nerve roots. Each neuron cell body has one axon which travels from the cell body to the distal target organ without interruption. Motor axons are termed efferent fibers because they transfer information away from the central nervous system to the skeletal, smooth and cardiac muscles and glands (Kandel, et al., 1991). Although there is only one output (*i.e.*, axon), each motor neuron cell body receives hundreds of inputs from neurons in the brain, other motor neurons in the ventral horn of the spinal cord, interneurons located within the spinal cord, and sensory neurons. The primary function of the motor neuron cell body, then, is to assemble all of the incoming information into one signal and then initiate firing of that signal down the axon (Kandel, et al., 1991). In contrast to motor neurons, sensory neuron cell bodies reside in the dorsal root ganglia, located just outside the spinal cord, and each extends one axon into the periphery. Sensory axons carry afferent information from the peripheral tissues back to the central nervous system. Therefore, the function of a sensory neuron cell body is to transmit the received input from the periphery to many other neuron cell bodies including motor neurons, interneurons located within the spinal cord, and ascending spinal cord neurons which transmit information to the brain (Kandel, et al., 1991).

Axons are the cell processes which connect the cell body to the distal target (*i.e.*, muscle or sensory organ). The cytoplasm inside each axon is termed axoplasm and is a viscous fluid containing microtubules and neurofilaments. These two components give the
axon a roughly uniaxial structure and are responsible for the intra-cellular transport of proteins to and from the neuron cell body (Madison, et al., 1992).

Two structural types of axon are present in all peripheral nerves: myelinated (Figure 1.1a) and unmyelinated (Figure 1.1b), differing in structure primarily with respect to axon diameter and the role of the Schwann cell. The Schwann cell is a partner with the axon and is the primary non-conducting cell in peripheral nerves. In myelinated axons, each Schwann cell is associated with only one axon (Figure 1.1a). The cytoplasm of the Schwann cell is repeatedly wrapped around the axon to form a myelin sheath which insulates the axon during electrical conduction. The thickness of the myelin sheath varies between axons depending on the diameter of the axon, with normal g-ratios (axon core diameter/fiber diameter) ranging from 0.65-0.8 (Fields, et al., 1989). Each Schwann cell serves a length of axon approximately 1-2 mm long, with the nucleus residing in the center of that distance. The locations where two Schwann cells meet along the axial length of the axon are termed nodes of Ranvier. The nodes are approximately 2 μm in length (Kandel, et al., 1991). The node of Ranvier is an important

![Figure 1.1 Schematic representations of (a) myelinated and (b) unmyelinated axons in the peripheral nervous system shown in cross-sectional orientation. (a) In the myelinated axon, the Schwann cell wraps its cytoplasm repeatedly around the axon core (axoplasm) to form the myelin sheath. The endoneurium encompasses both the axon and the Schwann cell and provides protective and structural support to the axon. (b) Many unmyelinated axons share the same Schwann cell and each has a single layer of Schwann cell cytoplasm surrounding it. The endoneurium encompasses the Schwann cell in the same way as in the myelinated axon case.](image)
site for propagation of action potentials along the axon (described below). Unmyelinated axons do not have the wrapped myelin sheath and are therefore, uninsulated. Unlike myelinated axons, one Schwann cell envelopes many unmyelinated axons (Figure 1.1b).

In mature, uninjured nerves, myelinated axons range from 1-16 μm in diameter for the rat and are responsible for all of the motor activity and a portion of the sensory activity of the peripheral nerves (Strichartz and Covino, 1990). Myelinated axons fall into two classes: A-fibers and B-fibers. A-fibers are the largest in diameter and carry primarily motor impulses to the muscles and also proprioceptive information (i.e., limb position) from the muscles and joints to the spinal cord (Strichartz and Covino, 1990). Many studies have shown that the conduction velocity of an axon is proportional to the axon diameter (Arbuthnott, et al., 1980; Hursh, 1939); hence, the A-fibers have the highest conduction velocities of all axons. Myelinated B-fibers have diameters ranging from 1-3 μm and carry information primarily to the autonomic system. Unmyelinated axons, termed C-fibers, are much smaller, 0.3-1.2 μm in diameter, and conduct primarily sensory impulses involved in pain, temperature and touch (Strichartz and Covino, 1990). C-fibers have very slow conduction velocities due to the lack of insulating myelin and the high resistance inherent from the small diameter of the fibers. Unmyelinated axons outnumber myelinated axons in all peripheral nerves. In humans, the ratio of myelinated to unmyelinated axons is 1:3 and in rats it ranges from 1:2 to 1:5 depending on the nerve. For example, in the rat sciatic nerve, there is one myelinated axon for every 1.9 - 2.5 unmyelinated axons (Lisney, 1989).

The primary function of a peripheral nerve axon is to carry an electrical signal, or action potential, between the target organ and the cell body. In motor neurons, the electrical signal is generated within the neuron cell body in response to information from other neurons and is then sent from the neuron cell body via the axon (efferent signal). In contrast, the electrical signal for sensory axons is generated in the periphery within special sensory receptors that
respond to various changes in their environment. Most sensory receptors produce electrical signals in response to mechanical stimulation (i.e., pressure or stretch) (Kandel, et al., 1991).

Nerve conduction is a result of ion diffusion across the axonal membrane. At rest, the axon is negatively charged and the surrounding extracellular material is positively charged, therefore, there is a resting potential across the axon membrane. When an impulse is generated, sodium ion channels in the membrane open and sodium ions (Na⁺) rush into the axon at the site of the signal. As the membrane begins to depolarize, additional voltage-dependent sodium ion channels open, resulting in further depolarization of the membrane (Kandel, et al., 1991). This local depolarization zone propagates along the axon as neighboring sodium channels open. Once the signal, or action potential, passes, potassium ion channels open and potassium ions (K⁺) rush out of the axon to restore the local resting potential. This is the basic mechanism in an unmyelinated axon. In myelinated axons, the myelin sheath insulates the axon so that there is no ion diffusion in the myelin covered regions. A high concentration of ion channels are present in the nodes of Ranvier allowing for a large influx of sodium ions (and outflux of potassium ions) at these locations which insures that the amplitude of the signal does not decay over the length of the axon. The diffusion of ions occurs only at the nodes of Ranvier where there is no myelin; therefore, the action potential ‘jumps’ from one node to the next; a process called saltatory conduction (Vander, et al., 1990).

In order for any functional outcome to occur following axon conduction, the axon must transfer the electrical signal information to the target via a synapse. For motor axons, the target is a muscle fiber and, for sensory axons, the targets are other neurons located in the spinal cord. At the neuromuscular junction, the terminal of a motor axon interacts with a muscle fiber through a synaptic cleft (Figure 1.2). As the electrical signal reaches the axon terminal, the pre-synaptic membrane of the axon depolarizes. This electrical depolarization of the membrane leads to a release of a chemical neurotransmitter, acetylcholine in the case of the neuromuscular junction, into the synaptic cleft. Hence, electrical energy is transferred into chemical energy.
**Figure 1.2** Schematic representation of the sequence of steps involved in signal transmission across a typical neuromuscular junction synapse. 1. The nerve impulse travels down the axon until it reaches the axon terminal. 2. The pre-synaptic membrane depolarizes releasing acetylcholine (●) into the synaptic cleft. 3. Acetylcholine attaches to acetylcholine receptors on the post-synaptic membrane. 4. Attachment of the acetylcholine opens sodium ion channels and sodium ions (●) rush into the muscle fiber. 5. The influx of sodium ions causes the muscle fiber to depolarize and fire a muscle action potential.

Acetylcholine receptors located on the post-synaptic membrane open transmitter-gated ion channels when acetylcholine binds to the receptor. Sodium ions rush into the muscle fiber through the opened ion channels causing a depolarization of the muscle fiber (chemical energy is transferred back to electrical energy) and hence, an action potential is generated within the muscle (Kandel, et al., 1991).

### 1.2.2 Non-Conducting Tissue

Three levels of connective tissue surround, support and protect the conducting fibers. The first level is the endoneurium which surrounds each individual axon and its associated Schwann cell (Figures 1.1 and 1.3). The endoneurium is composed of fibroblasts and fine,
loosely packed, type III collagen fibrils (Madison, et al., 1992; Rosen, et al., 1983). The primary functions of the endoneurium are to provide packing between nerve fibers (Thomas and Olsson, 1975) and support the individual axonal pathways (Rosen, et al., 1983). Axons are bundled together into fascicles which contain the axons, Schwann cells and the endoneurial tissue. A nerve can contain many fascicles (up to 100) and the fascicles are constantly changing size and shape along the length of the nerve (Sunderland, 1990). Fascicles range in size from 0.1 - 1 mm in diameter depending on the number of fascicles at a given location and the size of the nerve. At distal locations, the fascicles organize into nerve branches and then separate from the nerve trunk (Sunderland, 1990). Each fascicle is surrounded by the second level of connective tissue, the perineurium (Figure 1.3), which is composed of three concentric layers that form a dense sheath around the fascicle. The inner two layers are composed of

![Diagram of nerve tissue](image)

**Figure 1.3** Schematic representation of a cross-section of peripheral nerve tissue. Specifically, the non-conducting components are illustrated. The endoneurium surrounds each individual axon and fills the interaxonal space. Intrafascicular blood vessels, primarily capillaries, run parallel to the axons, however, they are not visible at this scale. The perineurium surrounds each fascicle and bundles the axons together. The epineurium provides a loose packing for the fascicles and contains the large, extrafascicular blood vessels. The diameter of a typical nerve trunk is approximately 1 mm in the rat.
flattened perineurial cells that form tight intercellular junctions to create a continuous cell layer and a basement membrane. The outer layer of the perineurium has thick, type III collagen fibers and a less ordered appearance (Madison, et al., 1992; Rosen, et al., 1983). The perineurium maintains an internal pressure in the fascicle, provides strength and elasticity to the nerve, and acts as a diffusion barrier (Sunderland, 1990). The final level of the connective tissue is the epineurium which is a loose matrix of thick, type I collagen fiber bundles and fibroblasts (Rosen, et al., 1983) (Figure 1.3). The major functions of the epineurium are to hold the fascicles together in one cohesive nerve trunk, and to provide cushioning against compressive trauma (Madison, et al., 1992).

The connective tissue structures also contain an abundant vascular supply that serves the peripheral nerve. Large blood vessels are contained within the epineurium (extrafascicular blood supply, Figure 1.3), while small capillaries run parallel to the axons within the fascicles (intrafascicular blood supply). The intrafascicular blood vessels have tight endothelial junctions which restrict certain molecules from entering the nerve fascicle. The purpose of this protein barrier is to reduce the likelihood of infection in the neural tissue, similar in concept to the blood-brain barrier (Madison, et al., 1992).

1.3 Requirements for Successful Regeneration

Successful regeneration of the peripheral nervous system requires the completion of three major tasks: survival of the neuron cell bodies after injury, growth of axons across the injury site and through the distal stump, and functional reconnection of the axons with appropriate distal targets (Madison, et al., 1992). At a minimum, these three tasks must be completed in order for function to be restored either partially or completely.

Survival of enough neuron cell bodies is essential for the ultimate return of function in the affected tissue. Previous studies have shown that the majority of motor neurons in the spinal cord survive peripheral nerve transection injuries; however, the number of neuron cell
deaths increases as the peripheral nerve injury moves closer in proximity to the spinal cord (Fu and Gordon, 1997; Lisney, 1989). Sensory neurons are more susceptible to cell death following injury, with up to 50% dying in response to axotomy (Fu and Gordon, 1997; Lisney, 1989).

The second task that must be completed is the growth of axons across the injury site and through the distal nerve stump. The mechanisms by which this axon growth occurs will be described in detail in Chapter 3. Briefly, this is the task which requires the axons to emerge from the proximal nerve stump, traverse the injury site, enter the distal nerve fascicles, travel through the distal nerve stump, and ultimately reach a distal target. In addition, once connected to an endpoint, the axon must grow in diameter in order to regain its functional capacity. Tubular devices and autografts are implanted within the injury site and, therefore, this is the primary task in which implants may directly affect regeneration.

The final task which must be completed is the formation of functional connections with the appropriate distal targets. This requires that each regenerating axon reach an appropriate target (i.e., sensory or muscle) and form a functional connection with that target. Studies have shown that motor axons have an affinity for growing toward muscle endpoints (Brushart, 1988; Madison, et al., 1996) and sensory axons have the ability to preferentially innervate sensory targets within the muscle (i.e., muscle spindles or stretch receptors) (Madison, et al., 1996). However, a motor axon must not only connect to a muscle endpoint but, more specifically, to the appropriate muscle in order to carry out functional instructions from the brain. It is important to note that, without completion of this final task, there will be minimal functional recovery.

1.4 Tissue Specific Analogs of the Extracellular Matrix

Certain analogs of the extracellular matrix (ECM) have been shown to possess surprising morphogenetic activity during healing of lesions in various anatomical sites. The
two of these analogs which have been studied most extensively are a skin regeneration template (SRT) and a nerve regeneration template (NRT) (Chang, et al., 1990; Yannas, 1995; Yannas, et al., 1989).

The first of these analogs, referred to as the skin regeneration template (SRT), has induced regeneration of dermis in full-thickness skin wounds in the guinea pig model (Murphy, et al., 1990; Yannas, et al., 1982; Yannas, et al., 1981; Yannas, et al., 1989), the porcine model (Orgill, et al., 1996) and in humans (Burke, et al., 1981; Heimbach, et al., 1988; Stern, et al., 1990; Yannas, et al., 1981). Since it is well known that the dermis of the adult mammal does not regenerate spontaneously (Billingham and Medawar, 1951; Billingham and Medawar, 1955), the SRT is required for dermal regeneration in all commonly encountered skin wounds which are sufficiently deep to have compromised the dermis. The SRT is currently used as a dermal regeneration treatment for patients who have sustained deep burns or deep mechanical trauma, including trauma from elective surgery, and who would otherwise have been treated with autografts (Heimbach, et al., 1988).

Another ECM analog, referred to as the nerve regeneration template (NRT), has induced regeneration of a functional peripheral nerve across a 15-mm gap in the rat sciatic nerve model (Yannas, et al., 1985; Yannas, et al., 1987). In this model, the highly porous ECM analog is used to fill the lumen of a tube, made either of silicone (non-degradable) or collagen (biodegradable); the nerve stumps are inserted into the tube and are prevented from being displaced further by two sutures at each stump. If the silicone tube is used without an ECM analog, the maximum gap distance which can be bridged by a functional peripheral nerve in this animal model is only 10 mm (Lundborg, et al., 1982). The structure of the NRT has been determined by selection of the network structure which resulted in maximum regenerative activity in the rat sciatic nerve model, using a gap length of 10 mm (Chang, et al., 1990; Chang and Yannas, 1992).
The structure of these biologically active ECM analogs has been characterized at the scale of the nanometer as well as at the scale of the micrometer. In the former scale, both ECM analogs referred to above are graft copolymers of type I collagen and chondroitin 6-sulfate which are cross-linked covalently and can therefore be described as insoluble macromolecular networks. At the larger scale, the analogs are highly porous matrices which are characterized in terms of the pore volume fraction, the average pore diameter and the average orientation of pore channel axes. Being insoluble, the ECM analogs cannot be isolated and characterized structurally using common biochemical techniques for the structural analysis of proteins. However, structural methodology which has been used to characterize synthetic polymeric networks, including infrared and Raman spectroscopy, rubber elasticity analysis of network structure, and various forms of microscopy, have been employed in the characterization of the ECM analogs. The structures of the SRT (Yannas, et al., 1989) and the NRT (Chang, et al., 1990; Chang and Yannas, 1992) (Table 1.2) have been identified by selecting the analogs of maximum activity from a large number of ECM analogs with related structure. Inspection of Table 1.2 shows that the NRT is significantly different from the SRT with respect to network cross-link density, average pore diameter and average orientation of pore channel axes. It is important to note that implantation of the SRT into a peripheral nerve lesion results in very poor regeneration. This further highlights that the ECM analogs, although similar, are highly tissue specific.

<table>
<thead>
<tr>
<th>Design parameter of ECM analog</th>
<th>Skin Regeneration Template (SRT)</th>
<th>Nerve Regeneration Template (NRT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I collagen/chondroitin 6-sulfate</td>
<td>98/2</td>
<td>98/2</td>
</tr>
<tr>
<td>Degradation half-life, weeks</td>
<td>1.5</td>
<td>6 - 8</td>
</tr>
<tr>
<td>Average pore diameter, μm</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>Pore channel orientation</td>
<td>random</td>
<td>axial</td>
</tr>
</tbody>
</table>

Table 1.2 Structural properties of the two regeneration templates which have been optimized. The skin and nerve regeneration templates have the same chemical composition but differ significantly in degradation rate, average pore diameter, and pore channel orientation (Yannas, et al., 1989)
Investigations are currently in progress to design similar ECM analogs, using the same systematic approach, that can be used as regeneration templates in other tissues. For example, an ECM analog, similar in composition to the SRT and the NRT, has been shown capable of regenerating the canine knee meniscus (Rodkey, et al., 1992; Stone, et al., 1990). Preliminary studies have been conducted using a variation of the SRT as an experimental substrate for regenerating tendon (Louie, et al., 1994). The findings suggest that implantation of this substrate altered the formation of scar tissue in ruptured Achilles tendon injuries. Further investigation is necessary to optimize the matrix properties and perhaps to incorporate tendon cells (tenocytes) into the experimental substrate to enhance the regenerative response (Louie, 1997; Louie, et al., 1997a; Louie, et al., 1997b). A similar approach is also being taken to design a substrate which can be used to study the possible induction of regeneration in the repair of articular cartilage defects. The findings in cartilage suggest a change in the chemical composition of the substrate from type I collagen to type II collagen, which would match the native collagen type for that tissue (Nehrer, et al., 1997a; Nehrer, et al., 1997b). In addition, cartilage, like tendon, will likely require the incorporation of cells (chondrocytes) into the matrix since the native tissue contains very few cells and is poorly vascularized (Nehrer, et al., 1997a). Finally, an ECM analog is being designed as a substrate for the regrowth of spinal cord axons across paralyzing injuries (Spilker, 1997; Spilker, et al., 1997; Spilker, et al., 1996). Each tissue brings its own specific regeneration challenges; however, using a systematic design approach, it has been proven that ECM analogs can be adapted into tissue-specific substrates which are capable of significantly enhancing regeneration.

1.5 Goal of this Research

This study comprised three major objectives: 1) to determine the extent and time course of recovery following regeneration over a period of slightly over 1 year (60 weeks), 2) to evaluate the effects of implant parameters on the mechanisms of axon regeneration, and 3) to examine the effects of implant parameters on the connective tissue response to injury.
Seven different device groups (with 9 animals per device group) were evaluated: matrix-filled large-pore collagen tube, matrix-filled small-pore collagen tube, matrix-filled silicone tube, unfilled large-pore collagen tube, unfilled small-pore collagen tube, unfilled silicone tube and autograft. An untreated gap was used as a control. The matrix was a collagen-glycosaminoglycan (CG) copolymer that was fabricated using an axial freezing bath and freeze-dryer. This procedure resulted in matrices with axially aligned pores, 20-50 μm in diameter, which are optimal for peripheral nerve regeneration (Chang, et al., 1990). The collagen tubes were biodegradable and retained their structural integrity in vivo for the period of the proposed study, as previously reported by others (Archibald, et al., 1995). The devices were implanted in a 10-mm gap in the rat sciatic nerve.

Following 6, 30, and 60 weeks of implantation, the nerves were retrieved and prepared for histological evaluation. Quantitative measurements of the regenerated myelinated axons (number and diameter) were made using digitized image analysis. Primary techniques for morphological evaluation included light microscopy and immunohistochemistry. Qualitative and quantitative histologic observations were made at four locations along the length of the regenerated nerve (-1, 5, 11, and 20 mm distances from the proximal cut). In addition, at 60 weeks, sensorimotor performance evaluations and in vitro electrophysiological measurements were performed on each animal prior to histological evaluation.

The bulk of the experimental findings have been organized into Chapters 2-4 according to the specific objective being addressed. In Chapter 2, I present the findings on the extent and time course of recovery following regeneration over a period of over 1 year, in Chapter 3, I address the effects of implant parameters on axon regeneration, and in Chapter 4, I report the findings of implant effects on connective tissue response to injury. Each of these chapters is a self-contained unit, encompassing its own Introduction, Methods, Results, and Discussion sections. In Chapter 5, I restate the conclusions from each chapter, tie the results together, and make brief recommendations for future study based on these findings.
Chapter 2: Long-Term Recovery of Peripheral Nerve Structure and Function

2.1 Introduction

2.1.1 Implant Strategies for the Regeneration of Peripheral Nerve

Tubes have been used to repair nerves injured by transection for over 100 years (Fields, et al., 1989). It has been suggested that this procedure, referred to as tubulation, serves a number of functions: allows regulatory factors and cells from the distal stump to remain in direct contact with the site of potential regeneration, provides direction for axons elongating across the gap, and isolates the wound space from exogenous cells and tissues (Madison, et al., 1992). Tubes used to bridge nerve defects have been fabricated from a variety of natural and synthetic polymers (Aldini, et al., 1996; Archibald, et al., 1991; Archibald, et al., 1995; den Dunnen, et al., 1993a; Robinson, et al., 1991; Tountas, et al., 1993); however, only a few studies have directly compared regeneration following implantation of tubes with varying compositions (Henry, et al., 1985; Madison, et al., 1987; Navarro, et al., 1996). While tubes have yielded promising results, the gap length that can be successfully bridged by an empty tube is limited. In particular, silicone tubes which have been implanted without being prefilled have facilitated the elongation of axons across 10-mm gaps but have been ineffective across longer gaps (Lundborg, et al., 1982). There is evidence that regeneration through nondegradable and impermeable tubular devices can be improved by using degradable (Aldini, et al., 1996; den Dunnen, et al., 1993a; Robinson, et al., 1991; Tountas, et al., 1993) or permeable tubes (Aebischer, et al., 1988; Jenq, et al., 1987; Li, et al., 1992).

Certain components of the extracellular matrix, including collagen, laminin and fibronectin, have also been shown capable of enhancing the regeneration of axons across tubulated nerve gaps (Bailey, et al., 1993; Bryan, et al., 1993; Glasby, et al., 1986; Gulati,
1988; Madison, et al., 1985; Madison, et al., 1988; Ohbayashi, et al., 1996; Rosen, et al., 1990; Yoshii, et al., 1987). Aside from increasing the number of axons crossing the gap, substrates have increased the maximum gap distance that can be bridged by axonal tissue from 10-mm to at least 15-mm (Madison, et al., 1988; Yannas, et al., 1987) as well as increasing the rate of axonal elongation (Madison, et al., 1985; Ohbayashi, et al., 1996). In preliminary studies, a collagen-glycosaminoglycan (CG) matrix, ensheathed by a silicone tube, facilitated regeneration of axons across a 15-mm gap in the rat sciatic nerve (Barnes, 1985; Yannas, et al., 1985; Yannas, et al., 1987). A series of CG matrices were also studied as substrates inside a silicone tube and it was observed that the average pore diameter and degradation rate affected profoundly the recovery of electrophysiological functional correlates in nerves regenerated across a 10-mm gap in the rat sciatic nerve (Chang, et al., 1990; Chang and Yannas, 1992).

2.1.2 Long-Term Recovery and Kinetics

In order to determine the feasibility of a device for treatment of peripheral nerve injuries, it is first necessary to measure the extent of regeneration that results from implantation of the device. A handful of investigators have studied long-term regeneration and have reported significant improvements in structure and function beyond six months post-operative (Archibald, et al., 1995; Tountas, et al., 1993). These findings suggest that the kinetics of nerve regeneration are such that a significant period of time is required to reach asymptotic values. Archibald and colleagues have reported the most extensive data in a primate model through 3.5 years (Archibald, et al., 1995). The functional performance of the nerve, which was regenerated through a collagen tube across a 5-mm gap in the median nerve, was equivalent with that of the autograft standard. Using serial electrophysiological data, they showed that the regeneration appeared complete after approximately 1 year. In other long-term kinetic studies, it has also been shown that significant qualitative histological (Gibson and
Daniloff, 1989; LeBeau, et al., 1988) and quantitative electrophysiological changes (Tountas, et al., 1993) take place between 6 and 12 months; however, the changes observed were dependent on the implanted device. In empty silicone tubes, degeneration of the newly regenerated axons was observed after six months (LeBeau, et al., 1988), whereas, axons regenerated through degradable polyglycolic acid tubes continued maturing after six months (Tountas, et al., 1993). In other long-term studies, the extent of recovery at a single long-term endpoint was reported (den Dunnen, et al., 1993b; Fields, et al., 1989). This is useful in the comparison of device performance but does not provide information regarding the kinetic changes over time that describe the regenerative process.

2.1.3 Project Goal

The objective of this study was to evaluate the effects of tube composition and presence of a specific CG substrate on the properties of nerves regenerated over 60 weeks across a 10-mm gap in the rat sciatic nerve. Large-pore collagen, small-pore collagen, and silicone tubes were implanted both empty and filled with CG matrix. The chemical composition and pore structure of the CG matrix were chosen based on the results of the preliminary optimization study (Chang, et al., 1990). A sciatic nerve autograft served as a control and the contralateral nerve served as a normal control. Histomorphometric analyses of the cross-sections of regenerated nerves at 30 and 60 weeks were performed to determine the effects of the substrate as well as mean pore diameter and chemical composition of the tube on the number and size distribution of axons regenerating across the mid-portion of the gap (i.e., 5 mm from the proximal stump). In addition, electrophysiological capacity at 60 weeks was measured and correlated with histological data. A study of early events (6 weeks) in the healing response has been reported elsewhere (Chamberlain, et al., 1998; Landstrom, 1994) and is incorporated in this study to develop a kinetic representation of the recovery of peripheral nerve structure.

Particular emphasis was placed on quantifying the large diameter fibers (≥ 6 μm) since, during regeneration, this subset of the fiber population plays a significant role in recovery of
electrophysiological function. Differences among groups of tubular implants were evaluated by comparing the number of fibers larger than a threshold value in each group. The threshold value chosen was 6 μm because of its importance histologically and electrophysiologically. Fibers larger than about 6 μm have likely reached a distal target and, therefore, can be considered relatively permanent (Aitken, 1949; Aitken, et al., 1947; Sanders and Young, 1944; Sanders and Young, 1946; Weiss, et al., 1945). Electrophysiologically, fibers with diameter of about 6 μm or larger represent axons in the “A” fiber range (Arbuthnott, et al., 1980; Strichartz and Covino, 1990). Since most electrophysiological measurements reflect the compound action potential of “A” fibers, evaluation of the large diameter fibers is a useful step towards eventual correlation of histological and electrophysiological measurements.

2.2 Materials and Methods

2.2.1 Peripheral Nerve Devices

Both types of collagen tube were fabricated from type I bovine tendon collagen and had an inside diameter of 1.5 mm (Dr. S. Batra, Integra Life Sciences, Plainsboro, NJ). Details of the procedure for molding the collagen tubes have been described previously (Archibald, et al., 1995; Li, et al., 1990; Li, et al., 1992). The large-pore collagen tubes have been reported to have walls with maximum pore diameter of 22 nm, a size which excludes transfer of proteins with molecular weight higher than 540 kD (Li, et al., 1992). The walls of the large-pore collagen tubes were approximately 650 μm thick and were comprised of collagen layers, arranged in a laminar fashion, that were loosely packed (Figure 2.1a). The large-pore collagen tubes were cross-linked by gaseous formaldehyde treatment in order to decrease the degradation rate following implantation (Archibald, et al., 1995). The small-pore collagen tubes have been reported to have walls with pore diameters of less than 4 nm (Li, et al., 1990). The small-pore collagen tubes (Figure 2.1b) had a compact arrangement of collagen and a wall thickness of approximately 80 μm. The silicone tubes (Silastic Medical Grade Tubing, Dow-
Figure 2.1 Environmental scanning electron micrographs (ESEM) of the collagen tubes. (a) The large-pore collagen tube has loosely packed layers of collagen in a laminar arrangement. The tube wall thickness, measured from the luminal surface (black arrow) to the outer surface (not visible), is approximately 650 µm. Scale bar = 100 µm. (b) The small-pore collagen tube has densely packed collagen layers and a wall thickness of approximately 80 µm. The luminal and outer surfaces are visible in this micrograph (white arrows). Scale bar = 20 µm.

Corning Co., Midland, MI) had an inside diameter of 1.5 mm and a wall thickness of 250 µm. All tubes were sterilized at 105°C for 24 hours under vacuum.

The collagen-glycosaminoglycan (CG) matrix copolymer was prepared as previously described (Chang, et al., 1990; Loree, et al., 1989; Yannas, et al., 1989). The CG matrix comprises fibrous, type I bovine hide collagen (USDA, Philadelphia, PA) and chondroitin 6-sulfate (Sigma Chemical Company, St. Louis, MO) in a 98/2 wt/wt. ratio, with a pore volume fraction of 0.95. To prepare the matrix, a CG suspension (collagen, chondroitin 6-sulfate, and acetic acid) was injected into silicone processing tubes and frozen under conditions which produced nearly optimal pore characteristics of the matrix, as determined by a 40-week electrophysiological study of several structurally related CG matrices in silicone tubes (Chang, et al., 1990; Chang and Yannas, 1992). Sublimation of ice crystals by freeze-drying left inside the silicone tube a cylinder of highly porous matrix with axially oriented pore channels, about 35 µm in diameter (Figure 2.2). The matrices were transferred to a 105°C oven under vacuum (Fisher Isotemp Vacuum Oven, Fisher Scientific, Boston, MA; VacTorr 150 Vacuum Pump,
Figure 2.2 Longitudinal environmental scanning electron micrograph (ESEM) of the CG matrix. The matrix has pore channels, approximately 20 - 50 μm in diameter, oriented parallel to the nerve axis (arrow). The nerve axis in this micrograph is from the upper left to the lower right (arrow). Note the collagen filaments, perpendicular to the nerve axis, bridging the pore channels. Scale bar = 20 μm.

