LONG TERM FUNCTIONAL AND MORPHOLOGICAL EVALUATION
OF PERIPHERAL NERVES REGENERATED
THROUGH DEGRADABLE COLLAGEN IMPLANTS

by

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ABSTRACT

Methods for engineering the regeneration of peripheral nerve in lesions have
generally focused on the implementation of tubes as implants to bridge the defect. A
previous study, in a transected rat sciatic nerve model, has shown that a highly porous
analog of the extracellular matrix was required to facilitate peripheral axon elongation
across a gap greater than 10 mm, ensheathed by a silicone tube. The current investigation
addresses the replacement of the silicone tube with a resorbable collagen device.

The collagen-glycosaminoglycan (GAG) matrix employed in this investigation was
a copolymer of type I collagen and chondroitin-6-sulfate with axially oriented pores, 5-10
μm in diameter. Six groups of rats (n=9 in each group) received tubular implants, which
were either empty or matrix-filled. Three tube types were studied: porous collagen
(maximum pore diameter, 20 nm), non porous collagen, and silicone. An additional
experimental group received an autograft (n=9), which represented the current clinical
repair technique. The sciatic nerve of adult female Lewis rats was transected and a tube
was inserted to bridge a 10-mm gap. The functional recovery of the animals was assessed
biweekly using a gait analysis technique. Additional functional tests included a muscle
mass measurement and a nociceptive test for mechanical stimulation. At 30 weeks post-
operative, the animals were sacrificed and resected nerves were prepared for quantitative
histology. Digitized nerve images, captured during light microscopy, were analyzed for
axon number and diameter distribution.

The formation of toe contractures limited the value of the gait analysis technique as
a tool for assessing function. The nociceptive test confirmed the return of some sensory
function in every implant group except the empty silicone tube. The morphological results
confirmed earlier findings that showed a significantly greater number of axons when
silicone tubes were filled with matrix. Comparable numbers of axons and large diameter
axons (≥ 6 μm) were found in the silicone and porous collagen tubes containing the
collagen-GAG matrix. In addition, the matrix-filled porous collagen tube was equivalent to
the autograft with respect to total number of axons and large diameter axons.

Results show that a resorbable porous collagen tube can be used, instead of
silicone, in the fabrication of a collagen-GAG matrix-filled device to engineer peripheral
nerve regeneration. The degradable collagen implant was also shown to be as effective as
the current clinical standard.

Thesis Supervisor: Ioannis V. Yannas
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CHAPTER 1: INTRODUCTION

1.1 STATEMENT OF PROBLEM

Peripheral nerves are the communication links that connect the brain and spinal cord to the rest of the body. Once a peripheral nerve is severed, the communication line between the brain and the tissue is interrupted, and sensory and motor function are lost. Depending on the severity of the injury, the result is partial to total paralysis in the affected tissue. Peripheral nerve injuries are the result of trauma, disease and certain surgical procedures.

Each year, there are over 200,000 cases of peripheral nerve injury which require surgical intervention. The current clinical treatments for severed peripheral nerves are dependent on the gap distance between the nerve stumps (Figure 1.1). If the two nerve stumps can be apposed, the nerve is repaired by directly suturing the two stumps back together. Clinical results show only a 25% recovery of motor function and a 3% recovery of sensory function when this technique is used. Generally, if the gap length is larger than 10 mm, the nerve ends cannot be apposed without creating tension in the nerve. Tension at the suture line leads to scar formation and poor regeneration of axons.

Therefore, for gap lengths greater than 10 mm, the current repair technique is the autograft. This procedure requires that an additional site be traumatized to remove an intact sensory nerve, which serves as the autograft tissue. Common nerves which serve as the donor site are the sural and lateral and medial antebrachial cutaneous nerves. The injured nerve is bridged by the autograft so that there is no tension in the wound site. With this type of treatment, only 20% of patients recovered motor function and none of the patients recovered sensory function. These clinical results, from the direct suture and autograft techniques, have prompted researchers to begin developing prostheses which traverse the
10 mm gap distance without requiring additional trauma and which improve the functional recovery.

1.2 NORMAL PERIPHERAL NERVE

To better understand peripheral nerve injury, it is important to have a background in the normal structure and function of peripheral nerves.

1.2.1 STRUCTURE OF NORMAL NERVE

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 nervous system. The conducting members of the peripheral nerve are axons, which are processes of the nerve cell body (neuron). The nerve cell body actually resides within the spinal cord and the process, or axon, reaches out into the periphery. Each nerve cell has one axon which travels from the cell body to the distal target organ without interruption. An axon can be as long as 1 meter in the human. The cytoplasm inside each axon is
referred to as the axoplasm and is a viscous fluid filled with microtubules and neurofilaments. These two components give the axon a roughly uniaxial structure, and are responsible for the intra-cellular transport of proteins.\(^4\)

The Schwann cell is a partner with the axon and is the primary non-conducting cell in peripheral nerves. Two types of axon are present in all peripheral nerves: myelinated and unmyelinated, differing primarily with respect to the role of the Schwann cell. In myelinated axons, the Schwann cell is associated with only one axon. The cytoplasm of the Schwann cell is wrapped around the axon to form a myelin sheath. Figure 1.2 is a schematic of a myelinated axon cut in cross section. Myelin is a protein which provides electrical insulation around each axon. The thickness of the myelin sheath varies between axons depending on the diameter of the axon. Each Schwann cell serves a length of axon approximately 1 mm long, with the nucleus residing in the center.\(^7\) The location where two Schwann cells meet along the length of the axon is a node of Ranvier. At the node, which is approximately 1 \(\mu m\) in length, the axon is uninsulated for a brief distance where

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**Figure 1.2** Schematic showing the cross section of a normal myelinated axon.
the two Schwann cells interdigitize. Figure 1.3 shows a typical myelinated axon in longitudinal section with the node of Ranvier visible. Unmyelinated axons do not have the wrapped myelin sheath and are therefore, uninsulated. One Schwann cell is associated with many unmyelinated axons (Figure 1.4).

In mature, uninjured nerves, myelinated axons tend to be larger, 1-15 μm in diameter, and are responsible for much of the motor activity of the peripheral nerves. Unmyelinated axons are much smaller, 0.5-2 μm in diameter, and conduct primarily sensory impulses. 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. In the rat sciatic nerve, there is one myelinated axon for every 1.9 - 2.5 unmyelinated axons.

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 (Figure 1.2, 1.3, & 1.4). The endoneurium is composed of

![Diagram of a myelinated axon](image.png)

**Figure 1.3** Schematic showing a longitudinal section of a normal myelinated axon.
fibroblasts and fine, loosely packed, type III collagen fibrils\textsuperscript{47,56}. The primary functions of the endoneurium are to provide packing between nerve fibers\textsuperscript{67} and support the individual axonal pathways\textsuperscript{56}. Axonal pathways range from 1 - 15 \( \mu \text{m} \) in diameter in the peripheral nerve of the rat. 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. Fascicles range in size from 0.1 - 1 mm in diameter depending on the number of fascicles at a given location. At distal locations, the fascicles organize into nerve branches and then separate from the nerve trunk\textsuperscript{65}. Each fascicle is surrounded by the second level of connective tissue, the perineurium (Figure 1.5), which is composed of three concentric layers that form a dense sheath around the fascicle. The inner two layers are composed of flattened perineurial cells and a basement membrane. The cells form tight intercellular junctions to create a single membrane. The outer layer of the perineurium has thick, type III collagen fibers and a less ordered appearance\textsuperscript{47,56}. The perineurium maintains an

\textbf{Figure 1.4} Schematic showing a cross section of a Schwann cell with its associated unmyelinated axons.
**Figure 1.5** Schematic showing the connective tissue in a nerve cross section. The scale bar refers to the fascicle sizes. The axons are not to scale (See Figures 1.2 & 1.4).

Internal pressure in the fascicle, provides strength and elasticity to the nerve, and acts as a diffusion barrier. The final level of the connective tissue is the epineurium which is a loose matrix of thick, type I collagen fiber bundles and fibroblasts (Figure 1.5). The major functions of the epineurium are to hold the fascicles together into one cohesive nerve trunk, and to provide cushioning against compressive trauma. The diameter of a typical nerve trunk is approximately 1 mm in the rat.

### 1.2.2 Function of Normal Nerve

The primary function of a peripheral nerve axon is to carry an electrical signal along its length. 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 site of the signal. This causes a local depolarization zone to form, which
propagates the signal along the axon. Once the signal, or action potential, passes, potassium ion channels open and potassium ions (K⁺) rush out to restore the local resting potential. The signal is carried along the membrane due to potential differences across it. 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 myelinated regions. The diffusion of ions occurs only at the nodes of Ranvier where there is no myelin. The action potential ‘jumps’ from one node to the next which is referred to as saltatory conduction.

The peripheral nervous system is composed of two types of nerve fibers: afferent and efferent. Afferent axons conduct electrical impulses from distal endpoints back to the nerve cell bodies within the spinal cord. Many afferent fibers are sensory and send signals to the brain regarding heat, pain, and nociception. Efferent fibers carry signals from the brain to the target organ. Efferent fibers innervate skeletal, smooth and cardiac muscle and glands.

1.2.3 LESION SITE

The anatomical site chosen most often, as well as in the experiment described here, to study peripheral nerve regeneration is the sciatic nerve. Figure 1.6 shows the anatomical location of the sciatic nerve in the hindquarters of a typical rodent. The sciatic nerve has several advantages as a lesion site. The first is that it serves an unambiguous target muscle: the plantar muscle of the foot. This muscle group is innervated only by the sciatic nerve and therefore, allows for reliable functional analysis. Secondly, the sciatic nerve is easily accessed for surgical procedures and elicits minimal trauma on the animal. The nerve is also long enough to study lesions as large as 10 mm in length.

1.3 REGENERATION MECHANISMS

When a peripheral nerve is severed, there are a variety of chemical and morphological changes within the nerve trunk as it attempts to regrow and restore
functional connections. If the cell body has not been severed, the nerve cell is still alive and viable. The cell body becomes swollen and undergoes chromatolysis, or release of the Nissl substance, which consists of large aggregates of rough endoplasmic reticulum. This process changes the priority of the cell body from production of neurotransmitters needed for synaptic activity to production of materials for axonal repair. This morphological change precedes the synthesis of growth-associated proteins (GAP’s), such as GAP-43, and cytoskeletal proteins, such as tubulin and actin, all of which are necessary for regeneration. After axotomy, the axon will degenerate proximally to at least the next node of Ranvier. This process is called traumatic degeneration and it occurs within hours.
of injury. The axonal tissue and myelin debris distal to the injury are completely phagocytosed by macrophages and Schwann cells by 4 days after injury, leaving only the endoneurial tubes in the distal stump. This process in the distal stump is known as Wallerian degeneration. Figure 1.7 is a schematic detailing the degenerative processes after an axon is severed.

Axons begin to regenerate from the proximal stump within hours of transection. A sprouting process occurs in which each axon divides into several axons that all begin to elongate. There can be many axon sprouts which are all supported by one cell body. As the axon sprouts grow, their distal tip, or growth cone, responds to haptotactic guidance clues along the endoneurial tube such as laminin and fibronectin. The growth cone also responds to chemotactic guidance from neurotrophic factors within the lesion site. Once an axon reaches a functional endpoint, the diameter of the axon will then increase and the other sprouts supported by that cell body will degenerate. The process of re-myelination seems to be coupled with the increase in diameter of the axon.

1.4 METHODS TO ENHANCE REGENERATION

In spite of all the activity within the wound site, if there is no surgical intervention, the end result of nerve transection is the formation of a neuroma. A neuroma is a bulb of unorganized axons which attempted to regrow but had no directional guidance, and therefore, formed into a tangled web. 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 and are very sensitive. Even though some axons have elongated, formation of a neuroma results in no functional connections. Because of this, many investigators are attempting to improve regeneration by introducing devices, substrate materials, growth factors and cells into the wound site.
1.4.1 ENTUBULATION OF THE NERVE STUMPS

In an effort to replace the nerve graft as a repair technique, many investigators employ a tube as their nerve regeneration device. The use of a tube has several advantages over the nerve graft technique. First, there is no donor site morbidity since no autograft
tissue is needed. The additional procedure to remove the autograft tissue is painful, creates a permanent dermal scar and leaves the patient with a loss of sensation in that location. The use of a tubular device eliminates this procedure. Second, the tube reduces the ingrowth of scar from neighboring tissues as well as limiting the amount of scar-producing fibroblasts that invade the wound site. In addition to keeping scar out of the wound site, the tube allows growth factors exuded from the damaged nerve stumps to collect within the chamber. Also Schwann cells, macrophages and perineurial cells migrate into the wound site and remain there because of the presence of the tubular device. A third advantage of tubulization is that there is less damage to the nerve stumps since the tube can be sutured into place with only one epineurial suture. This results in less traumatic degeneration at the proximal stump and is a simpler surgical procedure. The suture also prevents the rotation or displacement of the nerve stumps. Another important advantage is that the tube provides guidance for the regenerating fibers, leading them toward the distal stump. In a similar manner, the tube prevents the axons from escaping into the surrounding tissue. In addition to providing direction to the axons, the tube also appears to orient the vascular network, scar tissue and epineurium in the longitudinal direction. Finally, the tube serves as a chamber to introduce growth factors, substrate materials and cells into the wound site which may enhance the regeneration of axons through the nerve guide.

Tubulization techniques have been used for over one hundred years; however, there is still debate over the optimum tube material. The earliest tubes were based on decalcified bone and were used by Glück in 1880, without success, to repair peripheral nerves in rabbit and chicken models. Using similar tubes in 1882, Vanlair reported successful regeneration in the sciatic nerve of a dog model using a 3 cm gap. The use of many other natural and synthetic tube materials followed the success of the early tubular grafts. Naturally occurring tubes that were investigated included arteries, bone, dura, epineurium and perineurium, mesothelial tubes, and vein grafts. Non-degradable synthetic tubes
included magnesium, rayon, silicone, stainless steel and tantalum metal. Degradable tubes, both natural and synthetic, included collagen, polyester, polyglactin, and polylactate.

Selection of the optimum tube is a difficult task; however, investigators have begun to determine what requirements are necessary for a successful tube material. First, the tube must be biodegradable by the host organism. Second, the rate of resorption of the tube must be controllable such that the structural integrity of the tube remains intact until axons have elongated through it. The tube must be non-toxic and must inhibit the formation of fibrosis, neuroma, or ischemia. The tube must be flexible, suturable, and easily fabricated so that the dimensions of the tube can be varied depending on the implantation site.

Tube properties which still need optimization include rate of resorption for the tube, permeability of the tube material, and the nature of biodegradable material used. Studies comparing tube resorption rates have not been published. Most investigators focus on one resorbable tube and vary other aspects of the device, therefore, the effect of resorption rate is not well understood.

Permeability has been investigated by several researchers and differs from the classical view that the tube material should completely isolate the wound bed from the surrounding tissue. One study by Jenq and coworkers compared tubes which had 1.2 µm pores or 5.0 µm pores to impermeable silicone tubes after 8 weeks of implantation. The large pore tubes were permeable to cells and fluid and the small pore tubes were permeable to fluid only. The large pore (5.0 µm) tubes had a significantly higher percentage of successful regeneration and more axons per nerve than the small pore (1.2 µm) and impermeable tubes, which were comparable. The results of this study suggested that cells from the surrounding tissue enhance regeneration across an 8 mm gap in the rat sciatic nerve. Acrylic copolymer tubes permeable to proteins of molecular weight less than 50 kDa increased the number of myelinated axons compared to impermeable tubes after 8 weeks. The use of a selectively permeable membrane allowed passage of trophic factors from the external tissue into the wound and retention of important factors within the
wound, such as laminin which has a high molecular weight. Similarly, a collagen tube permeable to 540 kDa molecular weight proteins was as effective as the nerve graft across a 5 mm gap in the primate median nerve based on electrophysiological evaluation. These results suggest that permeable tubes enhance regeneration, however, the pore size which maximizes the response is unclear.

The resorbable material used for the tube is a major focus of current research. Biodegradable synthetic polymer tubes, including polyglycolic acid evaluated at 6 and 12 months, polylactic acid evaluated at 1, 3, and 6 months, PLLA/PCL copolymer evaluated at 18 months and polyurethane evaluated at 16 weeks, have all produced encouraging results. In particular, polyurethane tubes bridging an 8 mm gap in the rat sciatic nerve were reported to be significantly superior to the autografts histologically and the electrophysiological results were comparable. The use of naturally occurring tubes has produced mixed results. Intact femoral artery and femoral vein grafts have been used successfully at 12 weeks and 4 months respectively, however, jugular vein grafts restricted the elongation of axons due to production of extensive fibrous tissue after 3 months. Collagen nerve guides have given encouraging results in both rodent and primate models, in terms of histological and electrophysiological endpoints. The regenerative capacity of the collagen tubes was comparable to the autograft after 2 years of implantation and the direct suture repair technique after 4 years.

The geometry of the tube is also an important factor. Studies with silicone tubes showed that thinner, and thus more flexible, tubes resulted in less neuroma formation and more consistent bridging of the gap. The optimum diameter of the silicone tube was found to be 2-3 times the diameter of the nerve trunk. Smaller tubes constricted the lesion site and larger tubes led to connective tissue buildup, both of which resulted in inferior regeneration. The length of the tube is also important because if the tube was too short, the nerve stumps slipped out of the tube. Tubes that were too long resulted in thinning of the tissue cable near the midpoint suggesting inadequate blood supply.
Another important requirement for regeneration in the tubulization model is the presence of the distal nerve stump. Studies have shown that in the absence of the distal nerve stump, axons will not regenerate over a 10 mm gap length in either a silicone tube or in a degradable poly (DL-lactic acid) tube. In both instances, if the distal stump is present, axonal tissue will bridge the gap. Additional studies have investigated the possibility of using other tissue types as a substitute for the distal nerve insert. Williams, et al, found that patellar tendon and skin resulted in unsuccessful regeneration when used as distal inserts in the silicone tube. When an isolated piece of sciatic nerve was used as a distal insert, however, the regeneration was identical to that when the intact distal stump was inserted into the chamber. These results suggest that the distal stump provides essential chemical and cellular contributions to the wound site.