GCA/Precision Scientific, Chicago, IL) for cross-linking and sterilization (Yannas, et al., 1989; Yannas and Tobolsky, 1967). This treatment leaves the triple helical structure of collagen intact, provided that the moisture content of collagen prior to heat treatment is less than 1 wt. % (Yannas, 1972; Yannas, et al., 1989). Following this cross-linking procedure, the matrix has a half-life of approximately 4-6 weeks in the rat sciatic nerve. The CG matrix synthesized in this study differed in degradation rate, average pore diameter, and orientation of pore channel axes, but not in chemical composition, from a CG matrix which has induced partial regeneration of dermis in adult mammals (Yannas, et al., 1989).

In a sterile hood, the tubes were trimmed into 20-mm lengths and, if the prosthesis was matrix-filled, a 10-mm segment of CG matrix was inserted into the center of the tube. Each implant was placed in sterile phosphate buffered saline (Sigma Chemical Company, St. Louis, MO) prior to implantation, and was considered to be at least partly filled with saline when implanted.

2.2.2 Animal Model

Sixty-three adult female Lewis rats (Charles River Laboratories), 175-200 grams, were used in this study. The Lewis strain of rat was chosen because of its resistance to autotomy, or
self-mutilation, following sciatic nerve transection (Carr, et al., 1992). The animals were divided into seven experimental groups, with 9 animals per group, as follows: unfilled silicone tube (SI), matrix-filled silicone tube (SI/M), unfilled large-pore collagen tube (LC), matrix-filled large-pore collagen tube (LC/M), unfilled small-pore collagen tube (SC), matrix-filled small-pore collagen tube (SC/M), and autograft (AG).

Each animal was anesthetized using an intraperitoneal injection of sodium pentobarbital (Nembutal® Sodium Solution, Parke-Davis, 50mg/ml) with a dosage of 50 mg/kg. Once the animal was fully anesthetized, the surgical area was shaved with animal clippers and cleaned using an iodine sponge. The animal was placed in the prone position on the surgical board, arms and legs secured, with the legs in 30° abduction. A 4-cm incision was made parallel to and just posterior of the femur. The sciatic nerve was exposed and further anesthetized topically using a few drops of 1% Lidocaine placed directly on the nerve. The fascia surrounding the nerve was cut away so that the nerve was completely free from constraint.

For a tubular repair (Figure 2.3), the nerve was transected midway between the sciatic notch and the distal bifurcation using microscissors. The tubular prosthesis, either empty or filled with the CG matrix, was placed in the gap and the proximal and distal nerve stumps inserted 5 mm into each end of the tube, leaving a 10-mm gap in the center. The nerve was secured in place using two 10-0 sutures (Ethicon) at each end. For autograft repairs (Figure 2.3), a 10-mm segment of sciatic nerve was removed and then sutured back into place using 10-0 sutures, without rotation, thereby leaving the fascicles grossly aligned. This procedure for autografting differs from that used by several other investigators who rotated the transected nerve segment by 180° before suturing in place (Archibald, et al., 1995; Molander, et al., 1983) or used a contralateral or donor nerve for the graft tissue (Kim, et al., 1994; Robinson, et al., 1991; Rosen, et al., 1990), and suggests an anticipated higher potential for recovery for the autograft control used in the current study. The muscle and skin were closed using 4-0 sutures and skin staples as needed. The animals were placed back in their cages and were
monitored until fully alert. The animals were housed on wood chip bedding, 2 animals per cage, for the remainder of the experiment. Food and water was available ad libitum. The animals were monitored daily for signs of any abnormal behavior, such as insufficient grooming, lack of appetite, aggressive behavior and the appearance of autotomy.

In each experimental group, 6 animals were sacrificed at 30 weeks post-operatively by transcardial perfusion with mixed aldehydes and the sciatic nerve tissue was prepared for histomorphometric analysis. At 60 weeks post-operatively, the remaining 3 animals in each group were sacrificed by intraperitoneal injection with an overdose of sodium pentobarbital, the nerve was tested in vitro for electrophysiological response (see below), and the tissue was processed for histomorphometric analysis.

2.2.3 Sensorimotor Performance Evaluation

Prior to sacrifice and electrophysiological testing, a nociceptive test was performed at 60 weeks to assess the functional recovery of deep and cutaneous pain receptors using
mechanical stimulation. The stimulation, or pinch, was applied using small forceps and a consistent amount of pressure which was significant enough to cause response in the normal foot (Thalhammer, et al., 1995). For each animal, both the normal and implanted legs were tested. Typical responses which were considered positive for sensory function, included vocalization, withdrawal of the foot, and muscle flexion. Three response areas were tested on each foot, two for deep pain and one for cutaneous pain. For deep pain, the medial (first) toe and the lateral (fifth) toe were tested for sensory function. The medial toe serves as an internal control since it is innervated by the saphenous nerve and should be unaffected by sciatic nerve injury. The skin on the lateral toe was tested for a cutaneous pain response. An evaluation of muscle mass was made at 60 weeks using a circumferential measurement technique. Studies have shown that muscle tone correlates well with electrophysiological results (Chang, 1988). The circumference of both the normal and implant treated legs was measured at distances 20-mm and 30-mm proximal of the heel.

At 60 weeks post-operatively, each animal was anesthetized, and the sciatic nerve was exposed and freed from surrounding muscle and fascia. Two stimulating hook electrodes were positioned on the sciatic nerve at the hip, proximal to the lesion site, and were separated from the underlying muscle tissue with an insulator. Stimuli were applied over a range of signal durations (10 - 50 μs) and intensities. While stimulating in vivo, the animal was monitored for the appearance of gastrocnemius muscle twitch and toe flexion. At each signal duration, a threshold intensity was recorded.

2.2.4 Electrophysiological Procedures

Following the sensorimotor evaluation at 60 weeks, the sciatic nerve and distal nerve branches were dissected from the sciatic notch to the distal muscle inserts and placed in physiological Ringer's solution. Following removal of the nerve, the animal was given an overdose (100 mg/kg) of sodium pentobarbital via intraperitoneal injection. Using a dissecting microscope, connective tissue and fat were removed from the nerve; if present the tube was
carefully cut away. The prepared nerve was placed on an electrode array for in vitro recording of response to electrical impulses. The electrode array consisted of five silver electrodes; i.e., two stimulating, two recording and one ground, separated by fixed distances. Stimuli were applied at a signal duration of 50 μs. For A-fiber recording, the signal intensity was increased until the A-fiber peak was maximized. Once A-fiber recordings were complete, the signal intensity was further increased to stimulate the B-fibers and C-fibers. The recorded signal passed through a differential electrometer (Cat# AK-47UU, MetaMetrics), a 50x amplifier (Cat# AK-47S, MetaMetrics), and into a storage oscilloscope (Cat# 5113, Dual Beam Storage Oscilloscope, Tektronix). Compound nerve action potentials were recorded using Polaroid photographs of the oscilloscope tracings. Several aspects of the action potential were measured including: conduction velocity and peak to peak amplitude for all fiber types (i.e., A, B and C), refractory period to reach one-half amplitude during paired pulse stimulation (A-fibers), and steady state amplitude (stamina) during train stimulation at varying frequencies (10 - 100 Hz; A-fibers). Normal control values were obtained by testing the contralateral nerves under identical conditions.

2.2.5 Histomorphometric Procedures

Following sacrifice, the sciatic nerves were placed in Yanoff's fixative. A 2-mm segment of regenerated nerve, taken from the mid-portion of the gap, was post-fixed in 1% osmium tetroxide (Cat #0972A, Polysciences, Inc., Warrington, PA) and embedded in Epon (Poly/Bed 812 Embedding Kit, Cat #08792, Polysciences, Inc, Warrington, PA). Tissue sections suitable for histomorphometry were prepared by sectioning Epon-embedded samples on an ultramicrotome at a 1-μm thickness. The slides were additionally stained with toluidine blue (Cat# BP-107-10, Fisher Biotech, Boston, MA) to enhance the color of the osmium stain and then mounted and coverslipped (Cytoseal 60 Mounting Medium, Cat #8310-16, Stephens Scientific, Riverdale, NJ). The nerve sections were digitized into a Macintosh computer for image analysis using a video camera (Hamamatsu CCD Video Camera Module, Model XC-77,
Hamamatsu, Japan) connected to a light microscope (Olympus Vanox-T, Olympus, Japan). Low magnification images were captured to measure the cross-sectional areas of the regenerated tissue cables. High magnification images, representing 10% of the total tissue area, were selected randomly from the nerve cross-section and captured for axon counting and measurement.

The tissue cable areas and the number and size of myelinated axons were determined using the public domain software program NIH Image on a Macintosh computer (see Appendix B for calculations). Axons were counted in each high magnification image, and a density of axons generated. The density of axons was multiplied by the tissue cable area to obtain the total number of axons per nerve. For each fiber (i.e., axon core and myelin sheath) the image analysis resulted in values for the perimeter (P) which was used to calculate the idealized fiber diameter (D = P/π). This technique, which has been used previously (Archibald, et al., 1995), treated each axon as a circular cross-section and minimized errors caused by tissue shrinkage during processing (Karnes, et al., 1977). In addition, a total myelinated fiber area was calculated for each nerve and used to determine the N-ratio (total myelinated fiber area/total tissue cable area). The N-ratio has been used previously (den Dunnen, et al., 1996) and provides information regarding remodeling changes in the nerves over time.

2.2.6 Statistical Methods

Two-factor analyses of variance (ANOVA) were performed to determine the effect of tube type and presence of the CG matrix inside the tube on each outcome variable. If ANOVA indicated statistical significance, subsequent multiple comparisons were made using the Student Newman-Keuls test to determine differences between pairs of experimental groups (Zar, 1984). Following rejection of the null hypothesis of a one-factor ANOVA, Dunnett’s test was used to compare each tube group to the autograft control (Zar, 1984). Student’s t-test was used to determine if changes from 30 to 60 weeks, in each implant group, were significant. To correct for possible errors using multiple t-tests, the Bonferroni approximation for multiple
paired comparisons was used to adjust the critical p-value by dividing the experimental p value
(p = 0.05) by the number of t-tests performed (Lieber, 1994). Simple linear regression
techniques were used to study kinetic histological data, including ANOVA analyses to
determine the significance of the regression. Comparisons among regression slopes were
made using the Student Newman-Keuls procedure, following rejection of the null hypothesis
of an analysis of covariance (Zar, 1984). Statistical significance was accepted for p < 0.05.

2.3 Results

2.3.1 General Observations

Following the surgical procedure, the animals showed no severe signs of discomfort.
Autotomy was not observed in any of the animals, supporting previous conclusions on the
absence of self-mutilation following nerve transection in Lewis rats (Carr, et al., 1992). Over
the course of the 60-week experiment, 6 animals died of unknown causes, as follows: one
animal each in the LC (30 week) and AG groups (60 week), and two each in the SC (60 week)
and SC/M (60 week) groups. Due to small sample size (n=1) at 60 weeks, the SC and SC/M
groups were not included in the statistical analyses of electrophysiological and histological
data. At 30 weeks, a SI/M device in one animal displayed clinical signs of infection and,
therefore, this animal was not included in the quantitative analysis.

In animals sacrificed at 30 or 60 weeks, tissue was observed to have bridged the gap
between the nerve stumps in every lesion ensheathed by a collagen tube (LC, LC/M, SC, and
SC/M groups). The walls of the collagen tubes swelled during implantation, resulting in
reduction of the luminal area available for tissue cable formation to 75% and 15% of the
original value (1.77 mm²) for the large- and small-pore collagen tubes, respectively
(Chamberlain, 1996; Chamberlain, et al., 1997). The regenerated tissue cables completely
filled the resulting lumen of the large- and small-pore collagen tubes at 30 weeks (Table 2.1).
From 30 to 60 weeks, the area of the regenerated tissue cables was significantly reduced in
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<thead>
<tr>
<th></th>
<th>Total Tissue Cable Area (mm²)</th>
<th>Total Area Occupied by Myelinated Fibers (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 Weeks</td>
<td>60 Weeks</td>
</tr>
<tr>
<td>Normal</td>
<td>0.50 ± 0.02</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>SI</td>
<td>0.20 ± 0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>SI/M</td>
<td>0.37 ± 0.02</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>LC</td>
<td>1.34 ± 0.10</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>LC/M</td>
<td>1.27 ± 0.07</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>SC</td>
<td>0.24 ± 0.03</td>
<td>0.28</td>
</tr>
<tr>
<td>SC/M</td>
<td>0.32 ± 0.04</td>
<td>0.5</td>
</tr>
<tr>
<td>AG</td>
<td>0.41 ± 0.01</td>
<td>0.47 ± 0.05</td>
</tr>
</tbody>
</table>

Table 2.1  Morphological measurements (mean ± SEM) at 30 and 60 weeks of the total tissue cable area and the total area occupied by myelinated nerve fibers. In the SI group, only 3 of 6 animals at 30 weeks and 1 of 3 animals at 60 weeks had a regenerated tissue cable. The implant groups were as follows: unfilled silicone (SI), matrix-filled silicone (SI/M), unfilled large-pore collagen (LC), matrix-filled large-pore collagen (LC/M), unfilled small-pore collagen (SC), matrix-filled small-pore collagen (SC/M), and autograft (AG).

both the LC and LC/M groups (Table 2.1; p<0.01). At 60 weeks, all collagen tubes were still present, although they grossly appeared smaller in outside diameter, and fragmented, indicating that some degradation had occurred. A tissue cable bridged every SI/M device, while a tissue cable bridged the SI group in only 3 of 6 devices at 30 weeks, and 1 of 3 devices at 60 weeks. The tissue cables bridging the silicone tubes never completely filled the lumen of the tubes and the tissue cable areas did not change significantly from 30 to 60 weeks (Table 2.1; p>0.5).

At 30 weeks, there was no residual CG matrix observed in any of the tubuluted gaps. Previously, it was shown that the CG matrix resorption is nearly complete in the rat sciatic nerve by 6 weeks (Chamberlain, et al., 1998; Landstrom, 1994). Axons were present in the tissue at the midpoint of the experimental gap with 30 week (Figure 2.4a) and 60 week animals (Figure 2.4b); however, the axons were smaller in diameter and had thinner myelin sheaths than those seen in normal sciatic nerve (Figure 2.4c). At both 30 and 60 weeks, the qualitative amount of interaxonal connective tissue (endoneurium) was markedly increased and contained an increased number of blood vessels and Schwann cells compared to normal controls.
Figure 2.4  Histological micrographs of nerve tissue post-fixed with osmium tetroxide and stained with toluidine blue. The magnification for each micrograph is the same; scale bars = 10 μm. (a) Tissue regenerated through the mid-portion of a matrix-filled large-pore collagen (LC/M) implant at 30 weeks. Note the large number of axons in this cross-section with the majority of axons being small in diameter. The largest axons have diameters of approximately 7 μm. Many Schwann cells (S) are visible with some actively participating in myelination. (b) Tissue regenerated through the mid-portion of a LC/M implant at 60 weeks. Compared to 30 weeks, the axons are much larger (diameters up to 12 μm) and have thicker myelin sheaths. Also, fewer small diameter axons are visible. (c) Normal nerve tissue from the level of the lesion is shown as a control. Note the number of large diameter fibers and the thickness of the myelin sheaths compared to the regenerated nerves.
2.3.2 Histomorphometry

Total Number of Myelinated Axons

The results of a 6-week study in these laboratories (Chamberlain, et al., 1998; Landstrom, 1994), in which identical implants were studied in the same animal model as in the present study, were incorporated into an analysis of the kinetics of axonal regeneration. The total number of axons plotted as a function of time revealed two regions: a growth and a plateau region (Figure 2.5). In the tubulated groups, the number of axons per nerve increased up to 30 weeks and subsequent changes were not significant (p>0.3); in contrast, values at the autografted sites reached constant values after only 6 weeks (p>0.4).

![Graph showing the number of myelinated axons over time for different groups](image)

**Figure 2.5** Total number of myelinated axons as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. For each group, both a growth region and a plateau region were observed. In the tubulated groups, the number of axons per nerve increased up to 30 weeks and remained unchanged thereafter (p>0.3). In contrast, values at the autografted sites reached apparently constant values after only 6 weeks (p>0.4). LC = large-pore collagen.
At both 30 and 60 weeks, the number of myelinated axons inside the LC/M device was nearly three times that found in the normal control. All tubulated groups, excluding the SL device, had a comparable number of regenerated axons with the autograft control at 30 weeks; however, by 60 weeks, only the SL/M and LC/M devices were not statistically different from the autograft (p>0.4) (Figure 2.6). Two-way ANOVA indicated a significant effect of the CG matrix (p<0.001) and a significant effect of tube type (p<0.005) on the total number of myelinated axons at both 30 and 60 weeks. Filling the tubes with CG matrix significantly

![Graph showing the number of myelinated axons per nerve for each implant group at 60 weeks.](image)

**Figure 2.6** Total number of myelinated axons per nerve for each implant group at 60 weeks. The number of axons per normal sciatic nerve at the level of the lesion is shown for reference. All regenerated groups, except the unfilled silicone (SI) implant group, had more axons per nerve than normal nerve controls. The matrix-filled large-pore collagen (LC/M) implant group had the most axons per nerve, with nearly three times the number observed in the normal control. In the silicone and large-pore collagen tube groups, the presence of the matrix significantly increased the number of axons at 60 weeks (p<0.05). The implant groups were as follows: unfilled silicone (SI; n=3), matrix-filled silicone (SI/M; n=3), unfilled large-pore collagen (LC; n=3); matrix-filled large-pore collagen (LC/M; n=3), autograft (AG; n=2), unfilled small-pore collagen (SC; n=1), matrix-filled small-pore collagen (SC/M; n=1). NS = not significant.
increased the number of myelinated axons in the silicone (p<0.001) and large-pore collagen (p<0.05) tube groups (Figure 2.6).

**Fiber Diameter Distributions**

Normal nerve had a slightly bimodal fiber diameter distribution with peaks at 3.5 and 8.5 μm (Figure 2.7). In contrast, in all regenerated nerves, distributions were centered around a single mode of approximately 2.5 μm. Although distributions at 60 weeks were similar in shape to those observed at 30 weeks, there was a significantly greater number of larger diameter axons (Figure 2.7). The shift from 30 to 60 weeks of fiber diameters toward larger sizes was more pronounced in the LC/M (Figure 2.7) and AG groups (not shown).

![Figure 2.7](image)

**Figure 2.7** Axon diameter distribution for normal nerve and the matrix-filled large-pore collagen tube group (LC/M) at 30 and 60 weeks. The normal nerve distribution had a bimodal shape with modes at 3.5 and 8.5 μm; the bimodality is difficult to detect in this plot due to the large numbers of axons in the regenerated groups which compress the normal distribution. In the regenerated nerves, the overall shape of the distribution was similar for all groups. The modes of the regenerated distributions were 2.5 μm at both 30 and 60 weeks. From 30 to 60 weeks, a significant shift in the distribution toward the large diameter bins was observed. This shift was most pronounced in the LC/M and AG groups (not shown).
Normal nerve had a mean fiber diameter of $8.5 \pm 0.2 \mu m$. At 30 weeks, the mean fiber diameter for regenerated nerves in all groups was between 3.2 and 3.8 $\mu m$ (Table 2.2), with the exception of the SI group which had a significantly smaller mean fiber diameter ($2.6 \pm 0.1 \mu m$) than all other tubulated groups ($p<0.001$). By 60 weeks, mean fiber diameters had increased in every group and ranged from 3.6 to 4.4 $\mu m$ (Table 2.2). The increase in mean fiber diameter from 30 to 60 weeks was significant only in the LC/M group ($p<0.005$).

**Total Number of Large Diameter Fibers**

The term “large diameter fibers” (LDF) was used to refer to myelinated axons with fiber diameters (axon core diameter plus twice the myelin thickness) greater than or equal to 6 $\mu m$. In contrast to the total number of myelinated axons, the total number of LDF per nerve increased continuously at an approximately constant rate during the period of observation (6 to 60 weeks) in all tubulated groups; however, the increase in number of LDF from 30 to 60 weeks was significant only in the LC/M and LC groups ($p<0.05$) (Figure 2.8). Using linear

<table>
<thead>
<tr>
<th></th>
<th>Mean Fiber Diameter ((\mu m)) of all Fibers</th>
<th>Mean Fiber Diameter ((\mu m)) of Fibers Larger than 6 (\mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 Weeks</td>
<td>60 Weeks</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>2.6 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>SI/M</td>
<td>3.4 ± 0.1</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>LC</td>
<td>3.2 ± 0.1</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>LC/M</td>
<td>3.2 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>SC</td>
<td>3.4 ± 0.3</td>
<td>4.0</td>
</tr>
<tr>
<td>SC/M</td>
<td>3.7 ± 0.2</td>
<td>3.6</td>
</tr>
<tr>
<td>AG</td>
<td>3.8 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 2.2** Mean fiber diameters of all fibers and large diameter fibers (mean ± SEM) at 30 and 60 weeks. Mean fiber diameters are not reported for the SI group at 60 weeks since no axons were found crossing the center of the gap in this group. The implant groups were as follows: unfilled silicone tube (SI), matrix-filled silicone tube (SI/M), unfilled large-pore collagen tube (LC), matrix-filled large-pore collagen tube (LC/M), unfilled small-pore collagen tube (SC), matrix-filled small-pore collagen tube (SC/M), and autograft (AG).
Figure 2.8 Total number of large diameter myelinated fibers (≥ 6μm) as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. The total number of large diameter fibers increased continuously at an approximately constant rate during the period of observation (6 to 60 weeks). By 60 weeks, the best regeneration (LC/M and AG groups) resulted in only half the normal number of large diameter fibers. LC = large-pore collagen.

regression analysis, it was determined that a positive slope existed for all tubulated groups (r > 0.85; p<0.001) except the SI group (r = 0.02; p>0.5). The rate of increase in the number of LDF for the LC group (24 LDF/week) was significantly slower than that of the LC/M group (44 LDF/week) (p<0.01). The slope of the AG regression line (44 LDF/week) was not significantly different from the LC/M group (p>0.75).

All implant groups had significantly fewer LDF than normal controls at both 30 and 60 weeks (Figure 2.8). After 60 weeks, the AG and LC/M groups showed the largest number of LDF, over half those observed in normal controls (Figure 2.9). Only the LC/M group was not significantly different from the AG group (p>0.5); all other tube groups had significantly fewer LDF than the LC/M and AG groups (p<0.001).
Figure 2.9  Total number of large diameter myelinated fibers (≥ 6 μm) per nerve at 60 weeks. All of the regenerated nerves had significantly fewer large diameter fibers than normal nerve controls (p<0.001). In the case of the silicone and large-pore collagen tubes, the presence of the CG matrix significantly increased the number of large diameter fibers at 60 weeks (p<0.001). In addition, the LC/M and AG groups had significantly more large diameter fibers than all other groups at 60 weeks (p<0.001), and were not significantly different from each other (p>0.5). The implant groups were as follows: unfilled silicone (SI; n=3), matrix-filled silicone (SI/M; n=3), unfilled large-pore collagen (LC; n=3); matrix-filled large-pore collagen (LC/M; n=3), autograft (AG; n=2), unfilled small-pore collagen (SC; n=1), matrix-filled small-pore collagen (SC/M; n=1). NS = not significant.

Two-way ANOVA indicated a significant effect of matrix presence and tube type on the total number of LDF at both 30 and 60 weeks (p<0.05). The CG matrix significantly increased the number of LDF for silicone tubes and large-pore collagen tubes (p<0.005). After 60 weeks (Figure 2.9), tissue regenerated through the LC/M prostheses had significantly more LDF than all other tube groups (p<0.001).

A mean fiber diameter was calculated only for fibers larger than 6 μm. At 30 and 60 weeks, the mean fiber diameter for large fibers ranged from 7.0 to 8.0 μm and was
significantly lower than normal (10.3 ± 0.2 μm) in all regenerated nerves (Table 2.2). There were no significant increases in the mean fiber diameter of large diameter fibers from 30 to 60 weeks.

**Total Myelinated Fiber Area**

The total area occupied by myelinated fibers increased from 30 to 60 weeks in all experimental groups (Table 2.1; Figure 2.4 a & b); however, the increase was significant only in the LC/M, SI/M and AG groups (p<0.05). The total area occupied by myelinated fibers was significantly smaller than normal for all regenerated groups at 30 and 60 weeks (p<0.001). By 60 weeks, only the LC/M group was not significantly different than the autograft based on the amount of myelinated fiber area. The presence of the CG matrix and the type of tube implanted both had significant effects on the myelinated fiber area at 30 and 60 weeks (2-way ANOVA, p<0.001).

**2.3.3 Electrophysiology**

Electrophysiology at 60 weeks indicated that, in general, the regenerated nerves were able to conduct a compound nerve action potential which was similar in overall shape to a normal compound nerve action potential (Figure 2.10), but with a lower peak. In all regenerated nerves, the conduction velocities were slower and the amplitudes were lower than normal nerve. However, the regenerated A-fibers had refractory periods and responses to train stimulation that were not significantly different from normal nerve.

**A-Fibers**

The properties of the primary peak of the nerve action potential are the most commonly reported and represent impulses traveling along fibers larger than 6 μm, termed A-fibers (Arbuthnott, et al., 1980). The conduction velocities of A-fibers, measured using individual axon recording, typically range from 30-120 m/s (Strichartz and Covino, 1990) and
Figure 2.10 Typical oscilloscope tracings of A-fiber and B-fiber compound nerve action potentials for normal sciatic nerve and nerve regenerated through a LC/M implant at 60 weeks post-implantation. The A-fiber peak for the regenerated nerve has a significantly smaller amplitude than the normal nerve control. This was typical of all regenerated groups. In contrast, the conduction velocity of the regenerated nerve, although significantly slower than normal, was approaching normal values. The latency is measured along the x-axis from the stimulus to the peak and then combined with the constant distance between electrodes to determine conduction velocity. The dashed line indicating the B-fiber peak has been added on to the tracing for reference. Note that the normal nerve tracing has no visible B-fiber peak. In the regenerated nerves, the B-fiber peak was similar and visible in all groups. C-fiber peaks were observed in both normal and regenerated nerves; however, due to the relatively slow conduction velocity, the C-fiber peaks are not visible on this scale. The C-fiber peak would be located at a latency of approximately 17 milliseconds, with a relatively small amplitude of approximately 0.15 millivolts. The C-fiber conduction velocities and amplitudes were not significantly different for normal and regenerated nerves.
measurements on normal controls in this study were consistent with published data (Table 2.3). Although A-fiber conduction velocities for regenerated nerves approached normal in the LC/M and AG groups, all groups were significantly slower than normal (p<0.001; Table 2.3). Two-way ANOVA revealed a significant effect of tube type (p<0.001) and the CG matrix (p<0.001) on the A-fiber nerve conduction velocity. The LC/M group showed a significantly faster conduction velocity than all the other tube groups (p<0.025); and was the only group not significantly different from the AG control. The amplitude of the A-fiber peak was significantly lower than normal in all groups (Table 2.3). No significant differences were detected among the amplitudes of the regenerated nerve groups.

<table>
<thead>
<tr>
<th></th>
<th>Conduction Velocity (m/s)</th>
<th>Amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A Fibers</td>
<td>B Fibers</td>
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<tr>
<td>Normal</td>
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</tr>
<tr>
<td>S1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>S1/M</td>
<td>32 ± 1</td>
<td>4.5</td>
</tr>
<tr>
<td>LC</td>
<td>40 ± 4</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>LC/M</td>
<td>50 ± 2</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>SC</td>
<td>28</td>
<td>3.5</td>
</tr>
<tr>
<td>SC/M</td>
<td>40</td>
<td>5.9</td>
</tr>
<tr>
<td>AG</td>
<td>47 ± 3</td>
<td>5.7 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2.3 Electrophysiological results for each prosthesis group (mean ± SEM) at 60 weeks. Conduction velocity (m/s) and peak to peak amplitude (mV) are shown for 3 fiber types: A-fibers (myelinated fibers ≥ 6 μm in diameter), B-fibers (myelinated fibers ≤ 3 μm in diameter), and C-fibers (unmyelinated fibers < 1.3 μm in diameter). In the S1 group, two animals had no nerve cables, therefore no action potential was generated. In the third animal, a tissue cable was present and measured; however, no axons were found in the tissue cable and no action potential was measured. Data for the SC and SC/M groups were not included in the statistical analyses due to small sample size (n=1 in each group). B-fiber peaks were not observed in the normal nerve recordings (n=14); therefore, no values are given for normal B-fiber conduction velocity or amplitude. C-fiber peaks were not detected in the autograft group. The implant groups were as follows: unfilled silicone tube (S1; n=3), matrix-filled silicone tube (S1/M; n=2), unfilled large-pore collagen tube (LC; n=3), matrix-filled large-pore collagen tube (LC/M; n=3), unfilled small-pore collagen tube (SC; n=1), matrix-filled small-pore collagen tube (SC/M; n=1), and autograft (AG; n=2).
The refractory period was defined as the delay time between paired pulses for the second signal to reach one-half the original amplitude (Figure 2.11). Normal nerve had a refractory period of 3.0 ± 0.2 msec. Excluding the SI group, which had no measurable action potential, the regenerated nerve groups had average refractory periods that ranged from 3.5 - 3.9 msec. There were no statistical differences among implant groups (p>0.5) and none of the groups had significantly different refractory periods compared with normal nerve (p>0.1).