The mechanism of regeneration within a silicone tube nerve guide, with a 10 mm gap between nerve stumps and the distal stump intact, has been well studied. Following nerve transection and tubular repair, fluid fills the tube within one day. Within one week, a fibrin bridge completely crosses the gap and connects the proximal and distal stumps. At that time, fibroblasts and Schwann cells are found within 2 mm of the proximal and distal stumps, but not in the more central section. After two weeks, Schwann cells and fibroblasts are present in the entire 10 mm gap and unmyelinated axons begin appearing within 2 mm of the proximal stump. The axons grow at a rate of 1-2 mm/day. 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. By 4 weeks, unmyelinated axons reached the distal stump and myelinated axons were 7 mm into the wound site.

1.4.2 Use of Substrate Materials

Axonal regeneration is dependent, in part, on axonal guidance clues from the developing extracellular matrix in the wound site. Elongation of axons requires attachment
between the growth cone of the axon and a substrate material\textsuperscript{24}. Weiss concluded that the tubulization technique was successful, in large part, because it led to the formation of a longitudinal fibrin matrix which served as a scaffold for the migration of Schwann cells and axons into the wound bed\textsuperscript{74}. Many investigators have studied, with some success, the effect of implanted substrate materials on peripheral nerve regeneration. The substrates are usually delivered inside a tubular prosthesis and are used to bridge or fill the gap between the two nerve stumps. Examples of substrates used include the extracellular matrix proteins laminin, collagen and fibronectin.

Using a biodegradable tube (poly-D, L-lactate) filled with a laminin-containing gel, Madison, et al\textsuperscript{49}, showed that the laminin-containing gel significantly increased the rate of axonal elongation over tubes which were implanted empty. By two weeks, the filled tubes had regenerated nerve cables in the midportion of a 5 mm gap, while the empty tubes displayed no tissue. In addition to laminin, the gel contained type IV collagen and heparan sulfate which are other extracellular matrix components (Matrigel, Collaborative Research)\textsuperscript{49}. In a study using silicone tubes, the presence of a laminin-containing gel (Matrigel) or a collagen matrix (Vitrogen) significantly increased the maximum gap distance across which axons could regenerate\textsuperscript{48}. Rosen, et al, compared a polyglycolic acid tube filled with a type I collagen gel to the autograft in a 5 mm gap in the rat peroneal nerve after 1 year. The performance of the collagen gel implant was equal to the autograft as measured by axon counts and functional techniques; however, the autograft had larger axon diameters\textsuperscript{55}. A saline solution of fibronectin and laminin inside a silicone tube was also shown to enhance the number of myelinated fibers regenerating across a 5 mm gap in the rat sciatic nerve after 4 months\textsuperscript{6}. In contrast to these results, collagen (Vitrogen) and laminin (Matrigel) gels were found to impede the regeneration of axons after 12 weeks within semipermeable tubes (polyvinylchloride acrylic copolymer) across a 4 mm gap in the sciatic nerve of mice\textsuperscript{70}. These results indicate that the substrate performance is dependent
on the tube material. However, the great diversity of materials used to fabricate these prostheses has not led to simple rules for the design of a superior prosthesis.

1.4.3 ANALOG OF THE EXTRACELLULAR MATRIX

In an attempt to improve the healing response of dermal tissue, Yannas and coworkers developed an analog of the extracellular matrix (AECM) which was found to reduce the amount of contractile scarring and to regenerate near physiological dermis. The AECM was a highly porous copolymer of type I bovine hide collagen and chondroitin-6-sulfate. The activity of the AECM was dependent on the pore characteristics, degradation rate, and the density of epidermal cells seeded into the matrix. The optimum pores for the skin regeneration template were randomly oriented and between 20 μm and 125 μm in diameter. A maximum degradation rate for regeneration was defined as 140 enzyme units based on \textit{in vitro } degradation studies with bacterial collagenase. Matrices with faster degradation rates resulted in inferior results. A cell density of $5 \times 10^4$ cells/cm$^2$ was determined to be the minimum required for dermal regeneration$^{80}$.

The mechanism by which the matrix template suppresses the contractile response and leads to the production of near normal tissue is not well understood. A complex interaction occurs between the ECM analog and the cells and growth factors within the wound bed. Current studies are focusing on the ability of the matrix to inactivate cytokines which may normally lead to the formation of scar.

Success in the dermal model led to use of the matrix in regenerating other connective tissues. In a pilot experiment, the matrix, ensheathed in a silicone tube, was implanted in the rat sciatic nerve using a 15 mm gap length. A silicone tube implanted empty was used as a control. In the presence of the collagen-GAG matrix, both myelinated and unmyelinated axons traversed the gap as well as many blood vessels. In the empty tubes, there were essentially no axons or blood vessels within the gap. These results
suggested that the skin regeneration template could enhance regeneration in peripheral nerves\textsuperscript{81}.

Since the pore characteristics, degradation rate and cell density were crucial to performance of the skin matrix, an optimization study was conducted to determine the ideal parameters for the peripheral nerve matrix. Using electrophysiological measurements to assess the extent of regeneration, a family of matrices, ensheathed in silicone tubes, were tested in a 10 mm gap in the rat sciatic nerve. The structure of the matrices varied in three ways: pore orientation, pore diameter, and degradation rate. Cells were not used in the study. Matrices which had axially oriented pores resulted in successful regeneration 98% of the time, whereas, matrices which had radially oriented pores regenerated successfully only 50% of the time\textsuperscript{14}. Four different pore diameters were studied ranging from 5 µm to 300 µm. Electrophysiological results indicated that the small pore matrices (5 µm and 10 µm pores) were significantly superior to the large pore matrices (60 µm and 300 µm pores) with respect to distal motor latency, compound muscle action potential, and conduction velocity\textsuperscript{14}. Two variations of the degradation rate were studied. Rapidly degrading matrices had a molecular weight between crosslinks ($M_c$) of 60 kDa, and were untreated with regard to crosslinking. The slowly degrading matrices were glutaraldehyde crosslinked for 24 hours and had an $M_c$ of 12 kDa. The rapidly degrading matrices were significantly better than the slowly degrading matrices based on electrophysiological results. The slowly degrading matrix may be inferior because it physically impedes regeneration\textsuperscript{14}. These results suggest that an optimum nerve regeneration template which is rapidly degrading, and has axially oriented pores, 5 - 10 µm in diameter, enhances regeneration across a 10 mm gap.

1.5 Evaluation Techniques

An important aspect of a study of peripheral nerve regeneration is the choice of evaluation techniques. In this area of research, there is no definitive set of evaluation
methods that is accepted; therefore, there are many techniques that can be employed. Generally, a combination of techniques is the most effective in giving an overall assessment of regeneration. Most evaluation techniques fall into two categories: functional and morphological.

1.5.1 Functional

The value of a functional technique is that it gives information on the performance of the regenerated nerve cable. Electrophysiological methods are used to analyze the bulk capacity of the nerve to transmit a signal. Common parameters which are measured include nerve conduction velocity, amplitude, compound nerve action potentials, and compound muscle action potentials. Conduction velocity, amplitude and compound nerve action potentials give information about the ability of the nerve to conduct a signal, but this information does not translate directly to functional recovery. Inaccurate axonal connections give encouraging electrophysiological results, but do not result in functional recovery. Compound muscle action potentials measure motor function directly; however, they are susceptible to measurement errors\textsuperscript{25}.

In an effort to measure motor function directly, de Medinaceli developed an index based on measurements made from walking tracks. The technique involved recording footprints and comparing the experimental and the normal footprints based on simple linear distance measurements. The measurements were then combined by a weighted equation into a single index value between 0 and -100, where 0 is normal function and -100 is complete functional deficit. Initial experiments showed that after both crush and transection, the sciatic functional index (SFI) was -100 at 1 week post-operative. The animals that received the crush lesion recovered to normal SFI values by 25 weeks; however, the animals that received the transection lesion never recovered any function based on the SFI\textsuperscript{18}. Additional studies modified the weighted equation based on an extensive statistical analysis\textsuperscript{7}. Although this method is simple and reliable, it cannot be
used in the presence of any foot deformities such as chronic contractures and heel ulcerations\textsuperscript{17}. This technique provides an easy method of directly assessing motor functional recovery.

Sensory evaluation techniques are more subjective and less quantitative; and therefore, more difficult to interpret. Common sensory tests include response to painful stimuli and return of reflexes\textsuperscript{25}. Frequent handling of the animals has been shown to reduce the number of false positive responses by familiarizing the animal with the investigator and the test environment\textsuperscript{66}. Direct measurement of sensory response gives important information on nerve regeneration; however, the measurements must be taken carefully and the results interpreted with possible errors in mind.

1.5.2 Morphological

Morphological techniques evaluate the structural recovery of the peripheral nerve. The most common light microscopy techniques quantify the fascicle area, the total number of myelinated axons per cross section, and histograms of myelinated fiber size. These methods are time consuming, however, they give accurate assessments of the nerve structure\textsuperscript{25}. Using electron microscopy, unmyelinated axon counts and myelin thickness measurements can be made using careful sampling techniques\textsuperscript{24,25}. Although these techniques do not give direct information regarding functional return, they give objective, numerical assessment of the quality of regeneration and some morphological aspects can be correlated with functional tests.

1.6 Contractile Cells in Wound Healing

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. The myofibroblast is a modified fibroblast which is capable of generating force. The myofibroblast derived its name because it has the phenotypic characteristics of both the
fibroblast and the smooth muscle cell. The myofibroblast 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. All cells contain the β and γ isoforms of actin for cell motility; however, the α isoform is specific only to contractile cells. Like fibroblasts, myofibroblasts have a well developed rough endoplasmic reticulum and Golgi apparatus for protein synthesis. Additional ultrastructural features of the myofibroblast are pinocytotic vesicles and indented nuclei.

Myofibroblast contribution to normal healing has been well studied in wound closure of the dermis. The appearance of the myofibroblast coincides with the onset of contraction in 25 cm² area dermal wounds, with the population peaking at 3 weeks. After 8 weeks, the rate of contraction and myofibroblast content both decrease. By 16 weeks, no myofibroblasts are visible in the woundbed and contraction has ceased. It is unknown whether the cells originate from previous fibroblasts in the wound or from stem cells.

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. 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 and coworkers found that a collagen-GAG copolymer can delay and even eliminate wound contraction in the dermal model.

Myofibroblasts have been found to play a role in the healing of other connective tissues and in selected pathological conditions. In ligament, myofibroblasts have been found to re-establish in situ strain after transection injury. The mechanism by which this occurs is unclear; however, it could be a process termed ‘tractional structuring’ in which fibroblasts deposit matrix and then pull it along with them as they migrate. This process helps align the tissue and may contribute to the tensioning of the matrix during the healing process.
development. Pathological conditions in which myofibroblasts play a role include: Dupuytren's contracture, chronic alcoholic cirrhosis of the liver, hypertrophic scars, and the capsules that form around blood clots and silicone breast implants.

Contractile cells have not been identified in healing peripheral nerve; however, there is in vitro evidence that the application of mechanical force may play a role in axonal elongation. Dennerll and coworkers studied chick dorsal root ganglion neurons in vitro. Tension was applied to the neurons by a needle and in response, the neurons were shown to elongate. Within a specific tension range, the neurons behaved as viscoelastic solids. Above the threshold value, the axon elongated in addition to the viscoelastic behavior. The elongation occurred with no change in axon diameter, indicating axon growth. Similarly, if tension decreased below the lower threshold, the axon retracted. This suggested that elongation is a mechanically regulated response in developing axons. One in vivo theory, is that axonal elongation is caused by the tension of a migrating cell. This process is termed ‘towed growth’ and is similar in mechanism to the ‘tractional structuring’ described previously. The ‘towed growth’ theory was tested and confirmed in vitro by using tension to elongate axons whose ultrastructure remained normal.

1.7 PROJECT GOAL

The goal of this project was to evaluate the long term performance of a family of peripheral nerve devices based on functional and morphological evaluation techniques and rank order the devices according to performance. The current clinical repair technique, the autograft, was also evaluated and compared to the devices. The family of devices included: porous collagen, non porous collagen, and silicone tubes each implanted empty and filled with a collagen-GAG matrix. The manufacture of the collagen-GAG matrix was done according to existing protocols to obtain axially oriented, 5 - 10 μm diameter pores. This matrix structure was determined to be superior in previous studies. The long term goal was to replace the silicone tube with a resorbable, collagen tube, creating an entirely
degradable device. Two geometries of collagen tube were evaluated to begin determining optimum porosity and degradation characteristics. The devices were implanted in the rat sciatic nerve with a gap between nerve stumps of 10 mm. Functional recovery was evaluated biweekly using footprint analysis and once prior to sacrifice using a mechanical stimulation sensory test. Quantification of myelinated axon diameters and total number of myelinated axons per nerve provided the morphological evaluation at 30 weeks post implantation. Six week tissue sections were studied to determine if myofibroblasts play a role in peripheral nerve wound healing. Since tensile forces regulate the elongation of axons \textit{in vitro}, and the myofibroblast is a force generating cell, the myofibroblast could play the towing role in axonal elongation after injury. This provided rationale for studying myofibroblasts in peripheral nerve healing.
CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMAL MODEL

The animal model chosen for this experiment was the adult female Lewis rat. The Lewis strain of rat has shown resistance to autotomy, or self-mutilation, following sciatic nerve transection. A study conducted by Carr, et al\textsuperscript{12}, compared the appearance of autotomy in several strains of rat. The Lewis strain was the only strain which did not exhibit any degree of autotomy following both nerve transection with direct suture repair and transection with no repair. In contrast, the Carr study showed that the Sprague-Dawley strain, which was used previously in this laboratory\textsuperscript{15,35}, exhibited autotomous behavior in 71\% of the animals receiving nerve transection and direct suture repair. The Sprague-Dawley rats showed autotomy 100\% of the time when the nerve was transected and not repaired\textsuperscript{12}. Autotomy is a severe problem in peripheral nerve injury studies because it exposes the animal to infection and pain\textsuperscript{72}. In the current experiment, autotomy was additionally important because the gait analysis technique required that all toes remain intact for accurate footprint analysis. Using Sprague-Dawley rats, in the context of one study that used gait analysis, 56\% of the animals had to be sacrificed because the footprints were immeasurable due to autotomy\textsuperscript{73}. In an attempt to reduce autotomy, the Lewis strain of rat was chosen for this study.

The surgical lesion was a 10 mm gap in the sciatic nerve. This lesion is commonly used in peripheral nerve regeneration studies. Lundborg and coworkers found that the transected sciatic nerve regenerated across this gap distance when implanted with an empty silicone tube. Larger gaps, 15 mm and 20 mm, did not lead to axonal tissue crossing the gap in the silicone tube model\textsuperscript{44}. These results prompted investigators to study the 10 mm gap length extensively; therefore, there is a pool of knowledge regarding this particular lesion. In addition, larger gap lengths, such as 15 mm, cannot be accommodated in the
femoral region of the rat. Larger gaps require a cross anastomosis procedure to be performed in which the proximal portion of the right sciatic nerve is connected, with a tubular implant, across the back of the animal to the distal portion of the left sciatic nerve\textsuperscript{81}. This procedure results in confusing functional results and injury of both sciatic nerves. Because of these reasons, a 10 mm gap was chosen as the lesion. The gap was surgically generated in the sciatic nerve and an appropriate prosthesis placed within the gap.

The motivation for a long term study was to evaluate functional recovery over time, until it reached a plateau value. Electrophysiological evaluation of silicone tubes filled with collagen-GAG matrix (10 μm pore diameter, uncrosslinked matrix) showed a plateau in distal motor latency by 22 weeks, and in conduction velocity by 25 weeks\textsuperscript{15}. In addition, one long term study that used the gait analysis technique showed a plateau in sciatic functional index by 20 weeks following direct suture repair\textsuperscript{32}. The length chosen for this study was 30 weeks to ensure that a plateau could be reached and established.

Several experimental groups were evaluated, including four test groups and five control groups. The experimental grid is shown in Table 2.1. The test groups included porous collagen (PC) and non porous collagen (NPC) tubes, implanted both empty (e) and filled with a collagen-GAG matrix (m). The empty silicone tube (Se) was a control group which allowed comparison to other investigators and served as the current “best” tubular prosthesis. Similarly, the matrix filled silicone tube (Sm) allowed comparison to previous electrophysiological results\textsuperscript{15,51}. The autograft (AG) was evaluated as a positive control since it is the current clinical repair technique for gaps exceeding about 10 mm. A sham operation (sham) was performed in which the sciatic nerve was exposed but not damaged. The sham operation involved only muscular damage and this group was intended to evaluate effects of anesthetic and muscle trauma. The inhibited repair (IR) group was implanted with a special prosthesis designed to completely block regeneration. This group was expected to establish zero values for both functional and morphological recovery; and therefore, served as a negative control group.
To determine the number of animals in each group, a preliminary statistical analysis was performed. Using histological standard deviations from a previous study and estimated mean differences, a student’s t-test was performed to find the minimum number of animals necessary to achieve statistical significance (95% confidence). A similar calculation was made for the sciatic functional index using standard deviations from another long term study and estimated mean differences. Five animals were determined to be sufficient to distinguish between two experimental groups; however, the experiment was planned to account for animal death during the 30 week period and the possibility of smaller differences between the means (See Appendix B for sample calculation). In each of the tubular groups and the autograft, there were nine animals in each group, with the intent of collecting sciatic functional index and histological data from at least six animals. The sham (n = 5) and inhibited repair groups (n = 3) had less animals because the standard deviations were expected to be smaller (Table 2.1).