Steady state amplitude, reported as a percentage of the maximum amplitude during train stimulation at varying frequencies, was measured (Figure 2.12). At the highest frequency (100 Hz; Figure 2.12a), the regenerated nerves reached average steady state amplitude levels of 78 - 92% of the maximum amplitude. At 100 Hz, normal nerve had a steady state amplitude of

![Figure 2.11 Typical oscilloscope tracing used to measure refractory period of A-fiber action potential. This particular tracing is from a nerve regenerated through a matrix-filled large-pore collagen device (LC/M). Nine sets of paired pulses are shown superimposed in this figure. The action potential generated in response to the first pulse is at the left, and is identical for each of the nine sets of paired pulses. The action potential generated in response to the second pulse appears to the right following the delay time, which varied for each of the nine sets of paired pulses. The paired pulse with the shortest delay time (SD; delay = 2 msec) resulted in no action potential in response to the second pulse; conversely, the longest delay time (LD; delay = 7.5 msec) resulted in identical action potentials generated by the first and second pulses. The refractory period (RP), defined as the delay time necessary for the amplitude of the peak (RP peak) to regain one-half amplitude, was 3 milliseconds for this particular nerve.](image-url)
Figure 2.12 Typical oscilloscope tracings used to measure steady state amplitude response to train stimulation. This particular tracing is from a nerve regenerated through a matrix-filled large-pore collagen device (LC/M). Each ‘spike’ represents an action potential. Steady state amplitude (SSA) was measured as a percentage of the maximum amplitude (MA) during train stimulation at varying frequencies (10 - 100 Hz; A-fibers). (a) During high frequency train stimulation, 100 Hz, there was a decrease in the amplitude of subsequent action potentials until a steady state value was reached. (b) At lower frequencies, 10 Hz, there was no decrease in the amplitude of the action potentials; therefore, the steady state amplitude was equivalent to the maximum amplitude.

91% of maximum, which was not significantly different from the regenerated nerves.

However, at low frequencies (10 - 25 Hz), the regenerated nerves, like normal nerves, were able to sustain the maximum amplitude for each action potential generated (Figure 2.12b).

B-fibers

A secondary peak was present in nearly all regenerated nerves, reflecting the properties of B-fibers, myelinated fibers with diameters 1.3 - 3 μm (Arbuthnott, et al., 1980), which are known from single fiber recordings to have conduction velocities ranging from 3-15 m/s (Arbuthnott, et al., 1980; Strichartz and Covino, 1990). The action potential corresponding to these fibers was not detectable in any of the normal nerve controls (n=15). The B-fiber peak was visible in all regenerated nerves (Figure 2.10). The B-fiber conduction velocities and amplitudes were similar among groups (Table 2.3) with analysis of variance indicating no significant differences.
C-fibers

Unmyelinated fibers with the smallest diameter (< 1.3 μm) comprise the C-fiber group (Arbuthnott, et al., 1980) and generally have conduction velocities that range from 0.1-2.0 m/s (Strichartz and Covino, 1990). Action potentials for C-fibers were found in both normal and regenerated nerves; however, the C-fiber peaks were sometimes difficult to detect because of relatively small amplitudes. The C-fiber peak was not detected in any of the AG animals. For the remaining tube groups and normal nerve, use of ANOVA did not lead to detection of a significant effect of treatment on either the conduction velocity or amplitude of C-fibers (Table 2.3).

2.3.4 Sensorimotor Performance Evaluation

Pinch Test

Sensation was restored in both deep and cutaneous pain receptors of the lateral toe in all animals in the LC/M (n=3) and AG (n=2) groups. In the SI/M and LC groups, only 2 of 3 animals recovered deep pain sensation and all animals recovered cutaneous pain sensation. The SI group (n=3) showed no response to cutaneous or deep pain of the lateral toe which is consistent with the finding of no axonal regeneration across the gaps of these animals. All animals (n=14), including those in the SI group, responded positively to deep pinch of the medial toe. The medial toe is innervated by the saphenous nerve and, therefore, served as an internal control of the method.

Muscle Circumference

Measurement of the muscle circumference at 60 weeks indicated that all animals which had regenerated a nerve cable (SI/M, LC, LC/M, and AG groups) retained muscle circumferences at the thigh and gastrocnemius not significantly different from normal animals. In contrast, animals in the SI group which had no apparent regeneration had muscle
circumferences at both the thigh and gastrocnemius that were significantly smaller than all other treatment groups (p<0.05) and normal (p<0.01) indicating a significant amount of atrophy.

**Muscle Response to Electrical Stimuli**

In the LC/M, LC, SI/M and AG groups, the gastrocnemius muscle and the plantar muscles of the foot responded with visible movement to electrical stimulation of the sciatic nerve at a location proximal to the injury. In contrast, stimulation of nerves regenerated through the SI implants resulted in no response from either the gastrocnemius or the plantar muscles. These results were consistent with the lack of axons bridging the SI nerve gaps. The qualitative intensity of the muscular response of the regenerated nerves did not differ significantly from the response observed in the normal nerve controls.

**2.4 Discussion**

Previous studies have shown that filling a silicone tube with the CG matrix significantly enhances the regeneration across 10- and 15-mm gaps (Chang, et al., 1990; Chang and Yannas, 1992; Yannas, et al., 1987). In this study, filling silicone and large-pore collagen tubes with the CG matrix significantly increased the number of axons per nerve, the number of large diameter axons, the mean fiber diameter, and the A-fiber conduction velocity, compared to unfilled tubes. However, the effect of the matrix was most prominent in the silicone tubes where, in the absence of the CG matrix, regeneration was poor. The contribution of the matrix to the quality of regeneration may be due, in part, to providing a specific surface for the attachment and migration of cells. Previous studies have shown that, when other experimental parameters were kept constant, CG matrices with small pores, and subsequently more surface area, were more successful at facilitating elongation of axons than matrices with large pore diameters (Chang, et al., 1990). *In vitro* (Baron-Van Evercooren, et al., 1982; Bunge and Bunge, 1978; Ebendal, 1976; Tonge, et al., 1996) and *in vivo* (Torigoe, et al., 1996; Weiss,
1941) studies have provided evidence that axonal growth cones and Schwann cells migrate along collagen substrates using contact guidance mechanisms.

Among the tubes used in this study, the large-pore collagen tube yielded the most favorable results. The small-pore collagen tube performed nearly as well as the large-pore collagen tubes at 30 weeks, despite the structural collapse of the small-pore collagen tubes that limited the space available for regenerating axons; however, animal deaths at 60 weeks prevented electrophysiologic measurements and analysis of kinetic information obtained with the small-pore collagen tube groups. Because of this, no conclusions can be made on the effect of tube pore diameter on regeneration through collagen tubes. Although at 30 weeks, the SI/M group gave regenerates which were similar in structure to both matrix-filled collagen groups, by 60 weeks, the SI/M group was inferior to the LC/M group both in histological and electrophysiological measurements. Previous studies have shown that by one year post-implantation, axons regenerated through silicone tubes have begun to degenerate (LeBeau, et al., 1988). Although the cause of degeneration was unknown, the authors hypothesized that it may have been a result of either the tube material itself or the constriction of the proximal and distal nerve stumps due to long-term tubulization (LeBeau, et al., 1988). Constriction of peripheral nerve stumps by silicone tubes has also been reported to have led to clinical problems, including pain and loss of function, and the necessity to surgically remove the tubes (Merle, et al., 1989). The ultrastructure of axons regenerated through the SI/M device at 60 weeks was not evaluated in this study, however it is possible that the inferior results from this group at 60 weeks (compared to the LC/M group) may be a result of axon degeneration.

Although the collagen tubes in this study were undegraded at 60 weeks, signs of chronic nerve compression were not observed in the collagen tube devices; in contrast, regenerates in the large-pore collagen tube groups showed significant histological improvement from 30 to 60 weeks. Similarly, tubes fabricated from PLLA/PCL copolymer (den Dunnen, et al., 1993b) and collagen (Archibald, et al., 1991; Li, et al., 1992) that remained relatively undegraded for
at least one year, showed no signs of chronic nerve compression. If chronic nerve compression is indeed the cause of degeneration, these results suggest that the appropriate tube material may prevent the degeneration of axons and the constriction of the nerve stumps, thereby resulting in improved regeneration.

By 60 weeks post-operative, the LC/M device had the largest number of axons per nerve, significantly more large diameter axons, and a significantly higher A-fiber conduction velocity than all other tube groups. More importantly, the nerves regenerated through LC/M devices were not significantly different from AG nerves based on all measurements at 30 and 60 weeks. Conversely, all other treatment groups were inferior to the autograft after 60 weeks in at least two of the quantitative measures.

Equivalence with the autograft across a 10-mm gap was previously achieved by filling a collagen tube with a Schwann cell suspension (Kim, et al., 1994). A direct comparison between the two studies cannot be pursued further since Kim and colleagues used a sural nerve autograft as a control (Kim, et al., 1994) while, in this study, a sciatic nerve autograft was used. A few devices have led to regenerates which, across shorter gap distances (8 mm or less), were comparable with the autograft. Tubes fabricated from a copolymer of L-lactide and \( \varepsilon \)-caprolactone have resulted in functional and histological outcomes not significantly different from the autograft when bridging an 8-mm gap in the rat sciatic nerve (Robinson, et al., 1991). Collagen tubes, from the same manufacturer and with nominally similar characteristics as the large-pore collagen tubes used in the present study, have been shown to regenerate nerves with physiological responses equivalent to autograft controls across gaps of 4 mm in the rat and primate (Archibald, et al., 1991) and 5 mm in the primate (Archibald, et al., 1995). In the current study, a large-pore collagen tube (LC) did not perform as well as the AG over a larger gap distance (10-mm); however, filling the collagen tube with a CG matrix resulted in a regenerated nerve which had structural and functional properties not significantly different from that of the AG. The combined data suggest that, as the gap distance is increased, substrate
materials with specific structure become more important for achieving equivalence with the autograft.

Although return of axon structure and electrophysiological function in the LC/M group rivaled those found in the AG group, the nerves, in all groups, remained morphologically inferior to normal through 60 weeks. The LC/M and AG groups had nearly three times the number of axons as normal but only half the number of large diameter fibers. The mean fiber diameters of all fibers and of large diameter fibers were also reduced compared to normal. Other investigators have reported that axons regenerated through either silicone tubes or biodegradable tubes typically have axons with reduced diameter and that the regenerates conduct electric signals at reduced velocities even after 10 months (den Dunnen, et al., 1993b; Fields, et al., 1989). A-fiber conduction velocities for the AG and LC/M groups were nearly 75% of normal controls, but remained significantly slower than, and the amplitudes were less than half of, normal (Table 2.3). Only C-fiber nerve action potentials were not significantly different than normal.

The number and mean fiber diameter of large diameter fibers have been used previously to assess the extent of regeneration (Aitken, 1949; Sanders and Young, 1944), but are not commonly used in the current literature. We found that the number of large diameter fibers could be used to differentiate among groups and to assess the deviation from normal more effectively than the total number of axons. In addition, the mean fiber diameter of large fibers was considered more informative than the traditional mean fiber diameter because it was not affected by the small axon ‘sprouts’ that likely never reach a target organ. The emphasis on large diameter fibers enabled a search for correlations with the properties of the A-fiber peak of the nerve action potential. Linear correlations have been reported between amplitude of action potential and total number of axons per nerve cross section (Dellon and Mackinnon, 1989), as well as between conduction velocity and the mean fiber diameter (Boyd and Kalu, 1979; Cragg and Thomas, 1964; Dellon and Mackinnon, 1989; Hursh, 1939). In the current study, there
was no correlation between A-fiber amplitude, measured at 60 weeks, and the total number of axons ($r = 0.22$; linear regression analysis). There was however, a correlation between amplitude and the number of large diameter fibers ($r = 0.87$), consistent with the fact that the A-fiber action potential represents the signal carried only by axons larger than approximately 6 $\mu$m (Arbuthnot, et al., 1980). Regression analysis further revealed a linear relationship between A-fiber conduction velocity and mean fiber diameter of all fibers ($r = 0.88$), as well as between A-fiber conduction velocity and the mean fiber diameter of large diameter fibers ($r = 0.88$). The slope of this regression line suggests a scaling factor of 6 m/s/µm for A-fibers of the regenerated rat sciatic nerve, which compares favorably to previous determinations of 5.6-6 m/s/µm for A-fibers of the normal cat nerve (Boyd and Kalu, 1979; Hursh, 1939), 4.4 m/s/µm for A-fibers of the regenerated rabbit peroneal nerve (Cragg and Thomas, 1964), and approximately 7 m/s/µm for regenerated rat nerve (Dellon and Mackinnon, 1989). These data support the suggestion (Arbuthnot, et al., 1980; Strichartz and Covino, 1990) that large diameter fibers are responsible for the magnitude of A-fiber conduction velocity.

Measurement of the refractory period and amplitude during train stimulation provided some information about the function of ion channels, the structures responsible for generating and propagating action potentials along axons, in the regenerated nerves. The refractory period for regenerated A-fibers was not significantly different from normal in any of the experimental groups. This finding suggests that the ion channels in regenerated axons have a similar latent period and are able to resume function after stimulation in the same amount of time as ion channels in normal nerve. Similarly, the response of regenerated nerves to train stimulation suggest that they can carry rapidly firing signals, much like those essential for motor function. The regenerated nerves were able to sustain a steady state amplitude, as a percentage of the maximum amplitude, that was not significantly lower than normal nerve. Both of these findings suggest that the ion channels that are produced and inserted into the regenerating axon membrane perform physiologically similar to normal.
The action potential for B-fibers was not visible in normal nerve; however, regenerated nerves had B-fiber peaks that were similar among groups. Conduction velocities of 10-25 m/s have been recorded in normal cat nerve for B-fibers (termed group III fibers in Boyd and Kalu, 1979), 2-5 μm in diameter (Boyd and Kalu, 1979); however, B-fiber recordings from normal rat sciatic nerve have not been reported. The electrophysiological evidence for a B-fiber peak in regenerated nerves is consistent with the histological finding of many nerve fibers in the 2-3 μm diameter range (Figure 2.7). In addition, it is not surprising that no differences in B-fiber conduction velocity and amplitude were detected among groups since all regenerated groups contained an abundance of fibers 2-3 μm in diameter. We speculate that the B-fiber peak disappears over time in the compound nerve action potential as the regenerating axons grow in diameter and assimilate into the A-fiber peak. Measurement of the C-fiber peak revealed no differences between normal and regenerated nerves, indicating that the small diameter, unmyelinated axons responsible for this peak (Arbuthnott, et al., 1980; Strichartz and Covino, 1990) had recovered normal electrophysiological capacity.

Functionally, the nerves regenerated through all implants, except the SI group, were able to respond to sensory stimulation. In addition, electrical stimulation of the nerve proximal to the lesion resulted in visible muscle movement. These findings confirmed that functional connections were made between regenerating axons and muscle fibers, deep pain receptors and cutaneous pain receptors. The level of response and the apparent sensitivity of these connections did not differ significantly from normal. What is unknown, however, is whether the connections were made with the appropriate target organ which would allow the animal to carry out instructions from the brain.

In all groups, the total number of myelinated axons per nerve at the mid-point of the gap increased from the time of surgery to 30 weeks and then remained constant (p>0.3), indicating that no additional axons crossed the mid-point of the gap or became myelinated after that time (Figure 2.5). In contrast, the autograft curve reached its plateau by 6 weeks post-
operatively (p>0.4; Figure 2.5). Using a poly(DL-lactide-ε-caprolacton) nerve guide to bridge a 10-mm gap in the rat sciatic nerve, it was observed that the number of myelinated axons reached plateau values by 6 weeks (den Dunnen, et al., 1996). The plateau region of the curve, reported in this study and in others (den Dunnen, et al., 1996), likely coincided with a period of maturation, consisting of increasing axon diameters and thickening myelin sheaths of a constant number of myelinated axons.

The kinetic plot of the total number of large diameter axons increased relatively linearly between 6 and 60 weeks (Figure 2.8), with significant increases in the LC and LC/M groups between 30 and 60 weeks (p<0.05). If the linear increase continued beyond 60 weeks, the number of large fibers would reach normal levels by 110 weeks (about 2 years) for the LC/M group, but not until 200 weeks (about 4 years) for the LC group. In any case, however, the data must eventually reach a plateau, suggesting growth has ceased. It can be speculated that the maturation of axons could continue over several more weeks, resulting in a regenerated nerve more closely resembling normal; however, data over longer recovery periods are necessary to determine the location of the presumptive plateau.

An unexpected finding was the observation of tissue reorganization between 30 and 60 weeks. We observed three significant changes in the regenerated nerves from 30 to 60 weeks: an increase in the total number of large diameter fibers (LC and LC/M groups; p<0.05), an increase in the total cross-sectional area occupied by myelinated axons (LC/M, SI/M, and AG groups; p<0.05), and a decrease in the total tissue cable area (LC and LC/M groups; p<0.01). All three of these long-term changes, each of which is significant only in the LC/M group, apparently lead to a nerve trunk that is closer in structure to normal nerve. In contrast, the results of a short-term kinetic study, using poly(DL-lactide-ε-caprolacton) tubes to bridge 10-mm gaps in the rat sciatic nerve, suggested that maturation was complete by 10 weeks (den Dunnen, et al., 1996). Specifically, these data (den Dunnen, et al., 1996) showed a plateau of the N-ratio (myelinated axon area/total area) at 6 weeks post-operative, while our findings
show that significant increases in the N-ratio occur from 30 to 60 weeks in the LC and LC/M groups (p<0.01). Maturation of axons beyond six months has been reported (Archibald, et al., 1995; Tountas, et al., 1993); however, significant changes in the connective tissue of the nerve trunk have not been previously reported. Since the most vigorous remodeling occurred in the large-pore collagen tubes, future studies will address which tube property (permeability or tube composition) is responsible for the observations of long-term changes in the nerve trunk.
Chapter 3: Effects of Implant Parameters on the Mechanistic Steps of Axon Regeneration

3.1 Introduction

The peripheral nerve comprises two tissue components: the conducting tissue (i.e., axons) and the non-conducting tissue (i.e., connective tissue). This chapter will focus on the regenerative mechanisms of the conducting tissue and Chapter 4 will consider the mechanisms of connective tissue repair.

3.1.1 Mechanistic Steps of Axon Regeneration

The process of peripheral axon regeneration across a tubulated gap can be divided into five mechanistic steps (Dellon, 1990; Fawcett and Keynes, 1990; Fields, et al., 1989; Fu and Gordon, 1997; Seckel, 1990; Williams, et al., 1983):

1) Sprouting of axons outward from the proximal stump,

2) Elongation of axons across the gap between the two stumps,

3) Migration of the axons through the distal stump which terminates in reconnection of axons to the target organ (i.e., muscle or sensory organ),

4) Maturation of axons to a fully functional state, and

5) Pruning of non-functional axon sprouts.

These five steps occur serially in time for each individual axon, with each step dependent on completion of the previous step, and can be located spatially along the length of the nerve (Figure 3.1).

Prior to the onset of regeneration, both the proximal and distal nerve stumps undergo degenerative responses to injury (Figure 3.2). Immediately following transection injury, the proximal nerve stump begins a process of traumatic degeneration in which the cut axon reseals
its membrane and retracts back at least until the next node of Ranvier (approx. 0.5 mm). This process occurs within hours of transection injury (Fawcett and Keynes, 1990; Seckel, 1990). A different degenerative process, Wallerian degeneration, occurs in the distal stump following injury. This process consists primarily of phagocytosing the axoplasm and myelin of the disconnected axons and, as a result, preparing space in the distal stump for regenerating axons (Fawcett and Keynes, 1990; Seckel, 1990). Following transection, approximately 15-50% of peripheral neurons die due to the trauma of injury (Fu and Gordon, 1997; Lisney, 1989). It has been hypothesized that neurons with a relatively large volume of axoplasm have the best chance for survival; therefore, the majority of cell deaths are thought to be associated with small diameter, unmyelinated axons (Lisney, 1989). If the neuron survives the initial trauma of transection, the axon initiates will begin the mechanistic steps of regeneration.

Axon sprouting, the first mechanistic step in regeneration, is the process by which a neuron cell body extends several axon sprouts (rather than just one) following injury (Figure 3.3a). These sprouts extend initially into the freshly denervated proximal stump (Seckel, 1990). One neuron cell body can sprout as many as 100 daughter axons; however, typically
Figure 3.2 Schematic showing the degenerative events that follow injury to a peripheral nerve axon. (a) Structure of the uninjured neuron highlighting the location of the injury. (b) Following transection injury, traumatic degeneration occurs in the proximal stump with the axon retreating back to at least the next node of Ranvier. In the distal nerve stump, macrophages and Schwann cells participate in the phagocytosis of the axoplasm and myelin debris. After phagocytosis is complete, the Schwann cells will line the interior of the endoneurial tube, providing a favorable substrate for axon migration through the distal stump.

Only five sprouts are produced (Fu and Gordon, 1997). It has been observed that sprouting occurs within hours of injury, a period which is too short for sending and receiving a signal from the neuron cell body via chemical transport systems within the axon. This suggests that the sprouting response is mediated by the axon tip. In fact, severed axons that have been disconnected from the cell body still extend axon sprouts (Fawcett and Keynes, 1990).

After a neuron has sprouted multiple axons, the next step in the regeneration process is elongation of the axon sprouts across the injury site (Figure 3.3b). Elongation occurs by attachment and migration of the axon tip along a substrate material, a process termed contact guidance (Weiss, 1941; Weiss and Taylor, 1944). The chemical composition of the substrate is a crucial factor in facilitation of axonal elongation. The contact between axonal growth cone
Figure 3.3 Schematic representation of the five mechanistic steps of peripheral axon regeneration: (a) sprouting, (b) elongation across the 10-mm gap, (c) migration through the distal stump, (d) maturation, and (e) pruning.
and substrate, which provides the means for elongation, is hypothesized to occur at binding sites along the surface of the substrate. Collagen has been shown to support the elongation of peripheral axons in vitro (Baron-Van Evercooren, et al., 1982; Ebendal, 1976; Letourneau, 1975; Tonge, et al., 1996) and it has been suggested that axonal outgrowth occurs by progression of growth cones across collagen surfaces (Bunge and Bunge, 1978). In vivo, insoluble collagen matrices have been used to improve regeneration across nerve gaps bridged by tubular devices (Chang, et al., 1990; Ohbayashi, et al., 1996; Yannas, et al., 1987). In particular, an aligned type I collagen-glycosaminoglycan (CG) copolymer matrix enhanced the regeneration of axons across 10- (Chang, et al., 1990) and 15-mm gaps (Yannas, et al., 1987) in the rat sciatic nerve, when compared to empty tube controls.

Once an axon has traversed the gap and reached the distal stump, the axon must migrate through the distal stump before reconnecting to a distal target organ (i.e., muscle) to form a functional unit (Figure 3.3c). During Wallerian degeneration, Schwann cells (the primary support cell in the peripheral nerve) and macrophages remove the axonal and myelin debris through phagocytosis (Figure 3.2) (Fawcett and Keynes, 1990). Endoneurial tubes, the extracellular matrix structures that surround normal axons, are left relatively intact and serve as a favorable substrate for axonal migration (Dellon, 1990; Fu and Gordon, 1997). Schwann cells, which line the endoneurial tubes, provide additional support for the migrating axons and help direct axons toward a distal target (Fawcett and Keynes, 1990). The distal stump may begin to lose its favorable qualities over time and, following long-term degeneration (> 1 year), axons migrate poorly through the distal stump (Fu and Gordon, 1997).

If distal stump migration is completed and a reconnection is made, the regenerating axon enters the next step of regeneration which is maturation (Dellon, 1990; Sanders and Young, 1946; Weiss, et al., 1945). Axons mature in two ways: by increasing the axon diameter and increasing the myelin thickness (Figure 3.3d). Neuron cell bodies, located at the base of the spinal cord, produce new axoplasm which is transported down the axon, allowing
for radial growth of the axon (i.e., increasing the axon diameter) following formation of a functional connection. Some increase in axon diameter can occur in axon sprouts that are not connected to distal targets (Fu and Gordon, 1997); however, it has been shown that axons larger than 6 μm in diameter have likely reconnected to a distal endpoint (Aitken, 1949). It has also been demonstrated that the ultimate diameter of regenerating axons is determined by the diameter of the parent axon that sent it (Fu and Gordon, 1997). Therefore, growth in diameter of axons in the distal stump must be accompanied by resorption and reformation (remodeling) of the endoneurial tubes to provide space for axons that have regrown into inappropriate endoneurial tubes. Myelination begins within 1-3 weeks following injury (Fawcett and Keynes, 1990; Fields, et al., 1989), well before any functional connections are made. However, myelin sheaths thicken over time in proportion to the growth in axon diameter and, therefore, myelination is enhanced by the reconnection of axons to distal target organs (Dellon, 1990). Determination of whether a regenerating axon will become myelinated also appears to be regulated by the outgrowing axons, and not by the Schwann cells that are forming the myelin sheath (Fu and Gordon, 1997).

The final step in the axonal regeneration process is the degeneration of axon sprouts that have not reached a distal target organ (Dellon, 1990); a process termed ‘pruning’ (Figure 3.3e). Currently, it is believed that once a regenerating axon connects to an appropriate target, the neuron that sent it withdraws support from the other daughter axons, and eventually those under-nourished sprouts will degenerate. The mechanism by which pruning occurs is not well characterized (Dellon, 1990). The likelihood of pruning was observed in early studies based on a loss of axons over time (Aitken, et al., 1947). More recent studies have confirmed these findings and suggest that the process of pruning takes a significant amount of time (up to 2 years) to be completed (Henry, et al., 1985).

In summary, axonal regeneration involves five mechanistic steps: sprouting, elongation across the injury site, migration through the distal stump, maturation, and pruning.
The effects of tubes and substrate materials on these mechanisms have not been investigated in a systematic fashion. Knowledge of these effects may lead to the design of devices targeted to improve individual mechanisms. In addition, it is also not well understood how regeneration of axons through a tubular device differs from regeneration through an autograft repair. These issues are addressed in this chapter.

3.1.2 Project Goal

The objectives of this study were to determine the effect of implant parameters on the mechanistic steps of axon regeneration and to determine an approximate time course for each mechanistic step. Three implant parameters were evaluated: presence of the CG matrix (filled versus unfilled tubes), tube composition (silicone versus large-pore collagen tubes), and presence of a living tissue graft (autograft versus tubular repair).

To study the implant effects, large-pore collagen and silicone tubes were implanted, both empty and filled with a collagen-glycosaminoglycan (CG) matrix, to bridge a 10-mm gap in the adult rat sciatic nerve. The CG matrix was a highly characterized, insoluble network which has been shown to elicit biological activity within the regenerating peripheral nerve (Chang, et al., 1990; Chang and Yannas, 1992; Yannas, et al., 1985; Yannas, et al., 1987). In addition to the biomaterial implants, a sciatic nerve autograft was used as a control. Contralateral nerves were evaluated as normal controls. In this study, regenerated nerves collected at 6, 30 and 60 weeks post-wounding were evaluated to determine the time course of each mechanistic step. Histomorphometric analyses were performed to determine the number and size distribution of axons at five locations along the length of the nerve. Using these data, the effects of each implant parameter on the five mechanistic steps of axon regeneration were evaluated as follows:

1) The effects of implant parameters on sprouting were measured directly by comparing the number of axons in the proximal nerve stump at 30 weeks among groups. Since sprouting of axons occurs in the microenvironment of the proximal stump, and begins so quickly
after axotomy, it was hypothesized that the sprouting mechanism of regeneration is not affected by the type of device implanted.

2) Completed elongation, or the number of bridged axons, was evaluated by counting the number of axons in the distal sciatic nerve stump, 11-mm from the proximal cut, at 60 weeks. The process of elongation is mediated by the attachment of the axon growth cone to a substrate material; therefore, it was hypothesized that the presence of the CG matrix may enhance the number of axons which complete elongation, or reach the distal stump.

3) Comparisons among implant groups of the number of axons in the tibial and peroneal nerve branches at 60 weeks were used to infer differences in migration through the distal stump. Because the distal stump provides a favorable environment for axonal regeneration, it was hypothesized that migration through the distal stump was not directly affected by the implant parameters; however, indirect implant effects may be present due to cumulative effects from previous mechanistic steps.

4) Maturation was evaluated directly by comparing the number of large diameter fibers (≥ 6 \( \mu \text{m} \)) among groups after 60 weeks. It was hypothesized that only indirect implant effects would be observed on maturation, since maturation occurs after the matrix has degraded and the maturing axons are not in direct contact with the tube.

5) Pruning was evaluated by the change in the number of myelinated axons in the proximal sciatic nerve stump between 30 and 60 weeks. Since pruning occurs after degradation of the implants, it was hypothesized that the implant parameters would have no effect.