<table>
<thead>
<tr>
<th></th>
<th>Empty (Sample size, n)</th>
<th>Matrix-Filled (Sample size, n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test Groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Porous Collagen Tube</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Porous Collagen Tube</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td><strong>Control Groups</strong></td>
<td></td>
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</tr>
<tr>
<td>Silicone Tube</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Autograft</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Sham Operation</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Inhibited Repair</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1** Experimental grid showing the sample size for the test and control groups.
2.2 PERIPHERAL NERVE PROSTHESIS MANUFACTURING

The manufacturing process for the peripheral nerve prostheses involved several steps, including preparation of the collagen-glycosaminoglycan slurry, matrix production, and assembly of the final prostheses. Each step has been performed according to previously described protocols designed to produce the optimum matrix characteristics for regeneration in the rat sciatic nerve model.\(^{15,35,42}\)

2.2.1 PREPARATION OF THE COLLAGEN-GAG SLURRY

Preparation of the collagen-glycosaminoglycan (GAG) dispersion was the first step in manufacturing the nerve prostheses. Fibrous, type I bovine hide collagen (USDA, Philadelphia, PA) was ground in a liquid nitrogen cooled Wiley Mill (A.J. Thomas Co., Philadelphia, PA) with a 20 mesh screen. Using an analytical balance, 1.65 grams of collagen was measured and placed in a cooled (4°C) blender (Granco overhead blender, Granco Co., Kansas City, MO; Brinkman Cooler Model RC-2T, Brinkman Co., Westbury, NY) with 600 milliliters of 0.05 M acetic acid. After one hour of blending on the high speed setting, 120 ml of 0.11% w/v chondroitin-6-sulfate from shark cartilage (Sigma Cat# C-4384, Sigma Chemical Co., St. Louis, MO) was added dropwise to the blending dispersion over 15 minutes using a peristaltic pump (Manostat Cassette Pump, Manostat, NY, NY). The solution was blended for an additional 15 minutes, and then centrifuged (Model #CRU-5000, Damon International Equipment Co., Needham Heights, MA) at 2300 rpm for one hour under cooled conditions (4°C). A portion of the clear supernatant, 420 ml, was discarded and the remaining slurry was blended for 15 minutes on the low speed setting.

2.2.2 COLLAGEN-GAG MATRIX PRODUCTION

Prior to matrix production, silicone tubes (Silastic, Dow-Corning Co., Midland, MI), 1.5 mm inside diameter, were prepared by sealing one end of each 15 cm length with
silicone adhesive (Silastic, Dow-Corning Co., Midland, MI). Flexible PVC tubing, 0.125 inch inside diameter, was cut into 12 cm sections and vented with small holes spaced 1 cm apart for the length of the tube. One sealed silicone tube was inserted into each prepared PVC jacket. The collagen-GAG (CG) slurry was deaerated at -30mmHg with agitation for 10 minutes or until large bubbles were no longer visible. The slurry was drawn into a 10 cc syringe (Model #5604, Becton Dickinson Co., Rutherford, NH) and any air bubbles were expelled. A 25 gage needle (Model #25G1/2, Becton Dickinson Co., Rutherford, NH) was attached to the syringe and inserted into the plugged end of the silicone tube. The CG suspension was injected into the tube until a few drops emerged and then the free end was sealed with a tapered plug (pipette tip). Additional slurry was injected until the silicone tube became pressurized and completely filled the inside diameter of the PVC jacket. Once pressurized, the needle was removed and the PVC jacket simultaneously sealed using a tapered plug.

The prepared PVC jacket was then lowered, with its long axis in vertical position, into a -80°C freezing bath at $10^{-4}$ m/s until the tube was fully immersed in the bath. Figure 2.1(a) shows the freezing orientation of the final manufacturing assembly. A detailed schematic of the freezing apparatus (Loree 2000, MIT, Cambridge, MA) is shown in Figure 2.2. The apparatus consisted of a liquid nitrogen controlled cooling system and a gear train arrangement that allowed for variable lowering velocities. The heat transfer fluid inside the bath was silicone (Syltherm XLT Heat Transfer Liquid, Dow Corning, Midland, MI). The freezing parameters chosen were based on the conditions which produced the optimum characteristics of the matrix\textsuperscript{15,35,42}. The freezing temperature, -80°C, controlled the pore size of the CG matrix. Colder temperatures created smaller pore sizes and therefore a larger matrix surface area. Small pores, of 5-10 μm in diameter, were determined to be optimum for axonal regeneration based on electrophysiological data\textsuperscript{15,51}. This pore size was achieved by using a bath temperature of -80°C. Figure 2.3 shows a scanning electron micrograph of the CG matrix. Another parameter controlled by the
freezing conditions, was pore orientation. Quenching of the tube in the freezing bath created radial pores, which were not conducive to axon growth. Lowering the tubes into the bath slowly, at $10^{-4}$ m/s, caused dendritic growth in the axial direction. A detailed heat transfer analysis was performed to understand the freezing mechanics. Sublimation of the ice crystals left axially oriented pores which were optimum for axonal elongation.

Once the slurry-filled tubes were completely immersed, they were removed from the bath and the plugged ends cut off. This step exposed the ends of the tube to the freeze drier environment and allowed the ice to sublime out. The tubes were placed on their side on a cooled tray as shown in Figure 2.1(b). The tray was then placed in a -20 °C freeze drier (VirTis Genesis, VirTis, Gardiner, NY) chamber. Once the condenser reached -45°C, the chamber door was sealed and the vacuum pump was activated. After the chamber

![Figure 2.1](image-url)
reached a vacuum of 100 mTorr, the temperature was raised to 0°C. The tubes were left in
the freeze drier at 0°C and 100 mTorr of vacuum for 24 hours. The chamber temperature
was then raised to 25°C and the matrices were removed. The matrices were transferred to
foil packets and placed in a 105°C oven under vacuum for 24 hours (Fisher Isotemp
Vacuum Oven, Fisher Scientific, Boston, MA; VacTorr 150 Vacuum Pump,
GCA/Precision Scientific, Chicago, IL). This process dehydrothermally treated (DHT) the
matrix and served as a method of crosslinking and sterilization\(^2\). The packets were
removed from the oven and immediately sealed.

**FIGURE 2.2** Loree 2000 freezing apparatus\(^{42}\).
FIGURE 2.3 Scanning electron micrograph (SEM) of the collagen-GAG matrix for peripheral nerve. Scale bar = 10 μm.

2.2.3 PROSTHESIS TUBES

Two different geometries of collagen tubes were tested in this study: porous and non porous. The collagen tubes were obtained from Dr. Surendra Batra (Integra Life Sciences, Plainsboro, NJ). Both tube types were made from type I tendon collagen and had an inside diameter of 1.5 mm. The porous collagen tubes (Integra Life Sciences, Plainsboro, NJ) had an outer diameter of 3.0 mm and a wall thickness of 0.75 mm. Figure 2.4(a) is a detailed schematic of the tube dimensions. To control the in vivo rate of resorption, the porous collagen tubes were crosslinked by gaseous formaldehyde treatment. The maximum pore diameter of the porous collagen tubes was 215 Å, which corresponds to a protein of molecular weight 540 kDa. The tubes were freely permeable to proteins of molecular weight 68 kDa (pore size = 68 Å). Proteins which were shown to diffuse through the porous collagen tube included bovine serum albumin, β-galactosidase, glucose and myoglobin. The non porous collagen tubes (Integra Life Sciences, Plainsboro, NJ) had an outer diameter of 1.8 mm, a wall thickness of 0.15 mm (Figure
All dimensions are given in millimeters.

**Figure 2.4** Detailed schematic of the tube dimensions. For reference, the diameter of a typical nerve trunk is 1 mm. (a) Porous Collagen tube (PC) (b) Non Porous Collagen tube (NPC) (c) Silicone tube (S). Scale as shown.

2.4(b)), and were not crosslinked\textsuperscript{35}. The non porous tubes were not completely impermeable, but had a pore diameter of less than 38 Å. The tubes were freely permeable to small proteins, like glucose, with a molecular weight of 180 kDa (pore size = 7 Å). Larger proteins did not diffuse through the tubes\textsuperscript{38}. The silicone tubes (Silastic Medical Grade Tubing, Cat# 602-235, Dow-Corning Co., Midland, MI) had an inside diameter of 0.058 inches (1.47 mm) and an outside diameter of 0.077 inches (1.96 mm), leaving a wall thickness of 0.019 inches (0.25 mm) (Figure 2.4(c)). The tubes were flexible and were considered impermeable except to certain gas molecules.