3.2 Materials and Methods

Descriptions of the peripheral nerve devices, animal model, histomorphometric procedures, and statistical methods can be found in section 2.2. More detailed protocols for each procedure are located in Appendix A and sample calculations are presented in Appendix B for reference.
3.2.1 **Tissue Collection Procedure**

Following sacrifice at 30 or 60 weeks, the sciatic nerves were explanted from the sciatic notch at the hip to beyond the bifurcation point at the knee level, including the tibial and peroneal nerve branches proximal to the muscle insertion point (Figure 3.4). For tissue retrieved at 6 weeks, the sciatic nerves were explanted from the sciatic notch to the distal bifurcation; the tibial and peroneal nerve branches were not explanted. After explantation the tissue was placed in Yanoff's fixative. The nerves were sectioned into 2-mm segments and several segments were selected for analysis of the mechanistic steps of axon regeneration (Figure 3.5). The sectioning notation was slightly modified from a previous study (Landstrom, 1994) and can be cross-referenced to the notation used by Williams and coworkers (Williams, et al., 1983; Williams, et al., 1984). For example, the section from the center of the nerve gap in this study corresponded to the 'S5' section of Williams and

![Figure 3.4](image)

**Figure 3.4** Anatomy of the rodent hind limb showing the location of the sciatic, tibial, and peroneal nerves in relation to other anatomical structures.
Figure 3.5 Sectioning diagram for the explanted nerves at 30 and 60 weeks showing the locations and names of the tissue used in this study of axon regeneration mechanisms. Sectioning was identical for nerves explanted at 6 weeks; however, no tissue was collected beyond the distal bifurcation. For each tissue section, the distance from the proximal stump is given which indicates how far an axon must grow from the proximal edge of the nerve gap to reach that site.

coworkers. Terminology used to refer to the different tissue sections in this chapter are as follows: proximal sciatic nerve stump (-1 mm from the proximal cut), center of the nerve gap (5 mm from the proximal cut), distal sciatic nerve stump (11 mm from the proximal cut), tibial nerve branch (20 mm from the proximal cut), and peroneal nerve branch (20 mm form the proximal cut) (Figure 3.5). Data collected from the center of the nerve gap were reported in Chapter 2. The selected sections (Figure 3.5) were post-fixed in 1% osmium tetroxide (Cat #0972A, Polysciences, Inc., Warrington, PA), embedded in Epon (Poly/Bed 812 Embedding Kit, Cat #08792, Polysciences, Inc, Warrington, PA), and sectioned at a 1-μm thickness. The remainder of the histomorphometric procedure follows that described in section 2.2.5.

In addition to quantifying the total number of myelinated fibers, the total number of large diameter fibers (≥ 6 μm), and the total myelinated fiber area, the g-ratio was calculated for individual axons as a measure of myelination. Two diameters were measured: the axon core diameter and the fiber diameter (axon core plus twice the myelin thickness). For each axon, a g-ratio (axon core diameter/fiber diameter) was calculated and scatterplots of g-ratio
versus fiber diameter were made for each nerve. The linear regression slopes were compared among groups.

### 3.3 Results

#### 3.3.1 Total Number of Myelinated Fibers

**Proximal Sciatic Nerve Stump**

The total number of myelinated axons in the proximal sciatic nerve stump plotted as a function of time showed three regions: a growth, a plateau, and a decline region (Figure 3.6). The number of myelinated fibers increased significantly between 0 and 6 weeks (p<0.05), did

![Graph showing the total number of myelinated axons over time for different nerve groups.](image)

**Figure 3.6** Total number of myelinated axons in the proximal sciatic nerve stump as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. For each group, the number of axons had increased significantly by 6 weeks post-operative and did not change significantly between 6 and 30 weeks (p>0.6). The number of axons dropped in each group from 30 to 60 weeks; however, the decrease was significant only in the LC tube group (p<0.05).
not change significantly from 6 to 30 weeks (p>0.6), and then decreased from 30 to 60 weeks for all groups (Figure 3.6), except the unfilled silicone tube group (SI). The decrease in total number of axons from 30 to 60 weeks was significant in the LC group (p<0.05), however, the decreases in the SI/M (p=0.1), LC/M (p=0.15), and AG (p=0.3) groups were not significant. The SI group followed a similar pattern as the other implant groups through 30 weeks, however, from 30 to 60 weeks the number of axons increased in the SI group rather than decreasing; the increase was not significant (p=0.3).

At all time points, the number of myelinated axons in the proximal sciatic nerve stump was significantly higher than the normal control regardless of implant group (Figure 3.7; p<0.001). Two-way analysis of variance revealed no significant effects of the CG matrix (p>0.5) or tube composition (p>0.4) on the number of myelinated fibers in the proximal sciatic

![Proximal Sciatic Nerve, 30 weeks](image)

**Figura 3.7** Total number of myelinated axons in the proximal sciatic nerve stump at 30 weeks post-operative for all implant groups. Among the tubulated groups, there was no significant difference in the number of axons (p>0.4); however, all tubulated groups had significantly more axons than the autograft (p<0.05). NS = not significant.
nerve stump at 30 weeks (Figure 3.7). All tubulated groups; however, had significantly more myelinated axons at this location than the autograft repaired nerves (p<0.05).

**Distal Sciatic Nerve Stump**

The number of myelinated fibers in the distal sciatic nerve stump increased up to 30 weeks post-operative and subsequent changes were not significant (Figure 3.8; p>0.2). In all tubulated groups, an increase in the number of axons in the distal stump was observed between 30 and 60 weeks; however, the increase was not significant.

At 60 weeks, the number of myelinated axons in the distal sciatic nerve stump was significantly lower than normal for the SI group (p<0.01), which had no axons reaching the distal stump. Otherwise, all regenerated nerves had a similar number of axons to normal (Figure 3.9). Similarly, all regenerated nerves had significantly more axons in the distal sciatic

![Distal Sciatic Nerve](image)

**Figure 3.8** Total number of myelinated axons in the distal sciatic nerve stump as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. In each implant group, the number of axons in the distal sciatic nerve stump did not change significantly from 30 to 60 weeks.
Figure 3.9  Total number of myelinated axons in the distal sciatic nerve stump at 60 weeks post-operative for all implant groups. There were no significant differences among implant groups at 60 weeks based on the number of axons in the distal sciatic nerve stump. In addition, the number of axons was not significantly different than normal. The exception was the SI group which had no axons in the distal sciatic nerve stump. NS = not significant

nerve stump than the SI group (p<0.001); however, no other significant differences existed among the implant groups.

Tibial Nerve Branch

For all implant groups, the number of myelinated axons in the tibial nerve branch increased up to 30 weeks and did not change significantly thereafter (Figure 3.10; p>0.5).

By 60 weeks post-operative, the number of myelinated axons in the LC, SI/M and AG groups were not significantly different from normal (Figure 3.11; p>0.4). The SI group had significantly fewer axons than normal (p<0.001) and the LC/M group had significantly more axons in the tibial nerve than normal (p<0.05). Similarly, compared to the autograft, the LC/M
Figure 3.10 Total number of myelinated axons in the tibial nerve branch as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. The number of myelinated axons did not change significantly from 30 to 60 weeks in any of the implant groups.

group had significantly more axons (p<0.05) and the SI group had significantly fewer axons (p<0.001). The remaining groups were not significantly different from the AG (p>0.4). Two-way ANOVA revealed significant effects of the CG matrix (p<0.001) and tube composition (p<0.001) on the number of axons in the tibial nerve (Figure 3.11). The presence of the CG matrix significantly increased the number of axons in the tibial nerve in both the silicone (p<0.001) and large-pore collagen (p<0.05) tubes group compared to the corresponding unfilled tubes.

Peroneal Nerve Branch

The number of myelinated axons in the peroneal nerve branch increased up to 30 weeks and did not change significantly (p>0.2) thereafter for all implant groups (Figure 3.12). The
Figure 3.11 Total number of myelinated axons in the tibial nerve branch at 60 weeks post-operative for all implant groups. The presence of the CG matrix significantly increased the number of myelinated axons in the tibial nerve branch compared to the unfilled tubes (p<0.05).

The number of axons increased from 30 to 60 weeks in all groups, however, the differences were not significant.

At 60 weeks post-operative, the SI/M, LC/M and AG groups all had significantly more myelinated axons in the peroneal nerve than the normal control (Figure 3.13; p<0.01). The LC tube group was not significantly different from normal (p>0.2) and the SI group had significantly fewer axons than normal (p<0.001). All of the tubular implants, except for the SI group (p<0.001), had a similar number of axons to the autograft group. Both the presence of the CG matrix (p<0.001) and the tube composition (p<0.005) had significant effects on the number of axons in the peroneal nerve. The matrix-filled large-pore collagen (p<0.05) and silicone (p<0.001) tubes had significantly more axons than the corresponding unfilled tubes.
Figure 3.12 Total number of myelinated axons in the peroneal nerve branch as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. The number of axons in the peroneal nerve did not change significantly between 30 and 60 weeks for any of the groups, however, each group had an increased number of axons over that period.

Changes Along the Length of the Nerve

All of the regenerated nerves had a significant decrease in the number of axons between the proximal sciatic nerve stump and the center of the nerve gap (Figure 3.14; p<0.01). From the center of the nerve gap to the distal sciatic nerve stump, there was a significant decrease in the number of axons in the SI/M and LC/M groups (p<0.05). In contrast, the number of axons did not change significantly from the center of the gap to the distal nerve stump for the SI, LC, and AG groups (p>0.3). For all groups, the number of fibers that entered the distal nerve stump was not significantly different from the total number of axons reaching the tibial and peroneal nerve branches (p>0.4).
Figure 3.13  Total number of myelinated axons in the peroneal nerve branch at 60 weeks post-operative for all implant groups. The presence of the CG matrix significantly increased the number of myelinated axons in the peroneal nerve compared to the unfilled tubes (p<0.05). The SI/M, LC/M and AG groups all had significantly more axons in the peroneal nerve than normal (p<0.01). NS = not significant.

3.3.2 Fiber Diameter Distributions

Tibial Nerve Branch

Normal tibial nerve had a bimodal fiber diameter distribution with peaks at 2.5 μm and 8 μm (Figure 3.15) that was similar in shape to the distribution of normal sciatic nerve (Figure 2.7). In contrast, the diameter distribution of all regenerated nerves had a mode of approximately 2.5 μm at both 30 and 60 weeks. Between 30 and 60 weeks, the number of fibers in the large diameter bins increased significantly, but the result was still inferior to normal (Figure 3.15).
Figure 3.14 Total number of myelinated axons at 30 weeks as a function of distance along the nerve for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. The number of axons at 20 mm is the sum of the tibial and peroneal nerve branches. From the proximal edge of the gap to the distal edge of the gap, there was a significant decrease in the number of axons per nerve for all groups. In the case of the tubulated groups, only 20 - 25% of the axon sprouts crossed the gap and entered the distal nerve stump. Between the entrance to the distal stump and the nerve branches, very few axons were lost.

Peroneal Nerve Branch

The fiber diameter distribution of normal peroneal nerve (Figure 3.16) had no dominant mode and had a drop in the number of fibers larger than 11 μm, resulting in a much different shape than both the sciatic and tibial nerves (Figures 2.7 and 3.15). Again, the regenerated nerves had a mode around 3 μm; however, in the regenerated peroneal nerves, the number of fibers in the range from 6 - 9 μm was similar to normal at 60 weeks. In addition, the regenerated nerves at 60 weeks had a similar number of the largest diameter fibers (greater than 12 μm) compared to the normal nerve.
Figure 3.15 Fiber diameter distribution for normal tibial nerve and the matrix-filled large-pore collagen tube group (LC/M) at 30 and 60 weeks. The normal nerve distribution had two modes in the distribution at 2.5 and 8 μm. In the regenerated nerves, the overall shape of the distribution was similar for all groups. The mode of the regenerated distribution was 2.5 μm at 30 weeks, and increased to approximately 3 μm at 60 weeks. From 30 to 60 weeks, a significant shift in the distribution toward the larger diameter bins was observed.

3.3.3 Total Number of Large Diameter Fibers

The term “large diameter fibers” (LDF) was used to refer to myelinated axons with fiber diameters (axon core plus twice the myelin thickness) greater than or equal to 6 μm.

Tibial Nerve Branch

The total number of LDF in the tibial nerve of all implant groups increased continuously at an approximately constant rate during the period of observation (0 to 60 weeks); however, the increase in number of LDF from 30 to 60 weeks was significant only in the LC/M group (Figure 3.17; p<0.01). Using linear regression analysis, it was determined
that a positive slope existed for all tubulated groups ($r>0.8; p<0.01$), except the unfilled silicone tube group ($p>0.9$). The rate of increase in the number of LDF for the LC/M group (28 LDF/week) was significantly higher ($p<0.001$) than all other implant groups.

At 60 weeks, all implant groups had significantly fewer LDF fibers than normal tibial nerve (Figure 3.18; $p<0.001$). The LC/M group had significantly more LDF than all other implant groups, including the AG group (Figure 3.18; $p<0.01$). Two-way ANOVA revealed a significant effect of matrix presence ($p<0.001$) and tube composition ($p<0.001$) on the number of LDF in the tibial nerve branch.
**Figure 3.17** Total number of large diameter myelinated fibers (≥ 6 μm) in the tibial nerve branch as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. The number of large diameter fibers increased at an approximately constant rate from 0 to 60 weeks for all groups. The increase from 30 to 60 weeks; however, was significant only in the LC/M group (p<0.01).

**Peroneal Nerve Branch**

In the peroneal nerve branch, the number of LDF followed a similar pattern: increasing at an approximately continuous rate through 60 weeks post-operative (Figure 3.19). The increase in the number of LDF from 30 to 60 weeks was significant in the LC (p<0.01) and LC/M (p<0.005) groups, but not in the SI/M (p=0.1) and AG (p=0.15) groups. Each implant group had a positive slope between 0 and 60 weeks (r>0.85; p<0.001), except for the SI group (p>0.9). The slope of the LC/M group (12 LDF/week), determined using linear regression analysis, was significantly greater than the slopes of all other implant groups (p<0.001).
Figure 3.18  Total number of large diameter myelinated axons (≥ 6 μm) in the tibial nerve branch at 60 weeks post-operative for all implant groups. The LC/M group had significantly more large diameter fibers in the tibial nerve at 60 weeks than all other implant groups (p<0.01), but only half the number found in normal tibial nerve. NS = not significant.

All regenerated nerves had significantly fewer LDF fibers than normal peroneal nerve (p<0.001); however, the number of LDF in the LC/M group was over 75% of normal (Figure 3.20). The LC/M group had significantly more LDF than all other implant groups at 60 weeks (p<0.05). Both the CG matrix and the tube composition had significant effects on the number of LDF in the peroneal nerve (ANOVA, p<0.001).

Changes Along the Length of the Nerve

For the regenerated nerves, the number of LDF did not change significantly along the length of the nerve. There were no significant differences between the number of LDF in the center of the nerve gap and in the distal nerve tributaries (tibial nerve plus peroneal nerve) for any of the implant groups (p>0.2).
**Figure 3.19** Total number of large diameter myelinated fibers (≥ 6 μm) in the peroneal nerve branch as a function of time for the matrix-filled large-pore collagen (LC/M), unfilled large-pore collagen (LC), and autograft (AG) groups. For each implant group, the number of large diameter fibers increased at an approximately constant rate from 0 to 60 weeks. The increase in the number of LDF from 30 to 60 weeks was significant in both the LC and LC/M groups (p<0.01).

### 3.3.4 Total Myelinated Fiber Area

**Tibial Nerve Branch**

The total area occupied by myelinated fibers increased from 30 to 60 weeks in all experimental groups (Table 3.1); however, the increase was significant only in the LC/M group (p<0.05). In all experimental groups, the total area occupied by myelinated fibers in the tibial nerve was significantly smaller than normal (p<0.01). Two-way ANOVA revealed significant effects of both the CG matrix and tube composition (p<0.001) on the myelinated fiber area at 60 weeks. In addition, the interaction term of the ANOVA was not significant.
Figure 3.20 Total number of large diameter myelinated axons (≥ 6 μm) in the peroneal nerve branch at 60 weeks post-operative for all implant groups. The matrix-filled large-pore collagen tube group (LC/M) had significantly more LDF than all other implant groups (p<0.05) and had approximately 75% of the normal number of LDF. NS = not significant.

(p>0.25) indicating that the effect of the matrix was independent of tube type. By 60 weeks, the LC/M group had a significantly larger fiber area than all other implant groups (p<0.01).

All implant groups were superior to the SI device (p<0.001).

Peroneal Nerve Branch

From 30 to 60 weeks, all implant groups (except the SI group) had a significant increase in the total area occupied by myelinated fibers (Table 3.1; p<0.05). The LC/M group had a total area occupied by myelinated fibers not significantly different from normal peroneal nerve (p>0.2); however, all other implant groups had significantly smaller fiber areas than normal (p<0.01). The CG matrix and tube composition both had significant effects on fiber area at 60 weeks (ANOVA; p<0.001). The LC/M group had a significantly larger fiber area
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<th>Total Area Occupied by Myelinated Fibers (mm²)</th>
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<td>Tibial Nerve</td>
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<tr>
<td></td>
<td>30 Weeks</td>
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<tr>
<td>Normal</td>
<td>0.25 ± 0.01</td>
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<tr>
<td>SI</td>
<td>0.02 ± 0.02</td>
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<tr>
<td>SI/M</td>
<td>0.09 ± 0.01</td>
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<tr>
<td>LC</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>LC/M</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>AG</td>
<td>0.08 ± 0.01</td>
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<th>Tibial Nerve</th>
<th>Peroneal Nerve</th>
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<td>30 Weeks</td>
<td>60 Weeks</td>
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<tr>
<td>SI</td>
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<td>AG</td>
<td>0.08 ± 0.01</td>
<td>0.09 ± 0.01</td>
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Table 3.1: Morphological measurements (mean ± SEM) at 30 and 60 weeks of the total cross-sectional area occupied by myelinated nerve fibers in the tibial and peroneal nerve branches. The implant groups were as follows: unfilled silicone tube (SI), matrix-filled silicone tube (SI/M), unfilled large-pore collagen tube (LC), matrix-filled large-pore collagen tube (LC/M), and autograft (AG).

than all other implant groups (p<0.05) and all groups were significantly superior to the SI group (p<0.001).

3.3.5 Myelination

Calculation of the g-ratio for regenerated axons in the distal nerve tributaries at 60 weeks revealed no significant differences in myelination among implant groups. In addition, there were no significant differences between the average g-ratios of regenerated and normal nerve (Figure 3.21).

3.4 Discussion

The objectives of this study were twofold: 1) determine the kinetics of each mechanistic step of axon regeneration and 2) determine how individual implant parameters effect each mechanistic step of axon regeneration. In order to accomplish these objectives, the data were evaluated as they relate to each mechanistic step of regeneration. Based on the three time points evaluated in this study (6, 30, and 60 weeks) and the four locations along the length of
Figure 3.21 g-ratios (axon core diameter/fiber diameter) as a function of axon fiber diameter for (a) normal nerve and (b) nerve regenerated through matrix-filled large-pore collagen tubes (LC/M) at 60 weeks. (a) Normal nerve has a relatively flat regression line indicating that the myelin thickness is proportional to the fiber diameter. The distribution of fiber diameters is even with a similar number of fibers at each diameter. The g-ratios for the majority of normal axons ranged from 0.6 to 0.8; with an average g-ratio for the normal nerves of 0.75. (b) Representative plot of g-ratio versus fiber diameter for regenerated nerves. The regression slope was more positive than normal nerve indicating that, in general, smaller diameter fibers had thicker myelin sheaths and larger diameter fibers had thinner myelin sheaths than normal. A larger number of axons were concentrated in the small fiber diameter range compared to the larger diameters, consistent with the fiber diameter distribution of regenerated nerve (Figures 3.15 and 3.16). The g-ratios for regenerated nerves ranged from 0.55 to 0.8; with an average g-ratio of 0.77 in the LC/M group. The average g-ratios for regenerated nerves were not significantly different from normal nerve.

the nerve (proximal sciatic, distal sciatic, tibial nerve, and peroneal nerve), information on each mechanistic step was inferred (see section 3.1.2 for detailed description).

3.4.1 Kinetics of the Mechanistic Steps of Axon Regeneration

Changes in the total number of axons in the proximal sciatic nerve stump as a function of time (Figure 3.6) convey kinetic information about the first and last steps in the regenerative process: sprouting and pruning. The number of axons in the proximal sciatic nerve increased rapidly to a number significantly higher than normal by 6 weeks and did not change significantly between 6 and 30 weeks post-operative. These findings suggest that sprouting occurs very early in the regenerative process and is complete by 6 weeks (Figure 3.22). These
findings concur with previous reports that sprouting begins immediately following injury (Fawcett and Keynes, 1990); however, it has not previously been shown when sprouting is completed. From 30 to 60 weeks, the number of axons in the proximal sciatic nerve dropped in every implant group (Figure 3.6), however, the difference was significant only in the LC group (p<0.05). These findings suggest that some pruning had occurred by 60 weeks; however, previous studies have suggested that pruning takes up to 2 years (approximately 100 weeks) to be completed (Henry, et al., 1985). Since the decrease in the number of axons was not significant in all groups, and all regenerated nerves still had significantly more axons than normal at 60 weeks, it is likely that pruning was in the early phases between 30 and 60 weeks and may continue for several more weeks until completion (Figure 3.22).

The number of myelinated axons in the distal sciatic nerve provided information on the kinetics of completed elongation. For all groups, the number of axons in the distal sciatic nerve increased significantly from 0 to 30 weeks, and subsequent changes were not significant.

Figure 3.22 Axonal regeneration time line that places each mechanistic step of regeneration into an approximate temporal location. Sprouting occurred very early and was complete by 6 weeks post-operative. Elongation has been shown to begin within one week of injury, but was not complete until 30 weeks post-operative. Similarly, migration through the distal stump was not complete until after 30 weeks post-operative. Maturation began after approximately 6 weeks and was still ongoing at 60 weeks. Pruning had begun between 30 and 60 weeks, but would likely continue over several more weeks before completion.
(Figure 3.8). These results suggest that elongation across the gap was complete by 30 weeks and no additional axons reached the distal stump after that time. Although the differences were not significant, the tubulated groups all had increases in the number of axons from 30 to 60 weeks suggesting that elongation may take slightly longer than 30 weeks to be completed (Figure 3.22). The onset of elongation was not evaluated in this study, but must lag behind sprouting which begins immediately following injury. Other studies have reported that the fastest elongating axons cross the 10-mm gap by 4-6 weeks (Fields, et al., 1989; Williams, et al., 1983). The current study suggests that elongation of axons may continue over a much longer period, approximately 30 weeks, before a steady state number of axons was observed in the distal sciatic nerve. Therefore, although the fastest axons likely entered the distal sciatic nerve as early as 4-6 weeks following injury, elongation across the gap may have continued for many more weeks while more slowly moving axons crossed the gap until elongation was completed at 30 weeks.

The kinetics of migration through the distal stump were evaluated using the number of axons in the tibial and peroneal nerves. Migration through the tibial nerve branch was apparently complete by 30 weeks post-operative, with no significant changes thereafter in the number of myelinated axons (Figure 3.10). In the peroneal nerve, however, the number of myelinated axons increased from 30 to 60 weeks, although not significantly, suggesting that migration through the peroneal nerve was not yet complete at 30 weeks (Figure 3.12). Migration through the distal stump cannot begin until axons enter the distal stump, likely at around 4-6 weeks (Fields, et al., 1989; Williams, et al., 1983), and was complete by 30 weeks or shortly thereafter (Figure 3.22). Migration through the distal stump had a similar time scale to completed elongation across the gap which suggests that the last axons to enter the distal nerve stump never make it a significant distance into the distal nerve stump. Previous studies of prolonged denervation have shown that the distal nerve stump begins to lose its supportive qualities over time and, after approximately one year, less than 15% of axons entering the
distal stump will traverse the distal stump and reach a target (Fu and Gordon, 1997). Our findings suggest that conditions may become unfavorable for migration through the distal stump by approximately 30 weeks.

The next step in the regenerative process, maturation, was assessed not by the total number of myelinated fibers but instead by the number of fibers exceeding 6 μm in diameter, or "large diameter fibers" (LDF), total area occupied by myelinated fibers, and extent of myelination (g-ratio). The number of LDF increased fairly linearly up to 60 weeks for all implant groups in three locations: the center of the nerve gap (Figure 2.8), the tibial nerve branch (Figure 3.17) and the peroneal nerve branch (Figure 3.19). Similarly, significant increases in the total area occupied by myelinated fibers were observed between 30 and 60 weeks (Table 3.1). These findings suggest that the process of maturation may continue beyond 60 weeks post-operative (Figure 3.22). These findings contradict previous studies in a rat sciatic nerve model, with a silicone tube bridging the 10-mm gap, which suggested that maturation was complete by 12 weeks post-operative (Fields, et al., 1989; LeBeau, et al., 1988). However, significant electrophysiological improvements have been reported between 6 and 12 months post-operative in primate models (Archibald, et al., 1995; Tountas, et al., 1993), which also suggest that maturation of axons may be ongoing after 6 months. Results from the center of the gap and the tibial nerve branch in the current study suggest that if the number of large diameter fibers continued increasing at the current rate, the total number of LDF would reach normal by approximately 120 weeks in the LC/M group and not until 240 weeks in the LC group. In the peroneal nerve, however, normal levels of LDF would be reached much sooner; 85 weeks for the LC/M group and 160 weeks for the LC group. The data must, however, eventually reach a plateau value, suggesting that growth has ceased. It can be speculated from these data that maturation may continue for several more weeks until reaching a plateau; however, more data are necessary to confirm the location of the presumptive plateau.
3.4.2 Effects of Implant Parameters on Axon Regeneration

Effect of the CG Matrix Presence

Considering first the effect of the CG matrix on the mechanistic steps of axon regeneration, it was most useful to compare the results in the unfilled and matrix-filled collagen tube groups since both devices resulted in successful bridging of the gap in 100% of the cases. The silicone tube groups provided a less attractive comparison since the unfilled silicone tube group had few cases which resulted in tissue cable formation. For purposes of clarity, the following discussion will involve only comparison of the collagen tube groups.

The numbers of axons in the proximal sciatic nerve and distal sciatic nerve were not significantly different for the LC and LC/M groups (Figures 3.7 and 3.9); therefore, it can be inferred that the CG matrix had no effect on the first two steps of regeneration: sprouting and completed elongation (Table 3.2). The first significant effect of the CG matrix was observed in the migration step with significantly more axons reaching the tibial and peroneal nerve branches in the matrix-filled collagen tube group than the unfilled collagen tube group (Figures 3.11 and 3.13). The matrix-filled collagen tube group also had enhanced maturation of axons over the unfilled tubes with significantly more large diameter fibers and a significantly larger area occupied by myelinated fibers in both the tibial and peroneal nerve branches. Pruning was not impacted by the presence of the CG matrix (Table 3.2).

These results suggest that the CG matrix had impact only on events that occurred outside the nerve gap (migration through the distal stump) and after the matrix had been degraded (maturation). Differences between unfilled and matrix-filled implants did not exist in the number of axons completing elongation across the gap; however, elongation is the mechanistic step in which the CG matrix can have the most influence. The results of this study suggest that, given enough time (i.e., 30 weeks), a similar number of axons will successfully elongate across a 10-mm nerve gap implanted with either an unfilled or matrix-filled tube. It is hypothesized, therefore, that the CG matrix must increase the speed with which axons elongate
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<th>Significant Effect of Living Graft Tissue</th>
<th>Significant Effect of Tube Composition</th>
<th>Significant Effect of CG Matrix Presence</th>
</tr>
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<td>no effect</td>
</tr>
<tr>
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<td>√* (decreased in SI tube)</td>
<td>√** (increased)</td>
</tr>
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<td>√* (decreased in SI tube)</td>
<td>√ (increased)</td>
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<td>√ (increased)</td>
</tr>
<tr>
<td>Pruning</td>
<td>√ (decreased)</td>
<td>no effect</td>
<td>no effect</td>
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* Significant effect only when the tubes were unfilled.
** Significant effect only in the silicone tube, not the collagen tube.

Table 3.2 Table summarizing the effects of implant parameters on each mechanistic step in the regenerative process. The effect of living graft tissue reflects the differences between autografted and tubulated injuries. The effect of tube composition compares collagen and silicone tubes, and the effect of matrix presence compares filled and unfilled tubes. In some cases, the effect of the tube composition was dependent on matrix presence and vice versa. These were all related to the inferiority of the SI group to all other device groups. The true effects of each implant parameter are indicated by a (√) and occurred only when the effect was universal (i.e., the effect of the matrix was significant in both silicone and collagen tubes).

across the gap. The theoretical benefits of increasing elongation rate are threefold: axons migrate through the distal stump while the tissue is still favorable for regrowth, axons connect to distal endpoints before muscle atrophy results in the loss of targets, and axons reach the distal stump before barriers to regrowth, such as scar, can form. This hypothetical increase in elongation velocity may then lead to significantly more axons reaching the distal nerve branches, reconnecting successfully with targets, and eventually maturing to a fully functional state (as observed in this study).

Significant evidence exists in the literature which would support the hypothesis that the CG matrix may increase the elongation rate of axons. In particular, other substrate materials composed of collagen and laminin (Madison, et al., 1985; Ohbayashi, et al., 1996), have been shown to increase the rate of axonal elongation across gap injuries. These findings suggest...
that elongation rate can be influenced by factors external to the axon such as insoluble extracellular matrix components. In addition, the CG matrix provides a favorable chemical substrate (collagen) for attachment of nerve growth cones and a structure more conducive to elongation than the fibrin cable that forms following injury in an empty tube. The CG matrix is a highly porous material with a large amount of specific surface for attachment and migration of nerve growth cones. Previous studies have shown that increasing the specific surface of the CG matrix significantly improves electrophysiological recovery following tubulation of a 10-mm gap injury (Chang, et al., 1990; Chang and Yannas, 1992). In contrast, the fibrin cable formed in unfilled tubes is small in diameter and has a very low specific surface (Williams, et al., 1983). The axial pore orientation of the CG matrix has also been shown to significantly improve regeneration compared with radial-pore matrices, which suggests that the axially oriented structure of the matrix may guide axons toward the distal stump (Chang, et al., 1990; Chang and Yannas, 1992). Other substrate materials that are currently under investigation lack this highly characterized pore structure, and rely solely on the chemical benefits of the substrate material to enhance axon elongation. Another benefit of the CG matrix is that, like other implanted substrates, it is in place immediately following injury (0 days), whereas it takes at least 7 days for the fibrin cable to form across a 10-mm gap (Williams, et al., 1983). It has been observed that axons do not begin to migrate into an unfilled tube until the fibrin cable is formed (Williams, et al., 1983); therefore, elongation is delayed by one week in unfilled tubes. One study, which used a plasma-filled tube to induce formation of the fibrin cable by 1 day, reported that the rate of axonal elongation was significantly increased over unfilled tubes, which formed the cable in 7 days. By 4 weeks, the plasma-filled tubes had nearly twice as many axons at the mid-portion of the gap than the empty controls (Williams, 1987). These findings suggest that a substrate which is in place immediately following injury may increase the rate at which axons cross the gap by up to one week just by eliminating (or decreasing) the lag time between injury and elongation.
All of these factors, including the long-term superiority of nerves regenerated through matrix-filled devices (reported in Chapter 2), suggest that the elongation rate may be a critical factor in determining the ultimate outcome of regeneration. Our findings would suggest that speeding up elongation by as little as one week may have drastic effects on the ultimate outcomes of regeneration. Future studies will investigate if the matrix increases axon elongation rate across the gap and, if it does, by how much. Ultimately, it may be possible to screen future designs based on an elongation rate assay and predict the long-term outcomes of regeneration based on the data in the current study.

Effect of Tube Composition

The second implant parameter that was considered, tube composition, was most accurately evaluated by comparing the results of the matrix-filled silicone and large-pore collagen tubes, since both devices resulted in successful bridging of the gap in 100% of the cases. Comparing the unfilled tube groups was less attractive since the unfilled silicone tube group had few cases which resulted in tissue cable formation. For purposes of clarity, the following discussion will involve only the matrix-filled tube groups.

The matrix-filled silicone and large-pore collagen tubes had similar numbers of axons in the proximal sciatic nerve, distal sciatic nerve, tibial nerve branch, and peroneal nerve branch (Figures 3.7, 3.9, 3.11 and 3.13). Based on these findings, it was concluded that the tube composition had no effect on sprouting, completed elongation, and migration through the distal stump (Table 3.2). However, the LC/M group had significantly more large diameter fibers than the SI/M group in both the tibial and peroneal nerve branches (Figures 3.18 and 3.20), as well as in the center of the nerve gap (Figure 2.9), indicating a significant effect of tube composition on the maturation of axons. No differences were detected between the silicone and collagen tube groups on pruning.

The tube composition affected only one step in the axon regeneration process: maturation. The silicone and large-pore collagen tubes varied along three characteristics:
chemistry, permeability, and degradability. Since the large-pore collagen tubes were undegraded at 60 weeks, the difference between the silicone and collagen tubes must be a result of either the chemistry or permeability. It was hypothesized that axons traveling through matrix-filled silicone and collagen tubes would progress at the same elongation rate since most of the axon growth cones do not come into direct contact with the tube material. This hypothesis appears to be validated by the fact that similar numbers of axons reached the distal nerve branches in the silicone and large-pore collagen tubes. It would also be expected, therefore, that the two groups would show similar amounts of maturation; however, the silicone tubes were clearly inferior to the collagen tubes on this basis. Since a similar number of axons reached the distal stump in both tube types, the difference in maturation between the groups may be a result of non-axonal factors; namely the connective tissue response to the tubes.

Regenerated axons have been shown previously to degenerate after long-term (>1 year) tubulation with silicone tubes (LeBeau, et al., 1988). Moreover, clinical problems have arisen following implantation with silicone tubes, causing pain and loss of function, and necessitating removal of the silicone tube (Merle, et al., 1989). These symptoms have been attributed to chronic nerve compression which is caused by swelling of the nerve stumps in response to injury. In contrast, experimental findings with collagen (Archibald, et al., 1991; Li, et al., 1992) and PLLA/PCL (den Dunnen, et al., 1993) tubes have not reported deterioration in the quality of the axons or other symptoms associated with chronic nerve compression, despite the fact that these tubes were undegraded at the time of evaluation. Therefore, the lack of maturation observed within the silicone tubes in the current study may be a combination of decreased maturation and degeneration of a portion of the regenerated axon population.

Still, it is unclear why the tube composition would have such drastic effects on the maturation of axons. If chronic nerve compression is the cause of degenerative changes, the
large-pore collagen tubes would be expected to elicit a similar response since they are largely undegraded at 60 weeks and have the same inside tube diameter as the silicone tubes. Investigation of the connective tissue and cellular response to the tubes has led to the discovery of interesting differences between the silicone and collagen tubes which might help explain the effect of tube composition on axon maturation. Briefly, these differences include the formation of a contractile cell capsule in response to the silicone tube by 6 weeks post-operative which may impart constrictive hoop stresses on the nerve cable. It is hypothesized that hoop stresses, generated by this capsule, may prevent maturation of the axons in the long-term and result in the axon degeneration that has previously been reported. Also, the size of the regenerated nerve cable is reduced in the silicone tubes compared with the large-pore collagen tubes and may limit the space available for radial axonal growth. These findings would suggest, then, that the connective tissue which forms in response to the implant may play an active role in the successful development of axons into a fully functional state. These differences in connective tissue response to silicone and large-pore collagen tubes will be discussed in Chapter 4.

Effect of a Living Tissue Graft

The final implant parameter that was evaluated was the presence of a living tissue graft (autograft) as compared to the tubular devices. Based on the number of axons in the proximal sciatic nerve stump (Figure 3.7), the autograft had a significantly reduced amount of sprouting following nerve injury compared with all of the tubulated groups (Table 3.2). The autograft repair had a similar number of axons to at least one tubulated group in each of the following locations: distal sciatic nerve stump, tibial nerve branch, and peroneal nerve branch. These findings indicate that the presence of live bridging tissue had no effect on completed elongation across the gap or migration through the distal stump. Similarly, the number of large diameter axons was not significantly different from at least one tube group in each location; therefore, there was no effect of live bridging tissue on maturation. There was a decreased amount of pruning in the autograft group (only 7% of proximal nerve stump axons were pruned between
30 and 60 weeks) compared to the tubulated groups (37% of axons pruned, on average); indicating a significant effect of tissue grafts on pruning.

In contrast to the findings in tubular devices, the nerves implanted with an autograft had significantly fewer myelinated axons in the proximal sciatic nerve (Figure 3.7), averaging only 2 axon sprouts per injured myelinated axon compared to an average of 4-5 sprouts for the tubulated groups. This reduced degree of sprouting in the autograft was consistent with the reported sprouting of approximately 1.5 axon sprouts per injured myelinated axon following direct suture repair (Mackinnon, et al., 1991); however, reduced sprouting with autograft repair has not been previously reported. The significant difference in sprouting observed in the current study between tubular and autograft repairs suggests that the presence of a tube, regardless of its composition, leads to increased sprouting compared with an autograft repair (Table 3.2). The cause of this difference is unknown, however, at least two hypotheses can be proposed. First, it can be hypothesized that the autograft tissue contains less physical space for the entrance of axons since endoneurial tubes are filled with axonal material; hence, the number of sprouts that are produced is physically limited. A second hypothesis would propose that early contact with growth factors produced during Wallerian degeneration of the autograft (or the distal stump in the case of a direct suture repair) may signal the neuron cell body to discontinue axon sprout production, thereby reducing the number of axon sprouts. Perhaps the contact of the axon growth cone with growth factors originating in the distal stump alert the neuron cell body that the existing axon sprouts are moving in the right direction (i.e. toward a target organ) and eliminate the need for a larger population of sprouts which would provide a statistically better chance of finding a random target. Although clearly a difference exists between sprouting in the tubulated and autograft groups, it is unclear from this study whether more or less axon sprouts ultimately produce a better regenerated nerve.
3.4.3 Mechanistic Steps of Axon Regeneration

Sprouting

Sprouting was affected only by the presence of an autograft as compared to the tubulated groups (Table 3.2). However, it is unclear whether reduced sprouting results in a more efficient regenerative process, since the autograft was not superior to all of the other devices.

Completed Elongation Across the Gap

None of the implant parameters had a significant effect on the number of axons which completed elongation (i.e., bridged the gap) at 60 weeks post-operative (Table 3.2). These findings suggest that, as long as the implant resulted in formation of a tissue bridge, the same number of axons would cross the tissue bridge and enter the distal stump, regardless of treatment. This suggests that the important parameter of elongation is the rate at which axons travel across the gap, which will be the focus of future experiments.

Migration Through the Distal Stump

Migration through the distal stump was affected by the presence of the CG matrix, with more axons reaching the tibial and peroneal nerve branches in the matrix-filled tubes compared to unfilled tubes (Table 3.2). This supports the hypothesis that the matrix may have significantly enhanced the elongation rate, which would allow axons to avoid potential barriers in the distal stump such as scar formation, degeneration of the endoneurial tubes, and atrophy of the muscle synapses.

Maturation

Both the presence of the CG matrix and the tube composition had significant effects on the number of large diameter fibers and, hence, significantly affected maturation (Table 3.2). The effect of the matrix on maturation can be explained by the hypothesis that the matrix
increases the rate of elongation and thereby, increases the number of axons which connect to a distal target. In contrast, the significant effect of the tube composition cannot be reconciled based on axon regeneration mechanisms; therefore, it is hypothesized that connective tissue differences (addressed in Chapter 4) may be responsible for the effect.

A surprising finding was that the autograft group was inferior to the matrix-filled large-pore collagen tube group based on the number of large diameter myelinated fibers (≥ 6 μm) in the tibial and peroneal nerve branches. Recall that, at the center of the nerve gap, the AG and LC/M groups were not significantly different. The fact that significant differences between the AG and LC/M groups are present in the distal nerve branches was unexplained. The number of large diameter fibers was greater in the center of the nerve gap than in the distal branches for the autograft. This finding may suggest that axons do not need to be connected to an endpoint to grow large in diameter, as previously reported (Aitken, 1949). In all of the tubulated groups; however, the number of large diameter fibers in the center of the gap was not significantly different from the number of large fibers in the tibial plus peroneal nerves. In addition, these findings suggest that the off-the-shelf LC/M device may result in regeneration of a nerve that is superior to nerves regenerated through the current clinical treatment.

**Pruning**

Pruning was reduced in the autograft group, compared to the tubulated groups. This difference was likely due to the fact that autograft group had less axon sprouts and, therefore, less axons were available for pruning.
Chapter 4: Effects of Implant Parameters on the Connective Tissue Response to Peripheral Nerve Injury

4.1 Introduction

4.1.1 Spontaneous Healing of the Peripheral Nerve

The end result of unaided healing of peripheral nerve transection is the formation of a neuroma, a bulbous tissue containing unorganized axons and connective tissue (Archibald, et al., 1991). Even though some axons have elongated, formation of a neuroma results in no functional connections. This condition is the equivalent of scar in other connective tissues but, in contrast, it can be very painful because the axons are still receiving impulses from the spinal cord and are very sensitive. In addition, it has been shown that slight mechanical distortion of the neuroma can cause electrical excitation which likely also results in pain (Wall and Gutnick, 1974). Aside from the knowledge that the disorganized axons are surrounded by collagenous tissue, little is known about the cellular elements of the neuroma or the manner in which the wound closes after injury. These elements of neuroma formation will be addressed in this study.

4.1.2 Altered Healing Response in the Presence of a Tube

Neuroma formation occurs unless the axonal material of the nerve regenerates into the distal nerve stump (Dellon, 1990). Implantation of a tubular device into the wound site can reduce the likelihood of neuroma formation by directing the growth of regenerating axons toward the distal nerve stump and orienting connective tissue structures along the axis of the nerve (Fields, et al., 1989). However, implantation of a tube may not prevent neuroma formation if the injury is too severe. For example, use of a silicone tube leads to regeneration across gaps of 10-mm or less in the rat sciatic nerve; however, an increase in gap length to 15-mm results in neuroma formation in all cases (Lundborg, et al., 1982).
Tubulation techniques have been used for over one hundred years; however, there is still debate over the optimum tube material. The earliest tubes were decalcified bone and were used with limited success in the early 1880's. The use of many other natural and synthetic tube materials followed the success of the early tubular grafts. Naturally occurring tubes that have been investigated included arteries, bone, dura, and vein grafts, among others. Non-degradable synthetic tubes have included magnesium, silicone, and stainless steel. Degradable tubes, both natural and synthetic, have included collagen, polyester, polyglactin, and polylactate (see review in Fields, et al., 1989).

Selection of the optimum tube is a difficult task; however, progress has been made in defining the requirements necessary for a successful tube material. The tube must be biodegradable and have a rate of resorption that can be controlled such that the structural integrity of the tube remains intact until axons have completed elongation (Madison, et al., 1992). In addition, it must be non-toxic and must inhibit the formation of fibrosis, neuroma, and ischemia (Fields, et al., 1989). Finally, the tube must be flexible, suturable, and easily fabricated so that the dimensions of the tube can be varied depending on the implantation site (Madison, et al., 1992).

The events of regeneration within a silicone tube nerve guide, with a 10-mm gap between nerve stumps, have been well studied (Fields, et al., 1989; Williams, et al., 1983). Within one week after nerve transection and tubular repair, a fibrin bridge completely crosses the gap and connects the proximal and distal stumps (Fields, et al., 1989). At that time, fibroblasts and Schwann cells begin to migrate into the gap from both the proximal and distal nerve stumps (Williams, et al., 1983). Once Schwann cells and fibroblasts are present in the gap, axons emerge from the proximal nerve stump and elongate at a rate of approximately 1-2 mm/day (Seckel, 1990; Williams, et al., 1983). By the end of three weeks, vasculature has traversed the gap, unmyelinated axons have traveled 9 mm from the proximal stump, and myelinated axons are present in the first 5 mm of the gap (Williams, et al., 1983). By 4
weeks, unmyelinated axons have reached the distal stump and Schwann cells are actively myelinating axons within the gap (Williams, et al., 1983).

4.1.3 Complications Reported with Tubulation

It has been reported previously, in both animal studies and clinical situations, that implantation of a tubular device can lead to long-term complications, including pain and axon degeneration (LeBeau, et al., 1988; Merle, et al., 1989). The primary cause of these complications has been attributed to constriction of the nerve stumps by the tubular device which, in both cases, was made of silicone. It is hypothesized that the nerve stumps swell in response to injury and become constrained by the inside diameter of the tube implant. These findings would suggest that degradability of the tube is an important parameter to prevent these long-term complications.

4.1.4 Role of the Myofibroblast in the Healing of Connective Tissues

Wound contraction is an important part of wound healing because it brings the wound edges into approximation and facilitates wound closure. Myofibroblasts have been identified in certain connective tissues as important participants in the contraction of healing wounds, particularly in wound closure of the skin (Gabbiani, et al., 1971). The myofibroblast is a modified fibroblast which is capable of generating substantial contractile force. The myofibroblast derived its name because it has the phenotypic characteristics of both the fibroblast and the smooth muscle cell (Guber and Rudolph, 1978). It contains bundles of actin microfilaments, 4 - 8 nm in diameter, which are the same actin isoform (α isoform) found only in smooth muscle cells and pericytes (Gabbiani, et al., 1971). All cells contain the β and γ isoforms of actin for cell motility; however, the α isoform is specific only to contractile cells (Faryniarz, et al., 1996). Like fibroblasts, myofibroblasts have a well developed rough endoplasmic reticulum and Golgi apparatus for protein synthesis. Additional ultrastructural features include pinocytotic vesicles and indented nuclei (Gabbiani, et al., 1971).
Myofibroblasts apply force to the extracellular matrix through cell-matrix attachment sites which form the fibronexus. The fibronexus consists of intracellular actin filaments crossing the cell membrane and attaching to extracellular fibers (Faryniarz, et al., 1996). It has been proposed that it is not the force of each individual cell over a small distance that causes contraction, but rather an organized, three dimensional network of cells which exert forces at the macroscopic level (Yannas, et al., 1989). Yannas and coworkers found that implantation of a collagen-GAG copolymer can delay and even eliminate wound contraction in the dermal model (Troxel, 1994; Yannas, 1989).

Myofibroblasts have been found to play a role in the healing of other connective tissues and in selected pathological conditions (Desmouliere and Gabbiani, 1996). In ligament, myofibroblasts have been found to re-establish in situ strain after transection injury and may participate in the alignment of the regenerating collagen fibers (Faryniarz, et al., 1996). Pathological conditions in which myofibroblasts play a role include: Dupuytren's contracture (Gabbiani and Majno, 1972; Gelberman, et al., 1980), chronic alcoholic cirrhosis of the liver (Rudolph, et al., 1979), hypertrophic scars (Baur, et al., 1975) and the capsules that form around blood clots (Ryan, et al., 1973) and silicone breast implants (Rudolph, et al., 1978). In addition, myofibroblasts have recently been identified in the regenerating peripheral nerve after 6 weeks (Chamberlain, 1996).

4.1.5 Project Goal

The primary objective of this study was to determine the effect of implant parameters on the connective tissue response to peripheral nerve injury. A secondary objective was to determine if the connective tissue response to tube composition may have affected axon maturation, e.g., by reducing the axon maturation in the silicone tube groups (reported in Chapter 3). Three implant parameters were evaluated: the presence of the CG matrix (filled versus unfilled tubes), tube composition (collagen versus silicone), and the presence of a tube implant (tube groups versus untreated group). Six aspects of the connective tissue response
were evaluated: formation of a nerve trunk, nerve trunk diameter, tissue capsule thickness, presence of contractile cells aligned circumferentially in the tissue capsule, presence of contractile cells aligned axially in the nerve trunk, and presence of contractile cells aligned circumferentially within the collagen tube walls and around the outside of the tube (in both collagen and silicone tubes). For each connective tissue response, the effect of matrix presence, tube composition, and tube presence were evaluated.

Large-pore collagen and silicone tubes were implanted both empty and filled with a collagen-glycosaminoglycan (CG) matrix to bridge a 10-mm gap in the adult rat sciatic nerve. In an additional experimental group, a 5-mm segment of sciatic nerve tissue was removed, creating a 10-mm gap, and no treatment was administered; this provided a negative control group. Contralateral nerves were used as a normal control. Nerves were retrieved at 6, 30 and 60 weeks post-operative for evaluation. Histological procedures were used to examine the nerve trunk diameter and the tissue capsule thickness. Immunohistochemical techniques were employed to determine if contractile cells (myofibroblasts) were present in the tissue capsule, nerve trunk, and within the tube walls.

4.2 Materials and Methods

Descriptions of the peripheral nerve implants and the animal model for tubulated repair can be found in section 2.2. More detailed protocols for each procedure are presented in Appendix A for reference.

4.2.1 Animal Model for Spontaneous Healing

Six adult female Lewis rats (Charles River Laboratories), 175-200 grams, were used to evaluate the spontaneous healing of peripheral nerves. Each animal was anesthetized using an intraperitoneal injection of sodium pentobarbital (Nembutal Sodium Solution, Parke-Davis, 50mg/ml) with a dosage of 50 mg/kg. Once the animal was fully anesthetized, the surgical area was shaved and cleaned using an iodine sponge. The animal was placed on the surgical board
with arms and legs secured. The sciatic nerve was exposed and further anesthetized topically using a few drops of 1% Lidocaine placed directly on the nerve. A 5-mm segment of the sciatic nerve was removed from the midpoint between the sciatic notch and the distal bifurcation using microscissors. Removal of the 5-mm nerve segment and subsequent retraction of the nerve stumps resulted in formation of a 10-mm gap between the nerve stumps. The proximal and distal nerve stumps were each allowed to fall naturally onto the underlying tissue and no repair methods were employed. The muscle and skin were closed using 4-0 sutures and skin staples as needed. The animals were placed back in their cages and monitored until fully alert. The animals were housed on wood chip bedding, 2 animals per cage, for the remainder of the experiment. Food and water was available ad libitum. The animals were monitored daily for signs of abnormal behavior. At 6 weeks post-operative, the animals were anesthetized via intraperitoneal injection of sodium pentobarbital and then sacrificed by transcardial perfusion with mixed aldehydes and prepared for histological analysis.

4.2.2 Histological Techniques

Tissue Collection Procedure

Following sacrifice at 30 or 60 weeks, the sciatic nerves regenerated through tubular devices were explanted from the sciatic notch at the hip to beyond the distal bifurcation point at the knee level. After explantation the tissue was placed in Yanoff’s fixative and sectioned into 2-mm segments, of which several were selected for analysis of the connective tissue response to nerve injury (Figure 4.1). Two tissue segments from the proximal half of the nerve gap were retrieved and designated for paraffin embedding in either cross- or longitudinal section (Figure 4.1). These two tissue segments were embedded in paraffin (Paraplast Plus, Cat#48311-703, VWR Scientific, Boston, MA), cut on a microtome at an 8-μm thickness, and used for histological and immunohistochemical analysis. Additional tissue segments from the proximal nerve stump, center of the nerve gap, and distal nerve stump (Figure 4.1) were
Figure 4.1 Schematic outlining the tissue allocation for this study. Tissue sections from the proximal sciatic nerve stump, center of the nerve gap, and distal sciatic nerve stump were embedded in cross-sectional orientation in Epon and used to evaluate the nerve trunk diameter and tissue capsule thickness. A nerve segment, centered around 1 mm from the proximal stump, was embedded in longitudinal orientation in paraffin and used to evaluate the presence of axially aligned contractile cells in the nerve trunk. A nerve segment, centered around 3 mm, was embedded in cross-sectional orientation in paraffin. This section was used to evaluate the presence of contractile cells in the tissue capsule, nerve trunk, and within and around the tube walls.

embedded in Epon (as described in section 3.2.1) and used to evaluate the nerve trunk diameter and the tissue capsule thickness.

Following 6 weeks, the neuromas resulting from no treatment were retrieved and placed in Yanoff’s fixative. The entire proximal neuroma (n=2) and the entire distal neuroma (n=2) were embedded in paraffin in longitudinal section. The remaining proximal (n=2) and distal (n=2) neuromas were embedded in paraffin in cross-section. The neuromas were sectioned at an 8 μm thickness and used for histological and immunohistochemical analysis.

Histology

Sections embedded in paraffin were stained with hematoxylin and eosin and Masson’s trichrome according to standard protocols. The hematoxylin and eosin stain was used to
identify cells and Masson’s trichrome stain was used to determine collagen organization within the tissue.

Epon samples were used to quantify the nerve trunk diameter and the tissue capsule thickness in the proximal nerve stump, center of the nerve gap, and distal nerve stump. Nerve trunk diameters were determined using digitized images and image analysis software. The tissue capsule thickness was measured using a scaled reticule on the light microscope. The tissue capsule was defined by the following characteristics: circumferential orientation of cells and connective tissue, located around the perimeter of the nerve trunk (in cross-section), and contained no axons.

**Immunohistochemistry**

The immunohistochemical technique was intended to stain the α-smooth muscle actin isoform which is present only in myofibroblasts, smooth muscle cells and pericytes (Gabbiani, et al., 1971). Paraffin sections were cut to an 8-μm thickness using a microtome and placed on glass slides. The sections were deparaffinized and then treated with a 0.1% trypsin solution (Sigma Chemical Co., St. Louis, MO) for 60 minutes at room temperature to unmask antigen sites blocked by formalin fixation. The slides were quenched with a 3% hydrogen peroxide solution, followed by 20% nonimmune goat serum blocking solution. The slides were soaked in the primary antibody, a mouse monoclonal antibody against α-smooth muscle actin (#A2547, Sigma Chemical Co., St. Louis, MO; 1:400 concentration), for 2 hours at room temperature and then rinsed with phosphate buffered saline (PBS). As a negative control, the slides were treated with mouse serum (#M5905, Sigma Chemical Co., St. Louis, MO; 1:400 concentration) instead of the primary antibody. Any staining observed in the negative control tissue should indicate the amount of non-specific background staining. The biotinylated secondary antibody, goat anti-mouse immunoglobulin (#0529, Sigma Chemical Co., St. Louis, MO; 1:300 concentration), was applied for 1 hour at room temperature. Following a rinse in PBS, the peroxidase reagent, or avidin-biotin complex (#E2886, Sigma Chemical Co.,
St. Louis, MO), was applied for 20 minutes at room temperature. The chromogen solution (#AEC-101, Sigma Chemical Co., St. Louis, MO) was applied for 15 minutes to develop the color of the stain. The slides were then rinsed, mounted, and coverslipped with glycerol gelatin (#GG-1, Sigma Chemical Co., St. Louis, MO).

Three regions of the sciatic nerve sections were analyzed: within the tissue capsule, within the nerve trunk, and within and around the tube walls (Figure 4.2).

4.3 Results

4.3.1 Formation of a Nerve Trunk

Three classes of bridging tissue were observed within the gap: no bridging tissue, tissue cables, and nerve trunks. The result of treatment was ‘no bridging tissue’ when the two nerve stumps were not connected by any connective tissue structure. A tissue cable was defined as a connective tissue bridge that did not contain any axons. Nerve trunks were defined as connective tissue cables that contained myelinated axons. Verification of the

![Figure 4.2 Schematic of the areas analyzed for the presence of contractile cells. Three locations were studied: the tissue capsule, midportion of the nerve trunk, and within and around the tube walls. All locations were analyzed in cross-sectional orientation (as shown here) and, in addition, the midportion of the nerve trunk was analyzed in longitudinal section.](image)

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presence of myelinated axons in the nerve trunks was part of the analysis reported in Chapter 2. Regeneration was considered successful only when a nerve trunk formed within the gap.

The absence of a tube implant led to the formation of a cap over the ends of both the injured proximal and distal nerve stumps; this capping off of injured nerve tissue has been termed a neuroma (Dellon, 1990). In both the proximal and distal neuromas, there was a small amount of axial tissue growth into the nerve gap; however, no bridging tissue was observed (Table 4.1). Implantation of a tube did not completely eliminate neuroma formation. In 5 of the 9 animals implanted with an unfilled silicone tube (SI), a neuroma formed on the proximal nerve stump with similar gross characteristics to the untreated group (Figure 4.3a) and no bridging tissue was observed (Table 4.1). In 2 of the 9 animals implanted with a SI tube, a tissue cable bridged the gap; however, the bridging tissue was very thin and contained no axons (Figure 4.3b; Table 4.1). In the remaining 2 animals implanted with SI tubes, a nerve trunk formed between the nerve stumps; therefore, the success rate of regeneration through the SI devices was only 22% (Table 4.1). In contrast, nerve trunks which connected the proximal

<table>
<thead>
<tr>
<th></th>
<th>No Bridging Tissue</th>
<th>Tissue Cable (Connective Tissue only, No Axons)</th>
<th>Nerve Trunk (Connective Tissue with Axons)</th>
<th>Success Rate of Regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Tube</td>
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<td>0/5</td>
<td>0/5</td>
<td>0%</td>
</tr>
<tr>
<td>SI</td>
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<td>2/9</td>
<td>2/9</td>
<td>22%</td>
</tr>
<tr>
<td>SI/M</td>
<td>0/8</td>
<td>0/8</td>
<td>8/8</td>
<td>100%</td>
</tr>
<tr>
<td>LC</td>
<td>0/8</td>
<td>0/8</td>
<td>8/8</td>
<td>100%</td>
</tr>
<tr>
<td>LC/M</td>
<td>0/9</td>
<td>0/9</td>
<td>9/9</td>
<td>100%</td>
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</table>

Table 4.1 Frequency of occurrence of the three tissue types observed within the gap. Data are presented as the number of animals in each category over the total number of animals evaluated. The no tube animals had a 6 week survival time while the data for the tubulated groups are from 30 and 60 weeks post-operative. No bridging tissue occurred when the two nerve stumps were not bridged by any connective tissue. Tissue cables were defined as connective tissue cables that did not contain any axons and nerve trunks were defined as connective tissue that contained axons (Verification of the presence of axons in the nerve trunks was part of the Chapter 2 analysis). Regeneration was considered successful only when a nerve trunk bridged the gap. The success rate of regeneration is shown for each group.
Figure 4.3 Gross morphology of tissue retrieved after 60 weeks of implantation. The gross photographs are all at the same magnification; scale bar in (a) = 10 mm. Arrows indicate the proximal and distal edges of the gap in each photograph. (a) Proximal neuroma formed inside an unfilled silicone tube in an animal which had no bridging tissue. The distal tissue is not shown. (b) Tissue cable formed within an unfilled silicone tube that, following histological analysis, displayed no axons within the gap. (c) Nerve trunk regenerated through a matrix-filled silicone tube that contained myelinated axons. (d) Nerve trunk regenerated through a matrix-filled large-pore collagen tube that contained axons. In all cases, the tubes have been removed.

and distal nerve stumps were observed in all of the matrix-filled silicone (SI/M; n=8), unfilled large-pore collagen (LC; n=8), and matrix-filled large-pore collagen (LC/M; n=9) tube groups retrieved at 30 and 60 weeks, indicating a 100% success rate of regeneration for each group (Table 4.1; Figure 4.3 c&d).

4.3.2 Nerve Trunk Diameter

The nerve trunk diameter in the center of the nerve gap followed different kinetic paths, dependent on the tube composition (Figure 4.4). In the large-pore collagen tubes, both empty and matrix-filled, the nerve trunk diameter increased significantly from 6 to 30 weeks (p<0.01) and then decreased significantly between 30 and 60 weeks (p<0.01). In contrast, the nerve
Figure 4.4 Nerve trunk diameter in the center of the nerve gap for the matrix-filled silicone (SI/M), matrix-filled large-pore collagen (LC/M), and unfilled large-pore collagen (LC) tube groups plotted as a function of time. In the SI/M group, the nerve trunk diameter did not change significantly throughout the length of the study (6 to 60 weeks). In contrast, the LC and LC/M groups increased significantly from 6 to 30 weeks and then decreased significantly between 30 and 60 weeks.

trunk diameter in the silicone tubes did not change significantly over the entire period of the study, 6 to 60 weeks (p>0.4).