### 2.2.4 Prosthesis Assembly

For all tubular prostheses, the tubes, both collagen and silicone, were sterilized by dehydrothermal treatment at 105°C for 24 hours under vacuum. Empty tubular prostheses were prepared by simply cutting the tube with a scalpel to a length of 20 mm. For a matrix filled prosthesis, the tube was prepared in the same manner. To remove the matrix, the
silicone processing tube was carefully cut, using a scalpel, in the axial direction and the matrix was removed with forceps through the opening. The matrix was handled gently in order to preserve the pore structure. A 10 mm length of matrix was cut using a scalpel blade and inserted into the center of the prosthesis tube with forceps, leaving 5 mm on each end. Figure 2.5 is a diagram of the assembly procedure. Once assembled, the matrix fit snugly inside the tube, leaving no gap between the tube and the matrix. The matrix filled the entire lumen of the tube and was continuos throughout the tube.

The inhibited repair prosthesis was prepared by plugging one end of a silicone tube with Silastic adhesive (Medical Grade Silastic, Dow-Corning Co., Midland, MI). The adhesive was allowed to cure for 24 hours. The tubes were trimmed to a length of 15 mm which was defined from the free tube edge to the proximal edge of the silicone plug. For all prostheses, the entire assembly procedure was performed under sterile conditions in a laminar flow bench (Relialab, Tenney Engineering Inc., Union, NJ) using sterile gloves, mask, cap, and gown. After assembly, each implant was placed in a sterile specimen jar filled with phosphate buffered saline (Sigma Cat #P-3813, Sigma Chemical Company, St. Louis, MO).

2.3 SURGICAL IMPLANTATION AND ANIMAL CARE

Adult female Lewis rats (Charles River Laboratories), 175-200 grams, were housed at the Thorn building animal facility, Brigham & Women’s Hospital, Boston, MA. The animals were housed for one week prior to surgery to reduce the stress placed on the animal during travel. The surgical procedure was done in the Thorn building animal facility and was performed by Dr. Hu-Ping Hsu, an orthopedic surgeon who had prior experience with this procedure. The animal was anesthetized using an intraperitoneal injection of sodium pentobarbital (Nembutal Sodium Solution, 50mg/ml) with the dosage of 50 mg of solution per kg of animal. 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
Matrix Preparation

1M

Dehydrothermally Treated (DHT)
at 105°C for 24 hours

Collagen-GAG Matrix Silicone Processing Tube

Silicone Processing Tube was Cut with Scalpel

2M

Tube cut line

Matrix was Removed from Processing Tube with Forceps

3M

Silicone Processing Tube (discarded at this point)

Matrix was Trimmed with Scalpel to 10 mm

4M

Matrix Cut Line

Final Assembly

Matrix was Inserted into Tube with Forceps

1T

Dehydrothermally Treated (DHT)
at 105°C for 24 hours

Prosthesis Tube (Collagen or Silicone)

Tube was Trimmed with Scalpel to 20 mm

2T

20 mm Tube Cut Line

Matrix was Trimmed with Scalpel to 20 mm

FIGURE 2.5 Diagram of the prosthesis assembly procedure for a matrix filled tube.
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 subcutaneous tissue was cut away to release the underlying muscle tissue from the skin. The superficial *biceps femoris* was separated carefully using surgical scissors until the sciatic nerve was visible. Once the nerve was visible, the muscle was cut along the incision line, being careful not to disturb or damage the sciatic nerve. Retractors were placed inside the muscle to separate the wound edges. The nerve was now easily visible in the intramuscular septum between the deep *biceps femoris* and the *semimembranous* muscles. Figure 2.6 shows a schematic of the sciatic nerve and the surrounding muscles. The sciatic

![Diagram of the sciatic nerve and surrounding muscles](image)

**Figure 2.6** Anatomical schematic of the musculature of the rat hind leg.
nerve was 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, the nerve was bluntly transected midway between the proximal nerve trunk and the distal bifurcation using microscissors. The tubular prosthesis, either empty or filled with the collagen-GAG matrix, was measured and a mark placed 5 mm from each end of the tube. The tube was placed in the gap and the proximal and distal nerve stumps inserted 5 mm into each end, leaving a 10 mm gap. The error in the gap length was considered 1 mm or less. The nerve was secured in place using two 10-0 sutures (Ethicon) at each end. The sutures went through the epineurium and the tube and were tied with four single knots. Figure 2.7 shows a diagram of an empty and a matrix filled tubular prosthesis following implantation.

The inhibited repair prosthesis was surgically implanted using the basic procedure for a tubular implant, except only the proximal nerve stump was placed inside the tube. The distal stump was left resting on the underlying tissue. Figure 2.8 shows a schematic of the implanted inhibited repair prosthesis.

![Diagram showing an empty prosthesis tube (top) and a collagen-GAG matrix filled tube (bottom) after surgical implantation in the rat sciatic nerve.](image-url)
For an autograft, a 10 mm segment in the middle of the nerve was marked using a sterile pen. At the proximal end, 10-0 pre-sutures were placed on either side of the mark. The nerve was transected at the marked point using microscissors being careful not to cut through the pre-sutures. Once transected, the pre-sutures were pulled tightly and tied using four single knots. An additional 10-0 suture was placed at the proximal end and the entire procedure repeated for the distal marked point. This procedure created the least amount of rotation of the nerve so that the fascicles remain aligned. In contrast, many investigators use an autograft technique where the autograft tissue is rotated 180° and then sutured into place. Figure 2.9 shows the autograft configuration. For a sham operation, the nerve was left undamaged and the wound closed as follows.

Following implantation of the prosthesis or termination of the surgical treatment at the nerve site, the retractors were removed and the *biceps femoris* muscle closed using three 4-0 sutures (Ethicon). The skin was closed using two 4-0 sutures and three skin
Table 2.2 Table showing autotomy grading scale and the subsequent action taken.

<table>
<thead>
<tr>
<th>Scale</th>
<th>Translation</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No Autotomy</td>
<td>Monitor Daily</td>
</tr>
<tr>
<td>1</td>
<td>Autotomy of 1 toe</td>
<td>Monitor Twice Daily</td>
</tr>
<tr>
<td>2</td>
<td>Autotomy of &gt; 1 toe</td>
<td>Monitor 3-4 Times Daily</td>
</tr>
<tr>
<td>3</td>
<td>Autotomy of &gt; 3 toes</td>
<td>Sacrifice Animal</td>
</tr>
</tbody>
</table>

staples. Additional sutures were used if necessary. A numbered, metal ear tag was placed in the left ear of each rat for identification purposes. A small incision was made in the ear, using a scalpel, and the tag was inserted and secured. The tag number was recorded along with an animal number, the implant type and the pre-operative weight. One animal in each cage was marked with a triangular notch in the right ear for further identification. A sharp punching tool was used to create the notches while the animal was still anesthetized (Ear Punch, Cat #52-4645, Harvard Bioscience, South Natick, MA). The animals were placed back in their cages and monitored until they were 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 the appearance of any abnormal behavior, such as insufficient grooming, lack of appetite and aggressive behavior. In addition, the animals were checked for the appearance of autotomy and were treated based on the criteria shown in Table 2.2.

2.4 FUNCTIONAL EVALUATION METHODS

2.4.1 GAIT ANALYSIS TECHNIQUE

The gait analysis technique involved the measurement of footprint parameters from a walking track obtained for each animal at several time points. The walking tracks were obtained using the following simple procedure¹⁸. The animal was held by the investigator and its feet were dipped in water, being careful to only wet the hind feet. The rat was then
allowed to walk down a confined corridor (3" wide by 17" long) on a special pH sensitive paper toward a dark box. Each animal was trained to walk in the corridor pre-operatively, providing a normal functional value. At first, the animals investigated the corridor, but soon learned to run straight into the dark box. The animals were walked pre-operatively, and at weeks 1, 2, and every other week until 30 weeks.

The pH sensitive paper was prepared by dipping plain white Xerox paper in a 0.5% bromphenol blue solution (Sigma Cat #B-0126, Sigma Chemical Co., St. Louis, MO) in absolute acetone. The paper turned an orange/yellow color after it dried and was stored in tightly sealed bags to protect against humidity discoloration. When the paper came into contact with water, it turned a distinct blue creating blue footprints on a yellow background which were easy to identify.