At 30 weeks, there was a significant effect of tube composition (p<0.001) and no effect of matrix presence (p>0.3) on the nerve trunk diameter in the center of the nerve gap (Figure 4.5). The LC and LC/M nerve trunks were significantly larger in diameter than the SI and SI/M nerve trunks (Figure 4.5; p<0.001). The SI/M and SI groups had similar nerve trunk diameters (p>0.3); however, a nerve trunk formed in only 2 of 9 SI animals compared with 8 of 8 animals in the SI/M group. When a tissue cable, containing no axons, formed within the SI devices (in 2 of 9 animals), the diameter of the tissue cable was significantly smaller than all
Figure 4.5 Nerve trunk diameter in the center of the nerve gap for all implant groups at 30 weeks. Nerve trunks were defined as connective tissue bridging the nerve gap which contained axons. The collagen tubes had significantly larger nerve trunk diameters than the silicone tubes (p<0.001). The CG matrix did not have a significant effect on the nerve trunk diameter. However, nerve trunks formed in only 2 animals implanted with the SI device. When a tissue cable formed (2 of 9 SI animals), the tissue diameter was significantly smaller than the nerve trunks in all groups. In the untubulated group, no tissue bridged the gap; therefore, the nerve trunk diameter was undefined. NS = not significant.

of the nerve trunk diameters (p<0.01). In the untubulated animals, no nerve trunks were formed; therefore, no nerve trunk diameters could be measured.

In all experimental groups, including the neuroma group, the total tissue diameter in the proximal sciatic nerve stump was significantly increased (p<0.05) compared to normal nerve at 6 weeks (Figures 4.6 and 4.7; Table 4.2). At 30 and 60 weeks, the tissue diameter in the proximal stump remained significantly higher than normal for all tubulated groups (Table 4.2). There were no significant differences among implant groups in the tissue diameter in the proximal sciatic nerve stump at 6, 30, and 60 weeks (Figure 4.6; Table 4.2; p>0.4).
Figure 4.6 Nerve trunk diameter as a function of distance from the proximal stump for the matrix-filled silicone (SI/M) and large-pore collagen tube groups (LC/M) at 60 weeks post-operative. The two groups were not significantly different in the proximal and distal nerve stumps; however, the SI/M group had a significantly smaller diameter in the center of the nerve gap.

In the distal sciatic nerve stump, the neuroma group had a significantly larger tissue diameter than normal nerve at 6 weeks (Figure 4.7; Table 4.2; p<0.05). Similarly, at 30 and 60 weeks, the SI/M, LC, and LC/M groups had a significantly larger tissue diameter in the distal nerve stump than normal nerve (p<0.01). At 30 and 60 weeks, the tissue diameter was significantly smaller for the SI group than all other implant groups (p<0.001) and normal nerve (Table 4.2; p<0.01).

4.3.3 Tissue Capsule Thickness

The connective tissue layer, which was arranged circumferentially around the nerve fascicles and did not contain any axons, was termed the tissue capsule. The tissue capsule in
Figure 4.7 Tissue diameter as a function of distance from the proximal stump for the no treatment group, or neuroma, at 6 weeks post-operative. In the proximal neuroma, there was some hypertrophy of tissue around the cut end and then the tissue was capped off. There was no tissue bridging the gap between the two nerve stumps. The distal neuroma had more connective tissue proliferation, but the result was similar to the proximal stump: the tissue was capped off by a connective tissue layer.

normal nerve was the perineurium, a thin collagenous layer containing 1-2 cell layers which surrounded entirely each fascicle (Figure 4.8a). In the tubulated groups, the tissue capsule surrounded the entire nerve trunk since the nerve trunk within the tube was unifascicular. The tissue capsules which formed around nerve trunks regenerated through large-pore collagen tubes were thin collagen layers containing approximately 1-2 cell layers (Figure 4.8b). In contrast, the silicone tubulated groups had a much thicker tissue capsule that contained approximately 15-20 cell layers and was continuous around the entire perimeter of the nerve trunk (Figure 4.8c). In the animals which received no tubulation, a thick tissue capsule, similar to that observed in the silicone tube groups, formed around both the proximal and distal neuromas. However, instead of ensheathing the entire nerve trunk, the collagenous tissue
### Table 4.2

<table>
<thead>
<tr>
<th></th>
<th>Proximal Sciatic Nerve Stump</th>
<th>Distal Sciatic Nerve Stump</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 weeks</td>
<td>30 weeks</td>
</tr>
<tr>
<td>Normal</td>
<td>0.85 ± 0.02</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>Neuroma</td>
<td>1.4 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>SI</td>
<td>1.4 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>SI/M</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>LC</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>LC/M</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1</td>
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</table>

Tissue diameter in the proximal sciatic nerve stump (11 mm from the proximal cut) and the distal sciatic nerve stump (11 mm from the proximal cut) at 6, 30 and 60 weeks. The neuroma (no treatment group) was only evaluated at 6 weeks post-operative; therefore, no data are presented for 30 and 60 weeks. In addition, the diameters of the distal sciatic nerve stump were not available for the tube groups at 6 weeks. In the proximal nerve, all treatment groups had a significantly larger tissue diameter than normal nerve at 6, 30 and 60 weeks. In the distal neuroma, there was a significantly larger tissue diameter than normal. In contrast, by 60 weeks in the SI group, which had no regeneration of axons across the gap, the diameter was significantly smaller than normal. The implant groups were as follows: unfilled silicone tube (SI), matrix-filled silicone tube (SI/M), unfilled large-pore collagen tube (LC), matrix-filled large-pore collagen tube (LC/M).

A capsule capped off the neuromas, apparently preventing a tissue cable from bridging the nerve gap (Figure 4.9). The outer layer of the tissue cap was covered with elongated cells that were fibroblast-like in appearance.

The thickness of the tissue capsule at the center of the nerve gap did not change significantly for any group during the period of investigation, 6 to 60 weeks (Figure 4.10; p>0.3). At 30 weeks, the tube composition had a significant effect on the tissue capsule thickness (Figure 4.11; p<0.001); while the presence of the CG matrix had no effect (p>0.5). Both the SI and SI/M groups had significantly thicker tissue capsules than the LC and LC/M groups (p<0.001). The tissue capsule thicknesses of the LC and LC/M groups were not significantly different from normal (p>0.4). It was also observed that the thickness of the tissue capsule did not change significantly along the length of the nerve for any of the experimental groups (Figure 4.12; p>0.3).
Figure 4.8 Cross-sections of nerve tissue at 30 weeks post-operative, post-fixed with osmium tetroxide and stained with toluidine blue; scale bar = 50 μm. (a) Normal nerve tissue. The tissue capsule, perineurium (arrow), was a very thin collagenous layer containing only 1-2 cell layers. (b) Tissue from the center of the nerve gap (5 mm from the proximal cut) of a matrix-filled large-pore collagen tube (LC/M). Similar to normal nerve, the tissue capsule (arrow) was thin (approx. 4 μm) and contained only 1-2 cell layers. The undegraded collagen tube is visible (LC). (c) Tissue from the center of the nerve gap of a matrix-filled silicone tube (SI/M). The tissue capsule was thick (approx. 50 μm), and contained at least 15-20 cell layers at every location. In the silicone tubes, the tissue capsule buckled (arrow) at localized sites.
Figure 4.9 Longitudinal sections of the proximal and distal neuromas formed within untubulated gaps and retrieved after 6 weeks. The tissue is stained with Masson's trichrome; scale bar = 100 μm. The arrow indicates the direction of the nerve axis. (a) In the proximal neuroma, a thick, collagen tissue capsule (stained blue) surrounded the nerve tissue and then converged to form a cap at the end of the neuroma. The dense collagenous tissue formed at the end of the neuroma resembled fibrous scar tissue. The tissue capsule around the nerve stump was approximately 20 - 50 μm thick. The black box outlines the area of tissue shown under high magnification in Figure 4.15a. (b) In the distal neuroma, a similar collagen tissue capsule was visible, approximately 50 μm thick, which capped off the distal nerve stump. The black box outlines the area of tissue shown under high magnification in Figure 4.15b.
**Figure 4.10** Tissue capsule thickness in the center of the nerve gap for the matrix-filled silicone (SI/M) and matrix-filled large-pore collagen (LC/M) implant groups plotted as a function of time. The tissue capsule thickness did not change significantly from 6 to 60 weeks for either of the groups.

### 4.3.4 Contractile Cells

**Aligned Circumferentially within the Tissue Capsule**

The tissue capsules were analyzed using immunohistochemistry to determine if contractile cells were present. The smooth muscle cells lining the blood vessels stained positively, serving as a positive control for the α-smooth muscle actin staining. In normal nerve, the cells of the perineurium stained positively for α-smooth muscle actin (Figure 4.13). Similarly, in nerves regenerated through the large-pore collagen tubes (both unfilled and matrix-filled), a single layer of cells containing α-smooth muscle actin was observed at 30 and 60 weeks in some locations within the tissue capsules (Figure 4.14a). However, unlike
normal nerve, the layer of cells was not continuous around the entire nerve trunk. Early observations (6 weeks) in large-pore collagen tube groups indicated that no contractile cells were present in the tissue capsule (Chamberlain, 1996).

In the tissue capsules formed within the silicone tubes (both unfilled and matrix-filled), \(\alpha\)-smooth muscle actin positive cells were observed at both 30 and 60 weeks (Figure 4.14b); however, their appearance was much different than in normal perineurium. The thick tissue capsule was filled with at least 15-20 contractile cell layers that surrounded the entire perimeter of the nerve trunk. Similar findings were observed at 6 weeks post-operative, where all tissue capsules within silicone tubes contained contractile cells (Chamberlain, 1996). In some of the silicone tube tissue capsules evaluated at 30 and 60 weeks, there were cells located in the tissue
Figure 4.12 Tissue capsule thickness as a function of distance from the proximal nerve stump for the matrix-filled silicone (SI/M) and matrix-filled large-pore collagen (LC/M) implant groups at 30 weeks. The thickness of the tissue capsule did not change significantly along the length of the nerve for any of the implant groups.

capsule that had features which would identify them as myofibroblasts (indentated nuclei, long cell processes, located in buckled tissue capsule, etc.); however, they did not contain α-smooth muscle actin. It is suggested that these cells may have expressed the α-smooth muscle actin phenotype, participated in contraction, and then stopped producing the α-smooth muscle actin monomer once contraction was complete.

In the untreated group, contractile cells were observed within the tissue capsules which capped off both the proximal and distal neuromas (Figure 4.15). In the proximal neuroma, the cells located within the tissue capsule stained positively for α-smooth muscle actin along the entire length of the neuroma (parallel to the nerve axis); however, close to the capped end, almost all of the cells within the tissue cap stained positively (Figure 4.15a). It appeared as if
Figure 4.13 Cross-sectional serial micrographs of normal nerve tissue retrieved after 30 weeks and stained with (a) the α-smooth muscle actin antibody and (b) the corresponding negative control treated with mouse serum. Positive staining for α-smooth muscle actin is red/brown in color. Scale bars = 10 μm. (a) The cells of the normal perineurium (arrow) stained positively for α-smooth muscle actin indicating that they may have contractile properties. The blood vessel (B) is a positive control for the stain. (b) The negative control of the same tissue section shows no specific staining of the perineurial cells (arrow) or the blood vessel (B) and provides comparison for the amount of non-specific background staining to be expected with the immunohistochemical method.
Figure 4.14 Cross-sectional micrographs of nerve tissue retrieved after 60 weeks. Both micrographs were stained with the \( \alpha \)-smooth muscle actin antibody and viewed at the same magnification; scale bars = 10 \( \mu \)m. (a) Matrix-filled large-pore collagen tube device. The cells of the tissue capsule are stained positively; however the contractile cells (C) are not confluent around the entire nerve trunk. In all areas, the contractile cells were no more than 1-2 cell layers thick. (b) Matrix-filled silicone tube device. In contrast to the collagen tube, the SI/M devices had approximately 15-20 cell layers of contractile cells (C) around the entire perimeter of the nerve trunk.
Figure 4.15 Longitudinal sections from untreated animals retrieved at 6 weeks and stained with the α-smooth muscle actin antibody. The arrow indicates the direction of the nerve axis. (a) Tissue from the tip of the proximal neuroma (see Figure 4.9a for exact location). Almost every cell stained positively for α-smooth muscle actin in this region (both brown and red indicate positive staining). The cells along the edge of the tissue cap are myofibroblasts (M). An epithelial layer surrounding the tissue cap is not apparent at 6 weeks. (b) Tissue from the distal neuroma tissue cap (see Figure 4.9b for exact location). Myofibroblasts were abundant and the connective tissue appears to be buckling, which would suggest the cells are contracting. Buckling was consistently present in the silicone tubulated and no tube groups, but absent from the collagen tube groups, suggesting the buckling was not a histological artifact.
the tissue capsule converged (or contracted) until it ultimately met and formed a cap over the injured tissue. In the distal neuroma, the tissue cap was similar; however, the capping tissue surrounded the distal cut end with apparently less axial outgrowth of tissue. In other words, the capping tissue was not intermingled with the nerve trunk; rather, it appeared to form a distinct layer (Figure 4.15b). There was no apparent epithelial layer covering either the proximal or distal neuroma at 6 weeks post-operative.

**Aligned Axially within the Nerve Trunk**

In all experimental groups, cells containing α-smooth muscle actin were observed within the bulk of the nerve trunk at 30 and 60 weeks, aligned parallel to the nerve axis (Figure 4.16). In some cases, the contractile cells constituted as many as 20% of all non-neuronal cells. The contractile cells were observed to be aligned parallel with the nerve axis, in both cross-sections and longitudinal sections (Figure 4.16). There were no apparent differences in the presence or quantity of axially aligned contractile cells among groups, indicating that neither the presence of the CG matrix nor the tube composition had an effect.

**Aligned Circumferentially Within or Outside the Tube**

Contractile cells were observed within the walls of the large-pore collagen tubes at 30 weeks (Figure 4.17a). The long axes of the contractile cells were aligned in a circumferential direction. The cells did not form a continuous layer within the tube walls; however, in several locations, multiple cells had formed apparent connections (Figure 4.17a). Immediately outside the silicone tubes, a tissue layer (approximately 40 μm thick) was present at 30 and 60 weeks which, in all cases, contained approximately 15-20 cell layers composed of circumferentially aligned contractile cells (Figure 4.17b).
Figure 4.16 Nerves regenerated through a matrix-filled large-pore collagen tube implant at 30 weeks post-operative and stained with the α-smooth muscle actin antibody. Both micrographs are at the same magnification; scale bar = 10 μm. (a) Cross-sectional micrograph showing contractile cells (C) that have been cut transversely. The contractile cells are aligned in the same direction as the axons, coming out of the page in this case. (b) Longitudinal micrograph showing the axially aligned contractile cells (C) in longitudinal view. The arrow indicates the direction of the nerve axis.

Figure 4.17 Tissue sections retrieved after 30 weeks of implantation and stained with the α-smooth muscle actin antibody. Both micrographs are at the same magnification; scale bar = 10 μm. (a) Cross-sectional micrograph showing contractile cells (C) located between the lamellae of the large-pore collagen tubes. The contractile cells are aligned circumferentially. (b) Cross-sectional micrograph showing the contractile cells (C) in the tissue layer formed around the outside of the silicone tube implants. Again, the contractile cells are aligned circumferentially.
4.4 Discussion

4.4.1 Effect of Tube Presence on the Connective Tissue Response

Comparing the untreated group (neuroma) and the tubulated groups, the effect of tubulation was on the successful formation of a nerve trunk connecting the proximal and distal stumps (Tables 4.1 and 4.3). When the nerve stumps were not tubulated, a tissue cable or a nerve trunk never formed between the two nerve stumps, resulting in a success rate of regeneration of 0% in the untubulated group. However, implantation of a tube did not always lead to nerve trunk formation between the nerve stumps. In the SI group, the chances of a neuroma or tissue cable/nerve trunk forming were approximately equal (Table 4.1). The success rate of regeneration in the SI devices; however, was only 22% since the bridging tissue did not always contain axons (Table 4.1). These findings suggest that a tube implant can direct growth of a nerve trunk toward the distal stump; however, the length which a tube implant can bridge is limited. The observed limit of the unfilled silicone tube was 10 mm, as previously reported (Lundborg, et al., 1982). It was observed in the current study that, when a tissue cable or nerve trunk did not bridge the gap, the resultant tissue resembled the neuroma formed following no treatment in tissue cap shape, collagen composition, and presence of contractile cells.

4.4.2 Effect of Matrix Presence on the Connective Tissue Response

The observed effect of the CG matrix presence occurred in the SI groups, where the matrix resulted in more successful formation of a nerve trunk within the gap (Tables 4.1 and 4.3). Filling the silicone tube with the CG matrix increased the success rate of regeneration up to 100% from the 22% success rate in the unfilled silicone tubes. In Chapter 3, it was observed that the SI group had significantly fewer axons which completed elongation across the gap compared to the SI/M group. This effect of the CG matrix could not be explained solely on the basis of mechanisms of axon regeneration; however, the relatively low incidence
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<td>Contractile Cells Within and Outside the Tube Wall</td>
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Table 4.3 Table summarizing the effects of implant parameters on each connective tissue structure formed in response to nerve injury. Since the non-tubulated group did not form nerve trunks, the nerve trunk diameter, tissue capsule thickness, and presence of axially aligned contractile cells in the nerve trunk are not defined. Similarly, since no tube was present, analysis of the contractile cells within and around the tube walls is not applicable for the non-tubulated group.

of nerve trunks bridging the gap (22%) and the small nerve trunk diameters in the SI group may have limited the number of axons which completed elongation. There were no effects of the CG matrix on bridging the nerve gap or the nerve trunk diameter at the center of the gap in the large-pore collagen tubes. It follows that completed elongation across the gap was not significantly affected by the CG matrix in the large-pore collagen tube groups (Chapter 3).

It had been hypothesized that the presence of the CG matrix may have an effect on the presence of myofibroblasts aligned circumferentially within the tissue capsule and axially within the nerve trunk; however, no effects were observed on the number and orientation of contractile cells. This finding is unlike the effect of a similar CG matrix in dermal wounds, where the CG matrix alters myofibroblast formation and delays the onset of wound contraction.
(Troxel, 1994; Yannas, et al., 1989). However, in the SI groups, the increased incidence of successful regeneration in the SI/M group may suggest that the contractile forces are reduced in the presence of the CG matrix, eliminating the pinching off of tissue observed in the SI group which hypothetically led to neuroma formation. The thickness of the tissue capsule and the number of contractile cell layers, however, were similar for the SI and SI/M groups suggesting that the contractile forces generated by the tissue capsules may be similar for both groups. This observation may suggest that the presence of the matrix does not reduce the contractile forces; rather matrix presence leads to formation of more connective tissue within the gap prior to confluence of the contractile capsule, and that ultimately leads to a higher incidence of successful regeneration. Further study of the formation of the contractile capsule at survival times of less than six weeks is necessary to determine whether the matrix enhances the percentage of successful regeneration by reducing contractile forces or by increasing early tissue proliferation in the gap.

4.4.3 Effect of Tube Composition on the Connective Tissue Response

Of all implant parameters, the tube composition had the most prominent effects on the connective tissue response. The tube composition had a significant effect on formation of a nerve trunk (only in the case of the unfilled tubes), nerve trunk diameter, tissue capsule thickness, and number of contractile cells in the tissue capsule (Table 4.3). In the unfilled tubes, the silicone tube led to formation of a nerve trunk bridging the gap in only 22% of the animals, while the collagen tube had a nerve trunk that formed in all animals. Filling the silicone tube with CG matrix increased the percent bridging the gap to 100%, bringing it to the level of the matrix-filled large-pore collagen tube. The second effect of tube composition was on the nerve trunk diameter in the center of the nerve gap (Figure 4.5), where the silicone tubes had significantly smaller diameters. However, there was no effect of tube composition on the tissue diameter in the proximal and distal nerve stumps (Figure 4.6), suggesting that the tissue regenerated through the silicone tubes had a necking region in the center of the gap. There
were also significant effects of tube composition on the tissue capsule thickness and the number of contractile cells found in that capsule. The silicone tube groups had significantly thicker tissue capsules (Figure 4.11) and significantly more contractile cells in the tissue capsule (Figures 4.14) than the large-pore collagen tubes.

In Chapter 3, it was hypothesized that the effect of tube composition on axon maturation was due to differences in the connective tissue response. The findings in the current study appear to validate that hypothesis. The combination of necking in the nerve trunk at the center of the gap and the presence of a thick tissue capsule containing multiple layers of contractile cells suggest that the capsule is restricting nerve growth and may be applying contractile forces to the nerve trunk along its entire length. A similar contractile capsule has been reported to exert significant contractile forces around silicone breast implants (Rudolph, et al., 1978). Additional evidence which supports this constriction model includes the uniformly circular shape of the nerve trunk cross-sections and the observation of buckling in the tissue capsules of nerves regenerated through the silicone tubes. It is hypothesized that the restrictive forces caused by the contractile capsule are preventing the growth of axons in diameter, leading to the significant effect of tube composition on axon maturation.

Previous studies have shown that by one year post-implantation, axons regenerated through silicone tubes have begun to degenerate (LeBeau, et al., 1988). Although the cause of degeneration was unknown, the authors hypothesized that it may have been a result of either the tube material itself or the constriction of the proximal and distal nerve stumps due to long-term tubulation (LeBeau, et al., 1988). Constriction of peripheral nerve stumps by silicone tubes has also been reported to have led to clinical problems, including pain and loss of function, and the necessity to surgically remove the tubes (Merle, et al., 1989) (for additional discussion, see section 2.4). The results of the current study, however, suggest that the constriction of the nerve, causing pain and complications both experimentally and clinically, may have been located in the center of the nerve gap rather than in the nerve stumps and may be
eliminated by using a large-pore collagen tube. This would suggest that simply removing the silicone tube, the current clinical revision surgery (Merle, et al., 1989), may not eliminate the clinical symptoms of chronic nerve compression.

The collagen and silicone tubes differ along three parameters: chemical composition, permeability, and degradability. However, in the time period evaluated in this study, the collagen tubes remained undegraded; therefore, degradability was not a variable in the time scale of this study. The differences in the connective tissue response are, therefore, a result of either chemistry or permeability. The chemistry of the tube may be an important factor because, unlike silicone tubes, collagen tubes provide binding sites for many of the growth factors released from the nerve stumps following transection injury. Several growth factors commonly released after injury, including primarily TGF-β1, have been implicated in the formation of contractile scars in dermal wounds. It can be hypothesized that a collagen tube may eliminate the formation of a contractile capsule by binding large amounts of free cytokines in the wound fluid and, therefore, making them unavailable in the wound site. Similarly, the porosity of the tube may be an important factor by allowing diffusion of inflammatory cytokines out of the nerve gap. Hypotheses can be generated to support either tube property; however, further study will be required to determine which property is essential in eliminating the formation of the thick, contractile capsule and hypothetically reducing the nerve trunk diameter.

4.4.4 Aspects of the Connective Tissue Response

Formation of a Nerve Trunk

The absence of a tube implant led to the formation of a fibrous scar cap over the ends of the injured nerve stumps, preventing a tissue cable from forming between the two cut nerve ends. However, implantation of a tube did not completely eliminate neuroma formation. In 5 of 9 animals implanted with an unfilled silicone tube, a neuroma formed on the proximal nerve
stump with similar characteristics to that which formed in the absence of tubulation (distal neuromas were not analyzed) and no tissue bridged the nerve gap in these cases (Table 4.1). The matrix had an effect on successful bridging of the gap, but only in the case of the silicone tubes. Tissue bridged the gap in 4 of 9 SI devices; however 2 of the tissue cables had no axons. These tissue cables, observed at 30 and 60 weeks, were small in diameter and may have been fibrin cables, similar to those reported to form after one week of implantation with a silicone tube (Williams, et al., 1983). In the unfilled collagen tubes, nerve trunks bridged the gap in all cases; therefore, the presence of the CG matrix could not improve the success rate of regeneration. Similarly, the tube composition had a significant effect on successful regeneration when comparing the unfilled tubes, but no effect was observed when the tubes were matrix-filled (Table 4.3).

**Nerve Trunk Diameter**

The tube composition had a significant effect on the nerve trunk diameter with the collagen tubes having significantly larger diameters than the silicone tubes at the center of the nerve gap (Table 4.3). The presence of the CG matrix did not have a significant effect on nerve trunk diameter (Figure 4.5).

In the proximal nerve stump at 6 weeks, all groups, including the neuroma, had similar tissue diameters; all were significantly larger than normal (Table 4.2). Similarly, in the distal sciatic nerve stump the neuroma had a significantly larger diameter than normal nerve after 6 weeks (Table 4.2). This is in contrast to previous reports of tissue shrinkage in the distal nerve stump by an average of 40% in untreated lesions examined after survival times ranging from 6-70 weeks (Sunderland, 1990). However, the SI group, which had little or no regeneration of axons into the distal nerve stump, had a distal tissue diameter significantly smaller that normal at 30 and 60 weeks (Table 4.2; p<0.05), confirming previous results (Sunderland, 1990). These findings may suggest that in the short-term following injury (6 weeks), there is a proliferation of connective tissue in the distal stump leading to the increased tissue diameter.
This phase may be comparable to the granulation phase during skin wound healing. Between 6 and 30 weeks, however, significant remodeling may occur, resulting in shrinkage of the distal neuroma. This hypothetical remodeling process appears complete by 30 weeks, since no significant changes in tissue diameter were observed after that time. Contractile cells observed in the connective tissue capsule may also be participating in the shrinkage of the distal nerve stump.

**Tissue Capsule Thickness**

The thickness of the tissue capsule was affected only by the tube composition; the matrix presence had no effect (Figure 4.11 and Table 4.3). The silicone tube regenerates had significantly thicker tissue capsules than the large-pore collagen tube groups. In the untreated group, the tissue capsule that formed around the neuroma resembled the tissue capsules in the silicone tubes in thickness and collagen composition.

**Contractile Cells in the Tissue Capsule**

Clearly, the tube composition had a significant effect on the number of contractile cell layers found in the tissue capsule (Figure 4.14 and Table 4.3). In contrast, the presence of the CG matrix had no apparent effect on the number of contractile cell layers found in the tissue capsule. Contractile cells were abundant in the neuroma group; however, implantation of a tube did not always eliminate the presence of multi-layered contractile cells in the tissue capsule (contractile capsule was eliminated only in the collagen tube groups).

Contractile cells were observed in the normal nerve perineurium. This is consistent with ultrastructural studies that reported bundles of closely aggregated filaments, similar in appearance to the myofilaments of smooth muscle, in the cytoplasm of perineurial cells (Thomas and Olsson, 1975). Observations that the filaments were α-smooth muscle actin, however, have not been previously reported. Recently, authors have suggested that the observation of α-smooth muscle actin, identified with immunohistochemistry, is definitive
proof that the cells are capable of exerting contractile forces (Masur, et al., 1996). Unlike ultrastructural observations which identify the filamentous actin structures, the \( \alpha \)-smooth muscle actin immunohistochemical stain can verify that the actin within the cells is the \( \alpha \)-smooth muscle isoform which is capable of contraction and can identify the presence of the \( \alpha \)-smooth muscle actin monomer, which is the precursor of filament formation (Masur, et al., 1996). Therefore, the \( \alpha \)-smooth muscle actin immunohistochemical stain identifies cells which are capable of contraction, as well as those which are actively contracting.

The contractile cells observed in the tissue capsules of large-pore collagen tubes had a similar appearance to the perineurial cells which suggests that the regenerated nerves may be forming a nearly normal perineurium. The appearance of these \( \alpha \)-smooth muscle actin positive cells does not occur until 30 weeks post-operative. Previous observations at 6 weeks showed no contractile cells present in the tissue capsules formed in response to large-pore collagen tubes (Chamberlain, 1996). The nerve trunk diameters of large-pore collagen regenerates decreased significantly from 30 to 60 weeks; however, it is not apparent that the reduction was related to the appearance of contractile cells since no reduction in nerve trunk diameter was observed in the silicone tube groups. Because the contractile cells did not form a continuous layer in the LC and LC/M groups, it is unlikely that the cells could have reduced the nerve trunk diameter from 30 to 60 weeks by 15-25%. Rather, in the large-pore collagen regenerates, the reduction in diameter appeared to be a result of connective tissue remodeling.

**Axially Aligned Contractile Cells in the Nerve Trunk**

Implant parameters had no effect on the presence of axially aligned contractile cells in the nerve trunk (Table 4.3). The function of the axially aligned contractile cells is unknown; however, it has previously been hypothesized that they may assist axons in crossing the gap by providing tension in the substrate material (Chamberlain, 1996). There is significant evidence that, *in vitro*, axons elongate in response to axial tension applied to the axon growth cone (Bray, 1984; Bray, 1987; Dennerll, et al., 1989; Zheng, et al., 1991). Another possible
function of the contractile cells may be an attempt to close the wound by shortening the nerve gap. Tonge and coworkers observed that, after formation of a cellular bridge at 1 week, a non-tubulated 2-mm gap in the mouse sciatic nerve had been reduced in length by 18% (Tonge, et al., 1996). This may implicate the contractile cells in the shortening of the nerve gap. The contractile cells may also be responsible for aligning the newly synthesized collagen fibers and possibly the regenerated axons during healing. A similar process of collagen fiber alignment following transection injury has been described in the development and regeneration of ligament tissue (Faryniarz, et al., 1996).

Circumferential Contractile Cells within and around the Tube Wall

Implant parameters had no effect on the presence of contractile cells within and immediately outside the tube walls (Table 4.3). These results suggest that contractile cells can form in response to both the silicone and collagen tube surfaces, and may suggest that formation of a contractile capsule within the tube was affected more by tube permeability than tube chemistry. Permeability may be an important tube parameter because it allows transfer of inflammatory proteins out of the nerve gap and potentially neurotrophic growth factors from outside the tube into the gap. In the large-pore collagen tubes, the cells were found between the tube wall lamellae, whereas, in the silicone tube group the contractile cells were located in a tissue capsule surrounding the outside surface of the tube. The tissue capsule which formed on the outside surface of the silicone tubes was similar to the capsules reported around silicone breast implants (Rudolph, et al., 1978) and to those observed around silicone tube implants in the peripheral nerve at 6 weeks post-operative (Chamberlain, 1996).

4.4.5 Proposed Model of Peripheral Nerve Healing

Based on the results of this study, a model of peripheral nerve healing can be proposed which consists of two competitive forces: the axial forces created by the outgrowth of axons and Schwann cells from the proximal stump (as described in Chapter 3) and the constrictive
forces imposed by the tissue capsule attempting to close the wound. In the absence of an
implant, a tissue capsule, containing many contractile cells, formed around the nerve trunk and
ultimately capped off the cut nerve ends, bringing closure to the wound. Similar methods of
wound closure have been observed in other connective tissues, primarily skin (Yannas, et al.,
1989); however, this mechanism of wound closure has not been reported previously for the
injured peripheral nerve. When an unfilled silicone tube was implanted, the wound closed over
half of the time (5 of 9 animals) by forming a contractile cap similar to the neuroma, however,
in the remaining cases (4 of 9 animals), a tissue bridge formed between the nerve ends. When
a tissue cable or nerve trunk did bridge the nerve gap, a contractile capsule, similar to the one
responsible for capping off the neuroma, was observed surrounding the nerve trunk along its
entire length. Therefore, according to the proposed model of healing, in the SI group, the
constrictive forces prevailed over the axial forces in approximately half of the implants, and the
axial forces prevailed in the remainder of the implants. This balance may depend on the gap
length.

Filling the silicone tube with CG matrix resulted in the axial forces prevailing over the
contractile forces in all of the implants; however, the thick contractile capsule was not
eliminated. The contractile capsule still formed and apparently exerted contractile forces which
caused the nerve trunk to neck down to a significantly smaller diameter in the center of the
nerve gap (Figure 4.6). In Chapter 3, it was hypothesized that the CG matrix leads to a faster
rate of axonal elongation across the nerve gap. Presence of the CG matrix, therefore, may lead
to a more rapidly forming nerve trunk aided by a faster elongation of axons from the proximal
nerve stump and a faster ingrowth of cells from the distal stump. A faster outgrowth of axonal
material from the proximal stump would improve the likelihood of axial forces prevailing and,
therefore, lead to more successful bridging of the gap, as observed when the CG matrix was
added to the silicone tube.
The proposed model predicts that if the contractile forces are eliminated, a nerve trunk should form in every case and should have a uniform diameter along the entire length of the gap. Implantation of a large-pore collagen tube eliminated the constrictive capsule and, as predicted by the model, allowed the axial outgrowth to prevail in 100% of the cases. In addition, the nerve trunks formed within the large-pore collagen tubes did not exhibit necking in the center of the nerve gap. This model may be useful in the future design of implants to treat larger gap injuries since, as the gap length increases, the constrictive forces may become more likely to prevail over the axial forces.
Chapter 5: Conclusions

The current study of peripheral nerve regeneration set out to accomplish three objectives: determine the extent and time course of recovery over a period of 1 year, evaluate the effects of implant parameters on the mechanisms of axon regeneration, and examine the effects of implant parameters on the connective tissue response to injury.

Based on the results of the long-term study (Chapter 2), it was determined that implantation of a matrix-filled large-pore collagen tube device (LC/M) resulted in regeneration of axon structure and electrophysiological function that was not significantly different from the autograft across a gap distance of 10-mm. These results suggest that the LC/M implant can be used as an off-the-shelf replacement for the autograft as a treatment for peripheral nerve injuries. Another surprising observation was the significant amount of axon maturation that was observed between 30 and 60 weeks, suggesting that the regenerative process was not complete by 1 year post-operative. Future studies are necessary which follow the regenerative process to completion in order to determine the full time course of peripheral nerve regeneration.

The observation that one implant (LC/M) was superior to all other tubulated devices led to the study of the effects of implant parameters on axon regeneration (Chapter 3). It was determined that the presence of the CG matrix had significant effects on the number of axons completing migration through the distal stump and the amount of axon maturation. Based on these findings, it was hypothesized that the CG matrix must enhance the rate of axon elongation across the nerve gap, which may lead to the observed long-term effects of the matrix. Future short-term studies, which follow the progress of axons across the gap injury, are necessary to test this hypothesis. In addition, it was observed that the tube composition had a significant effect only on the maturation of axons, with the silicone tubes having reduced maturation. However, this difference could not be reconciled based on the mechanisms of
axon regeneration; therefore, it was hypothesized that the connective tissue formed in response to nerve injury may be responsible for the lack of axon maturation.

The study of implant parameters on the connective tissue response to injury (Chapter 4), revealed significant effects of tube composition and supported the hypothesis made in Chapter 3. In the silicone tubes, the combination of necking of the nerve trunk at the center of the gap and the presence of a thick tissue capsule containing multiple layers of contractile cells suggested that the tissue capsule may be applying contractile forces on the nerve trunk along its entire length. It was hypothesized that the restrictive forces generated by the contractile capsule prevented the growth of axons in diameter, leading to the significant effect of tube composition on axon maturation. It was also observed that this method of constriction was similar to the response of an untreated wound to nerve injury, hypothetically leading to the formation of a neuroma.

Although the LC/M device performed as well as the autograft, the results in both groups remained inferior to normal. The results of this study suggest future design improvements which may result in regeneration which surpasses the autograft and approaches normal. In addition, the findings outline more specific rules for the experimental design and evaluation of future devices.
Appendix A: Protocols

A.1 Collagen-Glycosaminoglycan (CG) Slurry Protocol


2. Turn on cooling system for blender (Granco overhead blender, Granco Co., Kansas City, MO) and allow to cool to 4 °C (Brinkman cooler model RC-2T, Brinkman Co., Westbury, NY).

3. Prepare 0.05M acetic acid (HOAc) solution: add 8.7 ml HOAc (glacial acetic acid, Mallinckrodt Chemical Co., Paris, KY) to 3 liters distilled water. (This solution has a shelf life of approximately 1 week).

4. Blend 1.65 g of collagen with 600ml of 0.05 M acetic acid on HIGH speed setting for 1 hour at 4 °C.

5. Prepare 0.11% w/v chondroitin 6-sulfate solution: dissolve 275 mg chondroitin 6-sulfate (from shark cartilage: no. C-4384, Sigma Chemical Co., St. Louis, MO) in 250 ml HOAc.

6. Calibrate peristaltic pump (Manostat Cassette Pump, Catalog no. 75-500-0.00, Manostat, NY, NY) to 40 ml/5 min.

7. Add 120 ml of chondroitin 6-sulfate solution dropwise to the blending collagen dispersion over 15 minutes using the peristaltic pump (maintain blender at 4°C).

8. Blend 15 minutes longer at 4°C.


10. Decant and discard 420 ml of clear supernatant. If supernatant is not clear, repeat step 9.

11. Reblend remaining solution on LOW speed setting for 15 minutes at 4°C.

12. Store in a capped centrifuge bottle at 4°C (will keep for up to about four months, re-blend 15 minutes on LOW speed, 4°C, before using if stored more than four weeks).
A.2 CG Matrix Manufacture Protocol

ONE TO TWO DAYS BEFORE FREEZING:

1. Prepare PVC jackets by cutting off 12-cm sections of flexible PVC tubing (0.125 inches ID, 0.25 inches OD) and straighten at 105°C for 2 hours. Puncture the tube with a needle at 90° intervals around the tube and spaced 1 cm apart for the length of the tube. (See Loree, 1988, for more details).

2. Flush silicone prostheses tubing (Dow-Corning model 602-235 medical grade Silastic 0.058 inch ID, 0.077 inch OD, Dow-Corning Co., Midland, MI) with deionized water (Deionizing Organic Adsorption System, Hydro Services and Supplies, Inc., Durham, NC) and cut off 15-cm lengths.

3. Seal one end of the silicone prosthesis tubes with silicone adhesive (Silastic, Dow-Corning, medical grade Silastic). Inject approximately 5 mm into the tube and allow the excess to stay on the outside of the tube. (The excess is important for adhesion and can be cut away later) Let cure for 24 hours at room temperature. Make plenty of tubes, approximately three times the number you plan to freeze, because some will not be usable.

4. Order a 160-liter liquid nitrogen tank from the MIT Cryogenic lab.

THE DAY OF FREEZING:

5. Dearne the CG suspension in a 1500 ml Erlenmeyer flask at -30mmHg for 10 minutes with agitation or until large bubbles are no longer visible.

6. Turn on the uniaxial freezing bath (Loree, 1988) and set the temperature to -80°C. It will take approximately 45 minutes for the bath to reach this temperature.

7. Insert each silicone tube into a prepared PVC jacket. Draw 10 cc of CG suspension into a 10 cc syringe (model 5604, Becton Dickinson Co., Rutherford, NH). Expel air bubbles. Attach a 25 gauge needle (model 25G5/8, Becton Dickinson Co., Rutherford, NH) to the syringe and insert carefully into the plugged end of the silicone tube. The needle should be inserted far enough so that 3-5 mm of needle is beyond the silicone plug (Figure A.1a).

8. Inject CG suspension until a few drops come out the free end. Plug the end of the PVC tube opposite the needle with a pipette tip. Insert another pipette tip into the needle end of the tube. The tip at this end should be secure, but not too tight (Figure A.1a). Inject additional slurry until the silicone tube is pressurized and expands to fill the entire PVC jacket. The end of the needle should be inside the PVC jacket to help prevent a pressure build up at the needle tip. When the tube is pressurized, carefully remove the needle and simultaneously press the pipette tip into the end of the tube. Pressure should be kept on the syringe plunger until the needle is completely out of the tube. Check to make sure the tube is still pressurized (Figure A.1b).

9. Attach the large gear to either motor on the freezing apparatus. Tape four prepared PVC jackets to the PVC hanger and place it on the gear train. Lower manually until the pipette tips are just touching the bath. Start the appropriate motor and let the tubes lower into the bath at a velocity of 10^-4 m/s (Figure A.2a). Watch to make sure the tubes are lowering and that they do not stick to any of the parts of the freezing apparatus.
10. Turn on the freeze drier (VirTis Genesis). Select program 3 on the automatic mode and start the program. (If the automatic mode is not working, the manual steps are listed below). Place the freeze drier tray in the freezer to cool.

11. When PVC jackets are fully immersed in the bath, turn off the motor and remove the tubes from the bath. Quickly separate the tubes and remove the pipette tips. Cut off the plugged end of the silicone tube and cut each PVC jacket approximately in half with an extremely sharp razor blade (Be sure to have plenty of new blades handy). Place the tubes on the cooled freeze drier tray (Figure A.2b) and put the tray either in the freezer, or in the freeze drier if it has reached -20°C. This step must be done as quickly as possible to ensure that the tubes stay completely frozen.

12. Place the tray containing the frozen tubes in the freeze-drier. Seal the chambers on the freeze drier and close the vacuum outlet tube. Check that the door is sealed shut when the vacuum comes on. After the program is finished, clear the chamber and allow the vacuum to totally release before opening the chamber door. Turn off the unit and remove the tray.

13. Prepare aluminum foil packets for the matrices and place each PVC tube along with the matrix in a packet. Leave one end of the packet open. Place the packets in the dehydrothermal treatment (DHT) oven at 105°C. Seal the chamber and turn on the vacuum pump. DHT the matrices for 24 hours. Remove the packets and quickly close them. The matrices are now sterile and must be handled using sterile procedure from this point forward.

Figure A.1 Schematic detailing the procedure for injecting the silicone processing tube with CG slurry. (a) Configuration of the processing tube prior to pressurization. (b) Final configuration of the tube assembly prior to lowering into the freezing bath.
Figure A.2 Schematic detailing the tube assembly orientation during (a) freezing and (b) sublimation.

**MANUAL FREEZE DRIER STEPS:**

1. Turn on the freeze switch and set the shelf temperature to -20°C.
2. Turn on the condenser (at the same time as step 1) and let it cool until it reaches -45°C.
3. When the shelf reaches -20°C, insert the tray containing the frozen tubes.
4. If the condenser is already below -45°C, turn on the vacuum switch and wait for the vacuum to reach 100mTorr. Make sure that the chamber door seals. It sometimes requires assistance in sealing.
5. Once the vacuum reaches 100 mTorr, turn on the heat switch and set the temperature to 0°C. Leave the product in the freeze drier for 17 hours at this temperature and pressure.
6. Set the temperature to 25°C and remove the product when the chamber has reached room temperature. Turn off the freeze drier.
A.3 Sterile Procedure and Implant Assembly Protocols

ONE TO TWO DAYS BEFORE GRAFT PREPARATION:

1. Sterilize the necessary implements:

**In Autoclave Bags:**

- Tool Pack: 1 jewelers forceps
- 2 regular forceps
- 1 large forceps
- 1 surgical blade holder
- 1 needle holder

1 magnifying glass + 1 ring stand clamp

1 specimen jar for each prosthesis (Put a small piece of autoclave tape on each jar for labeling purposes later)

1 500 ml glass bottle

(Always keep an extra tool pack and an extra set of specimen jars sterile in case of an emergency. It’s easiest to rotate the packs so that they don’t sit unused for too long)

**Wrap in Autoclave Paper:**

1 Teflon sheet for working area (Tape a ruler to the sheet with autoclave tape. This makes cutting easier in the sterile environment)

**Dehydrothemally Treat (DHT):**

Appropriate tubes for graft preparation. Procedure is the same as with the matrix (see section A.2 for details).

**Sterilize Liquid Using ZapCap and autoclaved 500-ml bottle:**

Phosphate Buffered Saline Solution (PBS)

2. Turn on the HEPA hood, at least 1 hour before working, preferably 24 hours prior.

**GRAFT PREPARATION:**

3. Bring necessary sterile implements to workbench:

- All items sterilized in step 1.
- Envelopes with CG matrices
- Envelopes with tubes
- 1 ring stand
- 4 #10 surgical blades
- 1 sterile pipette
- 1 sterile pen
- Whirl-Pak bags (1 per prosthesis plus 3; 1 for extra tubes, 1 for extra matrix, 1 for the pen)
4. Put on latex gloves, a cap, a mask and a clean, disposable lab coat. Wipe bench, metal frames and ring stand with 70% ethanol.

5. Using sterile technique, set up sterile field with Teflon pad. Open all tool packs and pour onto sterile field, including prostheses, scalpel blades, etc. Fill each jar with sterile PBS using the sterile pipette (if the prostheses are not going to be implanted for a week or two, fill jar with 70% ethanol for storage. Before surgery, rinse in two rinses of sterile saline solution immediately before surgery). Carefully open Whirl-Pak bags and stand on metal frames in hood.

6. Put on sterile gloves. Set up the magnifying glass on the ring stand so that the lens is approximately 6 inches from the tabletop.

7. Trim prosthesis tubes to 20 mm using scalpel (19 mm for the collagen tubes). Remove the matrix from the silicone processing tube by making a careful slit with the scalpel down the length of the silicone and gently pulling out the matrix with the forceps. Trim off any crushed or otherwise damaged matrix and cut remaining portion into 10-mm segments to be inserted into the center of the trimmed tubes.

8. Place prostheses into specimen jars with PBS, close jars tightly, label, place in Whirl-Pak bags, and close bags. Label each prosthesis by type: SI for unfilled silicone tube, SI/M for matrix-filled silicone tube, LC for unfilled large-pore collagen tube, LC/M for matrix-filled large-pore collagen tube, SC for unfilled small-pore collagen tube, and SC/M for matrix-filled small-pore collagen tube.
A.4 Surgical Protocol

SUPPLIES

1. Order animals: Adult, female Lewis rats, 150 - 175 grams, from Charles River Laboratories. Animals must arrive at least one week in advance of surgery to reduce the stress placed on the animal due to travel.

2. Sterilize the necessary items:
   
   1 metal bowl  
   gauze  
   1 surgical blade holder  
   1 micro-needle holder  
   1 micro-scissors  
   2 jewelers forceps  
   1 large forceps  
   1 large scissors  
   1 surgical (tenotomy) scissors  
   2 paper clip retractors  
   2 forceps  
   1 needle holder  
   animal skin staples  
   surgical lamp handle

3. Ready other sterile items:
   
   sterile table covering  
   scalpel blades (1 #15 blade, 1 #11 blade)  
   1 bottle of PBS  
   iodine sponge  
   sterile draping  
   Implants (sterilized and prepared as in section A.3)  
   1 bottle pentobarbital (Nembutal Sodium Solution), 50mg/ml

4. Ready other non-sterile items:
   
   surgical board  
   4 rubber bands  
   rat ear punching tool  
   numbered ear tags  
   microsurgery glasses  
   hair clippers

SURGICAL PROCEDURE

1. Weigh animal on an appropriately sized balance. Record the weight and determine anesthetic dosage based on the pre-operative weight.

2. Anesthetize animal with injection of sodium pentobarbital (50 mg of solution per kg of animal). Allow 10-15 minutes for anesthesia to take effect. Each animal reacts differently to the anesthetic and in some cases, more time may be required.

3. Meanwhile, arrange the surgical area so that the table is at a comfortable level for the surgeon, and the tools are conveniently located.

4. The surgeon should be steriley dressed in scrub shirt and pants, hat and mask.

5. When ready, shave the animal using the animal hair clippers from the base of the tail up to the middle of the back. The leg receiving the prosthesis should be shaved carefully and completely.
6. Place the animal on the surgical board in the prone position and secure the arms and legs to the board using rubber bands. The legs should be in 30° abduction. Place a piece of gauze under the appropriate thigh to elevate the leg slightly.

7. Clean the shaved portion of the animal vigorously with the iodine sponge to disinfect the area. At this point, the surgeon should put on the sterile gloves and remain sterile for the rest of the procedure. Cut a hole in the sterile draping small enough so that only the leg is exposed. Place the draping over the animal.

8. Using the #15 scalpel, make a 4 cm incision along the leg of the animal. Separate the skin from the muscle along the incision by cutting through the connective tissue with the surgical scissors.

9. Using the surgical scissors, separate the muscles until the sciatic nerve is visible. Carefully cut back the muscle along the skin incision line exposing the sciatic nerve.

10. Place the paper clip retractors inside the muscle to separate the wound edges. Anesthetize the nerve by placing a few drops of Lidocaine directly on the area. Cut away the fascia surrounding the sciatic nerve carefully so that the nerve is free from constraint.

11. For a tubular repair:
   Transect the nerve midway between the proximal nerve trunk and the distal bifurcation using microscissors. Measure the prosthesis and make a mark 5 mm in on each end. Place the tube in the gap and insert the proximal nerve stump 5 mm into the tube end, as marked. Secure the nerve in place by using two 10-0 sutures which travel through the epineurium and then through the tube. Tie the sutures with four single knots. Insert the distal nerve end 5 mm into the other end of the tube and secure in the same manner.

   For an autograft:
   Mark off a 10-mm segment in the middle of the nerve using the sterile pen. At the proximal end, place 10-0 pre-sutures on either side of the mark. Transect the nerve at the marked point using microscissors, being very careful not to cut through the pre-sutures. Once transected, pull the pre-sutures tightly and tie off using four single knots. Place another 10-0 suture at the proximal end of the autograft to secure the nerve. Repeat the same procedure at the distal nerve end.

   For a spontaneous repair (untreated group):
   Remove a 5-mm segment of the sciatic nerve from the midpoint between the proximal nerve trunk and the distal bifurcation using microscissors. Removal of the 5-mm nerve segment and subsequent retraction of the nerve stumps results in formation of a 10-mm gap between the nerve stumps. Allow the proximal and distal nerve stumps to fall naturally onto the underlying tissue and do not employ any repair methods.

12. Remove the paper clip retractors. Close the muscle using three 4-0 sutures. Close the skin using two 4-0 sutures and three skin staples.

13. Place the animals back in the cage and observe frequently until they are awake.
A.5 Sensorimotor Performance Evaluation Protocol

PINCH TEST PROCEDURE

1. Hold the animal firmly by the scruff of the neck, and allow the hind legs to hang freely.

2. Using forceps, pinch the inner toe and outer toe deeply on the normal foot to determine the type of response the animal will give to pain (vocalization, withdrawal of the foot, or muscle flexion).

3. Pinch the skin of the outer toe on the normal foot and monitor for type of response. With the cutaneous pain, pinch only the top layer of skin with the forceps.

4. Test the experimental foot in the same manner. Inner toe first, then outer toe and then the cutaneous region. Consider responses positive if they closely match the magnitude of the normal response. If a weaker response is detected, label it as a partial response.

MUSCLE MASS MEASUREMENT PROCEDURE

1. Weigh the animal and then anesthetize with an intraperitoneal injection of sodium pentobarbital (Nembutal Sodium Solution, 50 mg/ml) using a dosage of 50 mg of solution per kg of animal. Make this measurement prior to sacrifice.

2. Shave both legs of the animal using clippers.

3. On both the normal and experimental leg, mark two locations: 20 mm and 30 mm proximal to the heel.

4. At each mark, measure the circumference of the leg using a marked piece of string. Pull the string tight, but do not pinch or constrict the tissue. Measure the string linearly using a ruler.
A.6 Electrophysiology Protocol

RAT PHYSIOLOGICAL SOLUTION (RINGER'S) - STOCK SOLUTIONS

**Sodium Chloride (NaCl)**
2 M Stock Solution: Sodium Chloride (NaCl), 23.376 grams
Distilled Water, 200 mL

**Potassium Chloride (KCl)**
1 M Stock Solution: Potassium Chloride (KCl), 14.91 grams
Distilled Water, 200 mL

**Magnesium Sulfate (MgSO₄)**
0.5 M Stock Solution: Magnesium Sulfate (MgSO₄), 12.037 grams
Distilled Water, 200 mL

**Calcium Chloride (CaCl₂)**
1 M Stock Solution: Calcium Chloride (CaCl₂), 29.404 grams
Distilled Water, 200 mL

**Dibasic Sodium Phosphate (Na₂HPO₄)**
1 M Stock Solution: Dibasic Sodium Phosphate (Na₂HPO₄), 28.392 grams
Distilled Water, 200 mL

**MOPS Buffer**
0.5 M Stock Solution: MOPS, grams
Distilled Water, 200 mL

**Glucose**
1 M Stock Solution: Glucose, 36.04 grams
Distilled Water, 200 mL

**RINGER'S SOLUTION RECIPE** (for 500 mL final volume of Ringer's):

<table>
<thead>
<tr>
<th>Solution</th>
<th>Final Concentration</th>
<th>Volume of Stock Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>150 mM</td>
<td>37.5 mL</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>3.0 mM</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>5.0 mM</td>
<td>5 mL</td>
</tr>
<tr>
<td>Calcium Chloride</td>
<td>2.0 mM</td>
<td>1 mL</td>
</tr>
<tr>
<td>Dibasic Sodium Phosphate</td>
<td>5.0 mM</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>MOPS Buffer</td>
<td>5.0 mM</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 mM</td>
<td>2.5 mL</td>
</tr>
</tbody>
</table>

Fill remainder of 500 mL volume with distilled water.
Adjust to pH 7.3 using Sodium Hydroxide (NaOH).
ELECTROPHYSIOLOGICAL PROCEDURE

1. Set up the electrophysiological stimulating and recording equipment. The basic configuration is shown in Figure A.3. The stimulating pulse is generated in the stimulator (Grass S48 Stimulator, Grass Medical Instruments, Quincy, MA). The signal is sent simultaneously to the oscilloscope (5113 Dual Beam Storage Oscilloscope, Tektronix) and the stimulus isolation unit (Grass SIU5). The signal is passed through the stimulus isolation unit and sent to the nerve via the stimulating electrodes. The recording electrodes send the recorded signal through a differential electrometer (AK-47UU, MetaMetrics), an amplifier (AK-47S, MetaMetrics), and into the storage oscilloscope.

2. Prior to animal sacrifice, observe the qualitative extent of in vivo muscle twitch. Attach a stimulating probe to the proximal portion of the sciatic nerve (proximal to the injury site) and monitor the gastrocnemius and plantar muscles for twitch response. The stimulus can be given at varying levels to determine a stimulus threshold. Perform under anesthesia with sodium pentobarbital (Nembutal Sodium Solution, Parke-Davis, 50mg/ml; dosage = 50 mg/kg).

3. Dissect out the sciatic nerve and the distal branches while the animal is under anesthesia. Promptly euthanize the animal following dissection with an overdose of sodium pentobarbital (100mg/kg).

4. Using a dissecting microscope, clean the resected nerve by removing all excess tissue that is surrounding the nerve (i.e., fat and fascia). If the nerve is tubulated, also cut the tube away carefully. Place the nerve in Ringer's rat physiological solution.

5. Lay the prepared nerve in the electrophysiology chamber (Figure A.4) across the electrodes and secure the nerve at the ends. The chamber contains an electrode array consisting of six silver electrodes; however, use only the first five electrodes (i.e., two stimulating, two recording, and one ground). Fill the trough with Ringer's solution and cover the chamber to humidify the nerve.

6. Attach the banana plugs coming from the stimulus isolation unit, the differential electrometer, and the ground to the electrophysiology chamber.

7. Using a constant stimulus duration (50 μs), begin testing the nerve to see if an action potential can be detected. Start at a low voltage and increase the voltage until the amplitude of the action potential peaks, then increase the voltage by another 10% to reach the saturated action potential.

![Figure A.3 Block diagram showing the configuration of the stimulating and recording equipment in relation to the test nerve.](image)
Figure A.4 Sketch of the electrophysiology chamber used in this experiment.

8. Using a single pulse stimulus, record the A-fiber amplitude. Take a Polaroid photograph which will be used for measurement of the peak to peak amplitude and latency of the primary action potential (A-fibers). Measure and record the length of the nerve for calculation of the conduction velocity.

9. Measure the functional refractory period using a paired pulse. Using variable delay times on the storage screen, find the period when amplitude recovers to 50%, etc. Take a Polaroid photograph.

10. Stimulate the nerve at a frequency of 100 Hz to start, then reduce the frequency to 50 Hz, 40 Hz, 20 Hz, etc. for comparison of amplitude difference following train stimulation. Take a Polaroid photograph after each frequency.

11. Return to single pulse stimulation. Increase the voltage and look for the appearance of B-fiber and C-fiber action potentials. Take Polaroid photographs of any observed signals.
A.7 Animal Sacrifice and Tissue Processing Protocols

EQUIPMENT

- IV Stand
- IV bottle for perfusate
- IV tubing (2 sets)
- 3 way stopcock
- Pyrex dish
- 1 IV bag of saline, heparinized (20 units per milliliter)
- 18 gage needle
- Surgical instruments
- Specimen jars (1 for each animal)
- Pentobarbital
- Syringes (1 for each animal)

SOLUTIONS

Yanoff’s Fixative

Stock Solutions

**Stock A:**
- 1.67 grams Monobasic Sodium Phosphate $\text{NaH}_2\text{PO}_4$
- 8.95 grams Dibasic Sodium Phosphate $\text{Na}_2\text{HPO}_4$
- 960 ml Distilled $\text{H}_2\text{O}$
- 40 ml 25% Glutaraldehyde

**Stock B:**
- 4.0 grams Monobasic Sodium Phosphate $\text{NaH}_2\text{PO}_4$
- 8.95 grams Dibasic Sodium Phosphate $\text{Na}_2\text{HPO}_4$
- 900 ml Distilled $\text{H}_2\text{O}$
- 100 ml 38-40% Formaldehyde

Yanoff’s fixative is 1:1 mixture of stock A and stock B

10% Neutral Buffered Formalin

Stock B of Yanoff’s Fixative is 10% Neutral Buffered Formalin.

70% EtOH

SACRIFICE PROCEDURE

This perfusion sacrifice procedure was used for animals sacrificed at 6 and 30 weeks. The sacrifice procedure for 60 week animals was different and is described in section A.6.

1. Anesthetize animal with pentobarbital, 50 mg solution per kg rat, as in surgical procedure.

2. Fill IV bottle with Yanoff’s fixative.

3. Set up IV tubing from perfusate and saline, hang each solution from rack (reservoirs must be at least 100 cm above the animal).

4. Place the animal in the Pyrex dish and open the chest cavity.

5. Cut open the right auricle for blood drainage and position 18 gage needle into the left cardiac ventricle (through the right auricle).

6. Perfuse animal with 150 ml heparinized saline (20 units per ml) from reservoir to flush the remaining blood from the system. Follow with 300 ml cold perfusate.
Figure A.5 Sectioning diagram for the rat sciatic nerve and distal branches. L=Liquid Storage, P=Paraffin Embedding, and E=Epon embedding.

TISSUE PROCESSING PROCEDURE
This processing procedure was used for all animals (6, 30 and 60 weeks).

1. Explant the sciatic nerve and the proximal portions of the tibial and peroneal nerve up to the muscle insert. Clearly mark the distal portion of the tibial nerve with a suture for reference.

2. Place tissue into Yanoff’s fixative for 24 hours at 4°C.

3. Transfer tissue into 10% neutral buffered formalin solution for 24 hours at 4°C.

4. Remove tissue from formalin and rinse 1X in 70% EtOH.

5. Photograph the nerve to capture the gross morphology of the tissue.

6. Section the nerve into 2-mm segments according to Figure A.5. The sections that were used in this study were referred to as described in Table A.1.