Three footprint parameters were measured for analysis: print length (PL), toe spread (TS), and intermediate toe spread (IT) (Figure 2.10). The print length was measured as the linear distance between the third toe and the heel. Toe spread was defined as the linear distance between the first and fifth toes, and intermediate toe spread as the linear

![Image](image.png)

**Figure 2.10** Photograph of a normal rat footpad. The print length (PL), toe spread (TS), and intermediate toe spread (IT) are defined on the foot.
distance between the second and fourth toes. Each parameter was measured for the normal foot (Figure 2.11(a)) and the experimental foot (Figure 2.11(b)). A common deformity after sciatic nerve injury was the toe contracture, where the toes curl under the foot and become locked in that position\textsuperscript{17}. In the presence of a toe contracture, the print length could not be measured in the traditional manner. A method was developed to continue measuring these prints by measuring the partial print length, \(L\), from the heel to the proximal knuckle on the print. Since the toes were folded over, the length from the proximal knuckle to the end of the toe, \(\Delta L\), was also measured. By adding \(L\) and \(\Delta L\) a print length for contracture animals was calculated. The toe spread and intermediate toe spread were measured as usual. An example of a footprint resulting from a contracture is shown in Figure 2.11(c).

Each walking track was labeled with only the animal’s number to ensure that the measurements were not biased. Each measurement was made three times by a single observer and the average was used in all future calculations. Prior to making any

\[ \text{Figure 2.11} \quad \text{(a) Normal footprint (N) with the three footprint parameters, print length (NPL), toe spread (NTS) and intermediate toe spread (NIT), labeled as they would be measured. (b) Experimental footprint (E) with EPL, ETS and EIT labeled. (c) Experimental footprint with toe contracture. The measurements L and \(\Delta L\) are labeled. The sum of the two define the print length (EPL). Footprints are actual size and to scale.} \]
measurements, the observer became familiar and consistent with the measurement technique by using a self-evaluation test.\(^{10}\)

Once the footprints were measured, the parameters were used to determine a 'sciatic functional index' (SFI).\(^{18}\) Three factors were calculated by normalizing the footprint measurements: print length factor (PLF), toe spread factor (TSF), and intermediate toe spread factor (ITF). The print length factor was defined as

\[
PLF = \frac{EPL - NPL}{NPL} \quad (1)
\]

where EPL is the experimental print length (measured with or without contracture) and NPL is the normal print length. The toe spread factor was defined as

\[
TSF = \frac{ETS - NTS}{NTS} \quad (2)
\]

where ETS is the experimental toe spread and NTS is the normal toe spread. The intermediate toe spread factor was defined as

\[
ITF = \frac{EIT - NIT}{NIT} \quad (3)
\]

where EIT is the experimental intermediate toe spread and NIT is the normal intermediate toe spread. Combining the three factors into a weighted equation generates an overall functional index. A modified sciatic functional index equation was generated using statistical analysis and is now the most commonly used.\(^{7}\) The modified equation for the sciatic functional index is

\[
SFI = -38.3 \text{ (PLF)} + 109.5 \text{ (TSF)} + 13.3 \text{ (ITF)} - 8.8 \quad (4)
\]

where the PLF, TSF, and ITF are as defined in equations 1-3. The SFI was weighted so that normal function scores approximately -10 and no function scores approximately -110.
2.4.2 Other Performance Analysis Techniques

A nociceptive test was performed at 30 weeks to assess the functional recovery of deep and cutaneous pain receptors using mechanical stimulation. This test gave information regarding the degree of regeneration of sensory axons. 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. For each animal, both the normal and implanted legs were tested. The normal foot served as an internal control for the type of response each animal gives to painful stimuli. 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 skin on the lateral toe was tested for a cutaneous pain response.

An evaluation of muscle mass was made at 30 weeks using a circumferential measurement technique. Studies have shown that muscle tone correlates well with electrophysiological results. This technique was intended to quantify the degree of muscle tone and atrophy. Both legs of the animal were shaved to provide a better view of the muscle region. Marks were placed proximally 20mm and 30mm from the heel of each foot to indicate the measurement reference point. The circumference of the leg was measured and recorded at each of the four reference points. The statistical analysis of the muscle mass data was done using ‘Microsoft Excel’ software on a Macintosh computer. A two factor analysis of variance (ANOVA) was performed in which the factors were tube type and presence of the collagen-GAG matrix. Subsequent student’s t-test calculations were performed to evaluate differences between groups based on a 95% confidence limit.

A thorough investigation of the animal’s foot was also performed. The degree of toe contracture was noted as well as the appearance of any other foot deformities that may have been present. Heel ulcerations are common in animals with sciatic nerve transection.
<table>
<thead>
<tr>
<th>Score</th>
<th>Tissue Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No apparent deformity. Tissue appears normal.</td>
</tr>
<tr>
<td>1</td>
<td>Small ulcer. Heel is slightly red, but no swelling has occurred.</td>
</tr>
<tr>
<td>2</td>
<td>Heel is red and swollen somewhat. Does not seem to affect gait.</td>
</tr>
<tr>
<td>3</td>
<td>Ulcer is severe. Heel is very red and sore. Protrudes from the foot.</td>
</tr>
</tbody>
</table>

Table 2.3 Scale for grading heel ulcerations.

therefore, the degree of heel ulceration was scored based on the criteria shown in Table 2.3

Photographs were taken of the affected foot of each animal to give a permanent record of

the gross morphology of the foot and limb.

### 2.5 Animal Sacrifice and Tissue Processing for Morphological Study

#### 2.5.1 Animal Sacrifice Procedure

The animals were sacrificed using a transcardial perfusion to ensure prompt fixation

of the sciatic nerve, and to decrease the amount of myelin degradation experienced by the
tissue sections during the sacrifice. The animal was weighed, and then anesthetized with

the appropriate dosage (50 mg of solution per kilogram of animal) of sodium pentobarbital

(Nembutal Sodium Solution, 50 mg/ml) given by intraperitoneal injection. Once the animal

was sedated, the chest cavity was opened and the heart exposed. An 18 gage needle was

inserted into the heart on a diagonal trajectory from the right auricle into the left ventricle.

A cut was made in the right auricle to allow for blood drainage. Heparinized saline (20

units per ml) was perfused through the animal until the blood was flushed from the system.

The saline was followed by 300 ml of Yanoff’s fixative drawn from a suspended reservoir

100 cm above the animal.

#### 2.5.2 Tissue Processing

The sciatic nerve was explanted from the sciatic notch at the hip to beyond the

bifurcation point at the knee level, including portions of the tibial and peroneal nerve

branches. The tibial nerve was clearly marked with a suture at the distal endpoint to
distinguish between the tibial and peroneal branches after excision. The tissue was placed in Yanoff’s fixative and stored at 4°C for 24 hours, transferred into 10% neutral buffered formalin for 24 hours at 4°C, and then removed and rinsed in 70% ethanol. The nerve was photographed to capture the gross morphology of the tissue and then sectioned into 2 mm segments according to the diagram in Figure 2.12. The sectioning notation was slightly modified from a previous study and can be cross-referenced to the notation used by Williams and coworkers. For example, the ‘E6’ section in this study corresponded to the ‘S5’ section of Williams. Figure 2.12 shows both notations. Each tissue section was designated for one of three types of embedding medium: Epon, paraffin, and liquid storage.

Tissue sections designated for Epon embedding were soaked in 2% cacodylate buffered glutaraldehyde for 24 hours at 4°C, followed by 24 hours in 0.2 M cacodylate buffered sucrose solution at 4°C. After rinsing in cacodylate buffer, the tissue was post-fixed in 1% osmium tetroxide (Cat #0972A, Polysciences, Inc., Warrington, PA) for 2 hours at room temperature. The tissue was then dehydrated in graded alcohols, cleared in

![Diagram Figure 2.12](image-url)

**Figure 2.12** Sectioning diagram for the rat sciatic nerve, including proximal portions of the peroneal and tibial branches. Williams’ notation (S1, etc) is shown in parentheses.
xylene and embedded in Epon (Poly/Bed 812 Embedding Kit, Cat #08792, Polysciences, Inc, Warrington, PA). For paraffin embedding, the tissue was dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin (Paraplast Plus, Cat #15159-464, VWR Scientific, Boston, MA). Tissue designated for liquid storage was placed in 70% ethanol at 4°C.

2.6 **Histological Evaluation Methods**

2.6.1 **Qualitative Analysis Techniques**

The paraffin samples were cut on a microtome (Leitz Rotary Microtome 1512, Leitz Co.) into 6 μm thick sections, floated on a water bath (Lab Line Lo-Boy Tissue Float Bath, Baxter, Boston, MA) and placed on glass slides (VWR Superfrost Plus, Cat #48311-703, VWR Scientific, Boston, MA). Hematoxylin & Eosin (H & E) and Masson’s Trichrome stains were done according to standard protocols (See Appendix A for procedure and manufacturer information). Hematoxylin & Eosin staining was used to study the presence and type of cells within the regenerated nerves and around the tube edges. The sections were evaluated using light microscopy (Nikon Optiphot, Nikon, Japan) to determine if there was an acute or chronic inflammatory response to the prostheses. Masson’s Trichrome staining was used to study the collagen content within the regenerated nerves. The sections were evaluated using light microscopy to study the regenerated collagenous tissue.