7. Place each small nerve segment into an individual vial containing 70% EtOH. Process each according to the specific embedding protocol that applies (see sections A.8.1 and A.8.2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Distance from the Proximal Stump</th>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal sciatic nerve stump</td>
<td>-1 mm</td>
<td>E3</td>
</tr>
<tr>
<td>Center of the nerve gap</td>
<td>5 mm</td>
<td>E6</td>
</tr>
<tr>
<td>Distal Sciatic Nerve Stump</td>
<td>11 mm</td>
<td>E9</td>
</tr>
<tr>
<td>Tibial Nerve Branch</td>
<td>20 mm</td>
<td>E12</td>
</tr>
<tr>
<td>Peroneal Nerve Branch</td>
<td>20 mm</td>
<td>E14</td>
</tr>
</tbody>
</table>

Table A.1 Nomenclature used in the text to refer to the different nerve segments.
A.8 Histological Protocols

A.8.1 Epon Embedding Protocol

SOLUTIONS

Cacodylate Buffer (pH 7.4)

Stock Solutions

Stock A (0.2 M Sodium Cacodylate (mw 214)):

4.28 grams sodium cacodylate
100 ml of distilled water

Stock B (0.2 M HCl (mw 36.46)):

1.7 ml HCl
100 ml of distilled water

Composition of Buffer

25 ml of Stock A + 1.4 ml of Stock B (for pH 7.4)* + 73.6 ml of distilled water

*Volume of Stock B changes for different pH levels

Cacodylate Buffered Glutaraldehyde

2% Solution:

8 ml, 25% Glutaraldehyde
92 ml, Cacodylate Buffer (made as described above)

Cacodylate Buffered Sucrose Solution

0.2 M Solution:

6.846 grams, Sucrose (mw 342.3)
100 ml, Cacodylate Buffer (made as described above)

Osmium Tetroxide (Catalog #0972A, Polysciences, Inc., Warrington, PA)

1% Solution:

2 ml, 4% Osmium Tetroxide
6 ml, Distilled Water

Poly/Bed 812 Embedding Kit (Catalog #08792, Polysciences, Inc., Warrington, PA)

EMBEDDING PROCEDURE

1. Soak nerves in 2% cacodylate buffered glutaraldehyde overnight at 4°C.

2. Soak nerves in 0.2 M cacodylate buffered sucrose solution overnight at 4°C.

3. Rinse nerves 1 time in cacodylate buffer for 10 minutes at 4°C.

4. Fix in 1% osmium tetroxide for 2 hours at room temperature (in the hood).

5. Dehydrate nerves in EtOH:

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%</td>
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<tr>
<td>50%</td>
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<tr>
<td>70%</td>
<td>5 minutes</td>
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<tr>
<td>80%</td>
<td>5 minutes</td>
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<tr>
<td>90%</td>
<td>5 minutes</td>
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<td>95%</td>
<td>5 minutes</td>
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<tr>
<td>100%</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100%</td>
<td>5 minutes</td>
</tr>
<tr>
<td>100%</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>
6. Clear nerves in acetone 2 times for 5 minutes each.

7. Infiltrate in 1:1 acetone/Epon* mixture overnight at room temperature.

8. Infiltrate in 1:3 acetone/Epon mixture for 5 hours at room temperature.

9. Infiltrate in 100% Epon mixture overnight at room temperature.

10. Embed with 100% Epon in TEM molds. (Make well labels with pencil or on computer)

11. Let cure 24 hours at 60°C.

*During infiltration, the Epon mixture should NOT contain the hardener.
A.8.2 Paraffin Embedding Protocol

SOLUTIONS

Eosin Y (Catalog #E-511, Fisher Scientific, Fair Lawn, NJ)
Eosin Stock Solution: 1 gram, Eosin Y
1 Liter, 70% EtOH

Paraplast Plus Paraffin (Catalog #5159-464, VWR Scientific, Boston, MA)

EMBEDDING PROCEDURE

1. Stain nerves in Eosin for 1 minute. This makes the sections easier to identify during embedding and sectioning.

2. Dehydrate nerves in EtOH:
   - 70% 5 minutes
   - 70% 5 minutes
   - 80% 5 minutes
   - 90% 5 minutes
   - 95% 5 minutes
   - 100% 5 minutes
   - 100% 5 minutes
   - 100% 5 minutes

3. Clear nerves in xylene 2 times for 5 minutes each.

4. Put nerves in tissue cassettes, sandwiched by two blue sponges, and label the cassettes with the appropriate information in pencil.

4. Infiltrate in paraffin bath 2 times for 30 minutes each.

5. Embed in paraffin.

6. Cool and store in refrigerator.
A.8.3 Toluidine Blue Staining Protocol

Protocol for rat sciatic nerve tissue perfusion or drop-fixed in Yanoff's fixative (see section A.7 for solution recipe). Post-fixed with 1% osmium tetroxide. Epon embedded, sectioned at 1 μm thickness.

SOLUTIONS

Toluidine Blue Solution (Catalog #BP107-10, Fisher Biotech, Boston, MA)
1% Toluidine Blue Solution: 1 gram, Toluidine Blue Powder
1 gram, Sodium Borate Powder
100 ml, Distilled Water

Solution should be filtered and stored in a stopped bottle.

Cytoseal 60 Mounting Medium (Catalog #8310-16, Stephens Scientific).

STAINING PROCEDURE

1. Heat slides on hot plate to 60 - 80°C.

2. Stain with Toluidine Blue Solution for 30 - 60 seconds. Thicker sections will take less time.

3. Rinse in distilled water and allow to dry on hot plate for 2 - 3 minutes.

4. Mount with mounting medium.
A.8.4 Hematoxylin and Eosin Staining Protocol

Protocol for rat sciatic nerve tissue perfusion or drop-fixed in Yanoff's fixative (see section A.7 for solution recipe). Paraffin embedded, sectioned at 5-10 μm thickness.

SOLUTIONS

Gill's Hematoxylin  (Catalog #GHS-3-16, Sigma Chemical Co., St. Louis, MO)

Eosin Y  (Catalog #E-511, Fisher Scientific, Fair Lawn, NJ)
Eosin Stock Solution: 1 gram, Eosin Y
1 Liter, 70% EtOH

Weak Ammonium Hydroxide Water
Ammonium hydroxide, 2-4 Drops
Tap water, 500 mL

Cytoseal 60 Mounting Medium  (Catalog #8310-16, Stephens Scientific).

STAINING PROCEDURE

1. Deparaffinize and hydrate to water:
   xylene  2 minutes
   xylene  2 minutes
   100% EtOH 1 minute
   100% EtOH 1 minute
   95% EtOH 1 minute
   80% EtOH 1 minute
   70% EtOH 1 minute
   tap water 10 minutes

2. Gill's Hematoxylin for 4 minutes.
3. Rinse in distilled water.
4. "Blue" with weak ammonium hydroxide water.
5. Rinse in distilled water.
6. Counterstain with Eosin 1 minute 15 seconds.
7. Rinse in distilled water.
8. Dehydrate and clear:
   70% EtOH 1 minute
   80% EtOH 1 minute
   95% EtOH 1 minute
   100% EtOH 1 minute
   100% EtOH 1 minute
   xylene 2 minutes
   xylene 2 minutes

9. Mount with mounting medium.
A.8.5 Masson’s Trichrome Staining Protocol

Protocol for rat sciatic nerve tissue perfusion or drop-fixed in Yanoff’s fixative (see section A.7 for solution recipe). Paraffin embedded, sectioned at 5-10 μm thickness.

SOLUTIONS

**Bouin’s Solution**  (Sigma Chemical Co., St. Louis, MO)

**Weigert's Iron Hematoxylin Solution**
Solution A:  
1.0 gm, Hematoxylin crystal  
100.0 ml, 95% EtOH

Solution B:  
4.0 ml, Ferric Chloride, 29% aqueous  
95.0 ml, distilled water  
1.0 ml, HCl, conc.

Mix equal parts of solution A and solution B.

**Biebrich Scarlet Acid Fuchsín** (Catalog #B-6008, Sigma Chemical Co., St. Louis, MO)
Solution:  
90.0 ml, Biebrich scarlet, aqueous 1%  
10.0 ml, Acid fuchsín, aqueous 1%  
1.0 ml, Glacial acetic acid

**Phosphomolybdic-Phosphotungstic Acid**  (Catalog #2891, Baker, Phillipsburg, NJ)
Solution:  
5.0 gm, Phosphomolybdic acid  
5.0 gm, Phosphotungstic acid  
200.0 ml, Distilled water

**Aniline Blue** (Catalog # 02570, Polysciences Inc., Warrington, PA)
Solution:  
2.5 gm, Aniline Blue  
2.0 ml, Glacial acetic acid  
100.0 ml, Distilled water

**Glacial Acetic Acid**  (Mallinckrodt Chemical Company, Paris, KY)
1% Solution:  
1.0 ml, Glacial acetic acid  
100.0 ml, Distilled water

**Cytoseal 60 Mounting Medium**  (Catalog #8310-16, Stephens Scientific).

STAINING PROCEDURE

1. Deparaffinize and hydrate to water:  
   - xylene 2 minutes  
   - xylene 2 minutes  
   - 100% EtOH 1 minute  
   - 100% EtOH 1 minute  
   - 95% EtOH 1 minute  
   - 80% EtOH 1 minute  
   - 70% EtOH 1 minute  
   - tap water 10 minutes
2. Mordant in Bouin's solution for 1 hour at 56°C, or overnight at room temperature.

3. Cool and wash in running water until yellow color disappears (approx. 20 minutes).

4. Rinse in distilled water.

5. Weigert's iron hematoxylin solution for 10 minutes. Wash in running water 10 minutes.

6. Rinse in distilled water.

7. Biebrich scarlet acid fuchsin solution for 2 minutes. Save solution.

8. Rinse in distilled water.


10. Aniline blue solution for 15 minutes. Save solution.

11. Rinse in distilled water.


13. Rinse in distilled water.

14. Dehydrate and clear:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>1 minute</td>
</tr>
<tr>
<td>xylene</td>
<td>2 minutes</td>
</tr>
<tr>
<td>xylene</td>
<td>2 minutes</td>
</tr>
</tbody>
</table>

15. Mount with mounting medium.
A.8.6 α-Smooth Muscle Actin Staining Protocol

Protocol for rat sciatic nerve tissue perfusion or drop-fixed in Yanoff’s fixative (see section A.7 for solution recipe). Paraffin embedded, sectioned at 5-10 μm thickness.

SOLUTIONS (for staining approximately 25 slides):

**Phosphate Buffered Saline** (Catalog #P-3813, Sigma Chemical Co., St. Louis, MO)
Mix 1 Sigma Phosphate Buffered Saline (PBS) packet in 1 Liter Distilled Water (dH₂O)

**Trypsin** (Catalog #T-7409, Sigma)
Store trypsin powder in refrigerator.
0.1% Solution: Trypsin, 0.01 grams
PBS, 10 ml

**Hydrogen Peroxide, 30%** (Catalog #H-1009, Sigma)
Store stock solution in refrigerator.
3% Solution: 30 % Hydrogen Peroxide, 1 ml
dH₂O, 9 ml

**Goat Serum** (Catalog #G-9023, Sigma)
Freeze in 1 ml aliquots.
20% Solution: Goat Serum, 2 ml
Phosphate Buffered Saline, 8 ml

**Primary Antibody, Anti-α Smooth Muscle Actin** (Catalog #A-2547, Sigma)
Freeze in 25 μl aliquots.
1:400 concentration: Primary antibody, 25 μl
PBS, 10 ml

**Mouse Serum (used as Negative Control)** (Catalog # M-5905, Sigma)
Freeze in 20 μl aliquots.
1:200 concentration: Mouse Serum, 20 μl
PBS, 10 ml

**Secondary Antibody, Goat Anti-Mouse IgG** (Catalog #B-0529, Sigma)
Freeze in 33 μl aliquots.
1:300 concentration: Secondary Antibody, 33 μl
PBS, 10 ml

**ExtrAvidin Peroxidase Reagent** (Catalog # E-2886, Sigma)
Store in 100 μl aliquots in the refrigerator - DO NOT FREEZE!
1:50 concentration: ExtrAvidin Peroxidase, 200 μl
PBS, 10 ml

**AEC Staining Kit** (Catalog #AEC-101, Sigma)
Mix up 8 ml according to the package instructions.

**Mayer's Hematoxylin Solution** (Catalog #MHS-16, Sigma)

**Glycerol Gelatin** (Catalog #GG-1, Sigma)
STAINING PROCEDURE:

1. Put slides in green dipping rack. Hang rack from the side of a 2 liter beaker with enough xylene to cover the slides. Add stir bar and stir gently for 1 hour.

2. While slides are deparaffinizing, remove antibodies from the freezer:
   - 25 µl Primary Antibody
   - 33 µl Secondary Antibody
   - 20 µl Mouse Serum
   - 2 ml Goat Serum

   Also, remove trypsin from the refrigerator and allow to come to room temperature before opening to avoid adding moisture to the desiccated compound.

3. Mix up PBS in glass beaker with stir bar. Add 1 packet of PBS to 1 liter of distilled water. Stir for several minutes to mix.

4. Mix up trypsin with PBS to make 10 ml.

5. After 1 hour of xylene, remove the slides and deparaffinize as follows:
   - 100% EtOH 2 minutes
   - 100% EtOH 2 minutes
   - 95% EtOH 2 minutes
   - 80% EtOH 2 minutes
   - 70% EtOH 2 minutes
   - dH₂O 2 minutes
   - PBS 2 minutes
   - PBS 2 minutes

6. Gently dry slides, but not sections with Kimwipes. Circle sections to be stained with PAP pen. Add trypsin solution to circled sections. Do not let sections dry out between steps!!!

7. Incubate in trypsin solution (0.1%) for 1 hour at room temperature. Tip trypsin off slides into waste container.

8. PBS wash x 2, 2 minutes each.

9. Dry slides but not sections.

10. Incubate in hydrogen peroxide (3%) for 10 minutes.


12. Incubate with goat serum (20%) for 10 minutes.

13. Tip off excess serum, do NOT wash slides.

14. Incubate with primary antibody or mouse serum (negative control) for 2 hours at room temperature.
15. Tip off excess antibody. Be careful not to contaminate your negative control with the primary antibody. To avoid contamination in the rinse bath, use a transfer pipette to gently rinse sections individually before washing.


17. Incubate with the secondary antibody for 1 hour at room temperature.

18. Repeat steps 8 & 9.

19. Incubate with ExtrAvidin Peroxidase for 20 minutes at room temperature.


22. Rinse slides in dH₂O. While slides are in the dH₂O, mix up AEC according to instructions on the staining kit.

23. Dry slides and begin incubating in AEC solution. Begin timing after the first slide started. Watch how long it takes to dry the slides and apply the AEC solution. Try to keep that pace when you stop the development so that all slides stain similarly.

24. Incubate in AEC solution for 15 minutes.

25. Stop development by tipping AEC off slides into an appropriate waste container (AEC is a carcinogen!) and place the slides in a slide holder in dH₂O. Rinse in dH₂O ×2, 2 minutes each. Check slides for development. If some slides are not developed enough, repeat the AEC step for 2-5 minutes as needed.

26. Mayer’s hematoxylin solution for 15 minutes.

27. Running water bath for 15 minutes to develop the hematoxylin.

28. Coverslip with the warmed glycerol gelatin. If the gelatin hardens too early, place slides on 40 degree surface (like water bath edge) or in 57° oven for a few minutes to re-melt the gelatin.
A.9 Image Capture and Analysis Protocol

COMPUTER SETUP

1. Turn on the Power Macintosh computer. Open the ORL Microscope hard drive. Open the Applications folder. Open the Microscope Stuff folder. Open the Scion Image folder.

2. Open the application Scion Image.

MICROSCOPE SETUP

1. Turn the microscope on (green button on the right side). Microscope (Olympus Vanox-T, Olympus, Japan) should have video camera (Hamamatsu CCD Video Camera Module, Model XC-77, Hamamatsu, Japan) attached.

2. Select the white button (max) for light level (on right side, there are 3 light choices)

3. Locate the two knobs on the top portion of the microscope, one on the left and one on the right. Pull out both knobs to the TV position.

4. Insert a microscope slide into the scope and focus on the selected sample you want to image. Adjust to the appropriate magnification.

VIDEO BOX SETUP

1. Turn the Gain and Offset knobs completely to the left. This is essentially the off position.

2. Turn the video box on (Hamamatsu CCD Camera Control, Hamamatsu, Japan).

3. Watch the video level on the box at all times to make sure the "Normal" indicator light is on. If the "Over" light is on, there is too much light and it can damage the computer screen. If the "Over" light comes on, adjust the light on the microscope or the gain and offset knobs on the video box accordingly.

IMAGE CAPTURING PROCEDURE

1. Select Start Capturing from the Special menu (keystroke: ⌘G).

2. Turn the Gain and Offset knobs up to approximately the midpoint. Turn the offset knob up even more until the image is visible. Watch the light indicator on the video box for the over signal. The light can also be adjusted on the microscope to make the image visible on the screen. Again, watch the light indicator.

3. Position the image you want to capture on the screen. Focus the image and adjust the light to the appropriate level.

4. Select Stop Capturing from the Special menu (keystroke: ⌘G).

5. Save the image onto a Zip Disk (keystroke: ⌘S).

6. Repeat procedure to capture the next image.
IMAGE SELECTION PROCEDURE

1. Capture one low magnification image of the nerve trunk (as described above).

2. Measure the cross-sectional area of the nerve trunk (follow the Analysis Procedure outlined below). If there are multiple fascicles, measure each area separately and then sum the areas to get a total area.

3. Determine how many images are necessary to adequately sample the nerve (Table A.2).

4. Position the nerve so that the long axis of the nerve is on the Y axis (Figure A.6).

5. Divide the nerve into 4 quadrants (Figure A.6). Count the number of images from the top center of the cross-section to the bottom center of the cross-section (Y), and the number of images from the left center to the right center (X).

3. Begin imaging. Refer to Figure A.6 to see image placement. I find it easiest to start with image 5, the geometric center. Then move to quadrant 1 and capture all images in that quadrant. Then all images in quadrant 2, and so on. Use the values of X and Y counted in Step 5 to locate the images. For example, image 11 is 1/4 Y down from the top center of the cross-section and 1/4 X to the left.

4. Capture the appropriate number of images. If only 3 images are needed capture: 11, 41, & 5. If 5 images are needed capture: 11, 21, 31, 41, & 5. If 9 images are needed: 11, 12, 21, 22, 31, 32, 41, 42, & 5; and so on. Refer to Figure A.6 to see image placement.

<table>
<thead>
<tr>
<th>Fascicle Area Range (mm²)</th>
<th>Number of Images Necessary</th>
<th>Image Area Divided by Total Fascicle Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;0.25</td>
<td>3</td>
<td>≥10%</td>
</tr>
<tr>
<td>0.25&lt;A&lt;0.4167</td>
<td>5</td>
<td>10% - 16%</td>
</tr>
<tr>
<td>0.4167&lt;A&lt;0.75</td>
<td>9</td>
<td>10% - 18%</td>
</tr>
<tr>
<td>0.75&lt;A&lt;1.08</td>
<td>13</td>
<td>10% - 14%</td>
</tr>
<tr>
<td>1.08&lt;A&lt;1.417</td>
<td>17</td>
<td>10% - 13%</td>
</tr>
<tr>
<td>1.417&lt;A&lt;1.75</td>
<td>21</td>
<td>10% - 12%</td>
</tr>
<tr>
<td>A&gt;1.75</td>
<td>21</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>

Table A.2 Number of images necessary to describe nerve trunks of different cross-sectional area. Capturing the appropriate number of images results in sampling of at least 10% of the total tissue area, except when the area is larger than 1.75 mm². If tissue filled the entire inside diameter of the implant tubes, the area would be 1.77 mm²; therefore, in the majority of cases sampling is greater than 10%.
Figure A.6. Schematic showing the location of images around the nerve section.

IMAGE ANALYSIS PROCEDURE

1. Open NIH Image.

2. Open image file.

3. Go to the Analyze menu and select Set Scale. Select the appropriate unit of measure for the magnification of the image (i.e., millimeters for low magnification images and micrometers for high magnification images). Use Table A.3 to fill in the Known Distance and the Pixels entries. Table A.3 is calibrated for the microscope at the Brigham, with images captured in Scion Image. When the scale is set a diamond appears next to the file name on the top bar of the window.

4. Use Smooth and/or Sharpen from the Enhance menu to improve the quality of the image if needed. If you do not like the effect of these, choose Undo from the Edit menu before doing anything else.

5. From the Enhance menu, choose Arithmetic, Subtract. Subtract 3.

6. From the Options menu, select Density Slice. Threshold all the way to black so that no red should be showing in the image.

7. Select the pencil from the toolbar. Move the cursor over the red portion of the color scale and choose red as the color. (The paintbrush will appear red when the appropriate color has been selected).
<table>
<thead>
<tr>
<th>Magnification Power</th>
<th>Known Distance</th>
<th>Pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 mm</td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td>1 mm</td>
<td>243</td>
</tr>
<tr>
<td>10</td>
<td>0.9 mm</td>
<td>543</td>
</tr>
<tr>
<td>20</td>
<td>0.44 mm</td>
<td>539</td>
</tr>
<tr>
<td>40</td>
<td>220 μm</td>
<td>526</td>
</tr>
<tr>
<td>100</td>
<td>80 μm</td>
<td>485.5</td>
</tr>
</tbody>
</table>

Table A.3 Calibration values to set the scale for the appropriate magnifications on NIH Image. The magnification power refers to the objective that was used on the microscope at the Brigham & Women’s Hospital, Orthopedic Research Lab (Olympus Vanox-T, Olympus, Japan).

8. Circle the axons with the pencil on the OUTSIDE perimeter of the myelin sheath.

9. When all axons have been circled, go to the Analyze menu and select Options. Choose Area, Perimeter/Length and Headings.

10. Choose SAVE AS from the File menu and save the image as an edited file. Don’t forget to save at this point!!

11. Again in the Analyze menu choose Analyze Particles Enter the following:
   Minimum particle size: 5 pixels
   Maximum particle size 30000 pixels
   Label
   Outline
   Include
   Reset

12. Each axon will become filled and numbered as it is counted. Make sure that all axons are filled in. If an axon is not darkened, part of the perimeter is not complete, therefore, it has been measured inappropriately. Fix and repeat step 11.

12. Choose Show Results from the Analyze menu. Copy the results (keystroke: ⌘C) and paste them into Excel for analysis (keystroke: ⌘V).

14. Close the image. Do not save the changes to your edited file!
Appendix B: Calculations

The following is a stepwise procedure for analyzing the data obtained via image analysis. The protocol for obtaining images and the basic image analysis technique was outlined in section A.9. This section details how the numerical values were calculated from the raw data.

1. Measure the tissue area ($A_{tissue}$) and capture the appropriate number of images as described in section A.9.

2. Use image analysis program (NIH Image) to count the number of myelinated axons per image and measure the diameters. For each axon, the program will determine the area of the axon and the perimeter. Paste the raw data for all of the axons into a Microsoft Excel spreadsheet (as described in section A.9).

3. Calculate the diameter of each myelinated fiber using the following formula:

$$D = \frac{P}{\pi}$$

where $D$ is the fiber diameter and $P$ is the perimeter.

4. Calculate a diameter histogram for the axons of each image using Microsoft Excel. Highlight the column containing the fiber diameters. Select Data Analysis from the Tools menu. Select Histogram from the list of data analysis tools. Enter the Input Range, Bin Range, and Output Range. The Bin Range should be a separate column from the data and should contain integers from 1-12. Excel will output the number of axons in each diameter bin in the Output Range location.

5. Repeat steps 2 through 4 for all images captured for the nerve (from 3 - 21 images).

6. Once all images have been analyzed, calculate the myelinated axon density using the following formula:

$$AxonDensity = \frac{\sum_{i=1}^{N} (#axons)}{N*Area_{image}}$$

where $N$ is the total number of images, ($#axons$)$_i$ is the number of axons in image $i$, and $Area_{image}$ is the area of each digitized image. When images are captured according to the protocol in section A.9, the $Area_{image} = 8.334 \times 10^3 \text{ mm}^2$.

7. Calculate the total number of myelinated axons per nerve using the following formula:

$$Axons_{Nerve} = AxonDensity \times A_{tissue}$$

where $AxonDensity$ is as calculated in step 6 and $A_{tissue}$ is the total tissue area measured in step 1.
8. Determine the percentage of axons in each of the 12 size bins using the following formula for each bin:

\[
Percentage_x = \frac{\sum_{i=1}^{N} (# \ axons_x)_i}{Axons/Nerve}
\]

where \((#axons_x)_i\) is the number of axons in size bin \(X\) for image \(i\), \(N\) is the total number of images, and \(Axons/Nerve\) is as calculated in step 7.

9. Calculate the total number of large diameter myelinated fibers \((D \geq 6 \mu m)\) using the following formula:

\[
LgAxons/Nerve = \left( \sum_{X=6}^{12} Percentage_x \right) \cdot Axons/Nerve
\]

where \(Percentage_x\) is as calculated in step 8, and \(Axons/Nerve\) is as calculated in step 7.

10. To calculate an diameter distribution, multiply each \(Percentage_x\) by the \(Axons/Nerve\) to obtain the values for each bin.
Appendix C: Loss of Axons Entering the Distal Stump

An unexpected finding was the large number of axons that reached the distal sciatic nerve stump, but did not enter the distal nerve fascicles (Figure C.1). Few axons which did not enter the distal nerve fascicles reached 6 μm in diameter or larger; whereas, many axons entering the distal nerve fascicles were large in diameter (Figure C.2). This suggests that axons not incorporated into the distal nerve fascicles were not able to make functional target organ connections; hence, the important result of elongation across the gap was the number of axons that actually entered the distal nerve fascicles (Table C.1).

![Graph showing the number of myelinated axons versus distance from the proximal stump](image)

**Figure C.1** Total number of myelinated axons regenerated through matrix-filled large-pore collagen tubes at three locations along the length of the nerve. Approximately 19,000 axons reached the distal end of the nerve gap; however, only a fraction of those axons (38%) entered the distal nerve fascicles. Axons which did not enter the fascicles were presumed to never reach a target. All data and conclusions presented in Chapter 3 were based on the number of myelinated axons entering the distal fascicles as the measurement at this location.
Figure C.2 Fiber diameter distributions for axons regenerated through matrix-filled large-pore collagen tube implants (LC/M) at the entrance to the distal stump (11 mm from the proximal cut) at 30 weeks. Axons that entered the distal fascicles (light bars), in general, were larger in diameter than those that did not enter the distal fascicles (dark bars).

In the tubulated groups, only 40% of axons reaching the distal stump entered the distal nerve fascicles compare with 80% in the autograft group. The large cross-sectional area of the tubes compared to the normal nerve resulted in a significant amount of newly synthesized connective tissue surrounding the distal nerve fascicles and filling the tubular implants. In contrast, the autograft tissue and the distal nerve stump initially had the same cross-sectional area and, therefore, less connective tissue was available outside the nerve fascicles for errant growth of axons. Interestingly, the elongating axons showed no preference for growing into the distal nerve fascicles rather than the surrounding connective tissue. The axon densities within the distal fascicles and within the surrounding connective tissue were not significantly different. This phenomenon has also been observed by other investigators (Sunderland, 1990).
<table>
<thead>
<tr>
<th></th>
<th>Total Number of Axons Reaching the Distal Stump</th>
<th>Number of Axons Entering the Distal Sciatic Nerve Fascicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>$7,400 \pm 500$</td>
<td>$7,400 \pm 500$</td>
</tr>
<tr>
<td>SI</td>
<td>$2,800 \pm 2,500$</td>
<td>$700 \pm 600$</td>
</tr>
<tr>
<td>SI/M</td>
<td>$15,200 \pm 1,900$</td>
<td>$5,400 \pm 300$</td>
</tr>
<tr>
<td>LC</td>
<td>$13,300 \pm 3,200$</td>
<td>$5,500 \pm 900$</td>
</tr>
<tr>
<td>LC/M</td>
<td>$18,600 \pm 1,000$</td>
<td>$7,000 \pm 800$</td>
</tr>
<tr>
<td>AG</td>
<td>$12,600 \pm 1,300$</td>
<td>$10,000 \pm 1200$</td>
</tr>
</tbody>
</table>

**Table C.1** Total number of axons reaching the distal stump and the total number of axons entering the distal nerve fascicles at 30 weeks. Note that in each implant group, the number of axons that entered the distal stump was a fraction of the number reaching the distal stump. Unfilled silicone tube (SI), matrix-filled silicone tube (SI/M), unfilled large-pore collagen tube (LC), matrix-filled large-pore collagen tube (LC/M), and autograft (AG).

These findings provide rationale for modifying the design of the implant tube. Since a large number of axons which bridged the gap did not enter the distal nerve fascicles, it may be advantageous to provide additional direction for the axons via a funnel-shaped tube, with the distal end of the tube having a diameter equivalent to the diameter of the normal nerve trunk. This design modification may eliminate the errant growth of axons and result in more efficient regeneration. However, it is possible that a funnel-shaped tube may restrict the growth of many axons prior to reaching the distal nerve stump. In addition, previous studies using silicone tubes have shown that tubes which have a diameter equal to the normal nerve trunk result in poor regeneration compared to larger tubes (1.5 times the nerve diameter) (Ducker and Hayes, 1968). However, the findings in the current study suggest that use of a large-pore collagen tube, rather than a silicone tube, may prevent the constrictive problems previously associated with silicone tubes (Chapter 4). Therefore, use of a large-pore collagen tube, with diameter equal to the normal nerve trunk, may provide more accurate direction of axons to the distal stump without the degenerative side effects. Future experimentation is necessary to determine if a funnel-shaped tube can improve regeneration.
References