2.6.2 **Quantitative Analysis Technique**

Epon samples, which were post-fixed in 1% osmium tetroxide (OsO₄), were sectioned 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 slides were observed using light
microscopy to ensure that the entire cross section of the nerve was present and that the section was not oblique. The cross sectional area of the tissue and the remaining tube were measured using a grid in the light microscope. Most of the sections were elliptical; therefore, a major and minor axis for the tissue and the outer tube edge were measured, to obtain the tissue and total areas, respectively. The areas, both tissue and total, were calculated using the area formula for an ellipse,

$$A = \frac{\pi \times \text{major} \times \text{minor}}{4}$$

where, major and minor represent the axes. If the tissue filled the entire lumen of the tube, the tube area was calculated by subtracting the tissue area from the total area ($A_{\text{tube}} = A_{\text{total}} - A_{\text{tissue}}$). If the tissue did not fill the tube, a third set of axes were measured for the inner tube edge and then this area was subtracted from the total area to give the tube area.

For normal and autograft tissue, the tissue area was outlined by the epineurium. The nerves were digitized into the 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). The software program 'DIGIT' (July, 1993 version) was used to grab images from the video box (Hamamatsu CCD Camera Control, Model C2400, Hamamatsu, Japan) and digitize them into a Macintosh computer. The number of images to be captured was dependent on the tissue cross sectional area ($A_{\text{tissue}}$). The images captured were intended to account for approximately 10% of the total nerve area. This provided adequate sampling of the tissue cross section. Table 2.4 indicates the number of images necessary for particular tissue areas.

Since the density of axons in the regenerated nerves was not uniform across the tissue, the images were not selected in a totally random manner. Instead the nerve was divided into four quadrants and images were selected from within those quadrants. First, the nerve was positioned so that the long axis was vertical in the case of cross sections.
which had an elliptical shape. Then, the nerve was divided into four quadrants of approximately equal size. The first four images were taken, one from each quadrant, to ensure equal representation from all parts of the cross section. The fifth image was taken from the geometrical center of the cross section. If the fascicle was small enough (according to Table 2.4), these were the only images taken. If additional images were necessary, one image was taken from each quadrant to add 4 images to the total. This was continued as needed according to the area of the specimen up to a maximum of 21 total images, or 5 in each quadrant. The images were always collected in groups of four so that each section of the nerve was equally represented, therefore, 5, 9, 13, etc. images were collected. Figure 2.13 shows a schematic of two characteristic nerve cross sections and the possible images that would be collected from them.

Once the nerve images were digitized into the computer as described, the analysis was done using the public domain software program ‘NIH Image’ (available from the Internet by anonymous ftp from zippy.nimh.gov, or on floppy disk from NTIS, 5285 Port Royal Road, Springfield, VA 22161, part no. PB 93-504868) on a Macintosh computer. The scale of the images was determined by digitizing an etched scale slide into the program and determining the number of pixels per micron. This conversion factor (6.024 pixels/micron) was used for every digitized image. The outer diameter of each axon was highlighted in the density slice mode, using interactive enhancement as needed. Axons which were touching the edge of the image were not counted since exact measurements of

<table>
<thead>
<tr>
<th>Fascicle Area (mm²)</th>
<th>Number of Images Necessary</th>
<th>Image Area/Total Fascicle Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;0.355</td>
<td>5</td>
<td>≥10%</td>
</tr>
<tr>
<td>0.355&lt;A&lt;0.639</td>
<td>9</td>
<td>10% - 18%</td>
</tr>
<tr>
<td>0.639&lt;A&lt;0.923</td>
<td>13</td>
<td>10% - 14%</td>
</tr>
<tr>
<td>0.923&lt;A&lt;1.207</td>
<td>17</td>
<td>10% - 13%</td>
</tr>
<tr>
<td>1.207&lt;A&lt;1.49</td>
<td>21</td>
<td>10% - 12%</td>
</tr>
<tr>
<td>A&gt;1.49</td>
<td>21</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>

**Table 2.4** Table indicating the number of images taken for a given fascicle area.
FIGURE 2.13 Schematic showing the technique for dividing the nerve into quadrants and selecting random samples from within each quadrant. (a). A representative diagram of a porous collagen tube implant. The cross section is very nearly round and the area is large, calling for the maximum number of images (21) to be sampled. (b). A representative diagram of a non porous collagen tube implant. The cross section is very elliptical and the area is usually small enough that either 5 or 9 images is satisfactory.

those axons were impossible. Once all axons were correctly labeled, the program was used to count each axon and to report its area and perimeter. The program shaded each axon as it was counted and labeled it with a number which was used to connect the axon to its area measurement. Figure 2.14 is an example of a small portion of a regenerated nerve image, before and after it has been analyzed by the program.

Using the data generated by the program, the density of axons in each nerve was calculated using the following formula,

\[
Density = \frac{\sum_{i=1}^{n} (# \text{axons})_i}{n * A_{\text{image}}}
\]  

(2)
where, \( n \) is the number of images, \( A_{\text{image}} \) is the image area and \#\text{axons} \ is the number of axons in each image. The total number of axons per nerve was then calculated as follows,

\[
\frac{\text{axons}}{\text{nerve}} = (\text{Density}) \cdot (A_{\text{tissue}})
\]

where, \( \text{Density} \) is as calculated in (2) and \( A_{\text{tissue}} \) is as calculated in (1). The diameter of each axon was calculated using the formula for hydraulic diameter;

\[
D_h = \frac{4 \cdot A_{\text{axon}}}{P_{\text{axon}}}
\]

where, \( D_h \) is the hydraulic diameter of the axon, \( A_{\text{axon}} \) is the area of the axon, and \( P_{\text{axon}} \) is the perimeter of the axon. Hydraulic diameter is a formula used in fluid mechanics to define the size of a cross section of a nonsymmetric shape. This formula was chosen since most of the axons were not circular, it gives a conservative estimate of the diameter of each axon.

![Image of a nerve](image.png)

**Figure 2.14** Image of a nerve regenerated through an implant based on a matrix-filled porous collagen tube at 30 weeks. (a). The original image as digitized into the computer. (b). The analyzed image after the axons have been measured and counted. Note that the axons touching the top edge are not counted, this is because this small inset is from the top of the image. The axons on the side and bottom are counted because they do not come from the image edge.
A two factor analysis of variance (ANOVA) was performed, using ‘Microsoft Excel’, to study the statistical significance of quantitative histological measurements. The factors were tube type and presence of the collagen-GAG matrix at 30 weeks post-operative. Subsequent student’s t-test calculations were performed to evaluate differences between groups based on a 95% confidence limit\(^3^9\).

2.6.3 IMMUNOHISTOCHEMISTRY TECHNIQUE

The immunohistochemical technique was intended to stain the \(\alpha\)-smooth muscle actin isoform which was present only in myofibroblasts, smooth muscle cells and pericytes\(^2^7\). Immunohistochemical staining was done on tissue samples collected during a previous experiment with an endpoint of 6 weeks\(^3^5\). The experimental groups for that study were identical to those used in this study (See section 2.1) using the same surgical procedure and surgeon (See section 2.3). The animal sacrifice procedure and tissue allocation were also the same (See section 2.5).

The staining protocol used was a modified version of a protocol used on dermal tissue\(^6^9\). The antibody diagram for the procedure is shown in Figure 2.15. Paraffin sections were cut to a 6 \(\mu\)m 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 37\(^\circ\)C to unmask antigen sites blocked by formalin fixation. The slides were treated with a 3% hydrogen peroxide solution, followed by 20% nonimmune goat serum which served as the blocking agent. The primary antibody was a mouse monoclonal antibody against \(\alpha\)-smooth muscle actin (#A3682, Sigma Chemical Co., St. Louis, MO). The primary antibody attached to the antigen, which in this case was the \(\alpha\)-smooth muscle actin filaments. The slides were soaked in the primary antibody for 2 hours at 37\(^\circ\)C and then rinsed with phosphate buffered saline (PBS). The biotinylated secondary antibody, goat anti-mouse immunoglobulin (#B0529, Sigma
FIGURE 2.15 Antibody diagram for the α-smooth muscle actin stain.

Chemical Co., St. Louis, MO), attached specifically to the primary antibody and was applied for 1 hour at 37°C. Following a rinse in PBS, the peroxidase reagent, or avidin-biotin complex, (#E2886, Sigma Chemical Co., St. Louis, MO) was applied for 1 hour at room temperature. The avidin-biotin complex bound to the biotin on the secondary antibody. The chromogen solution (#IMMH-2, Sigma Chemical Co., St. Louis, MO) was applied for 15 minutes and added the colorization through an oxidation-reduction reaction with the avidin-biotin complex. The slides were then rinsed, mounted and coverslipped (Aqua PolyMount, Cat #18606, Polysciences Inc, Warrington, PA).

Three regions of the sciatic nerve sections were analyzed: adjacent to the tube surface in cross section (both inside and outside), within the defect site in cross section, and within the defect site in longitudinal section. Figure 2.16 is a schematic showing the three locations. Tube surface sections were of interest to determine the effect of tube
material on myofibroblast formation. The defect site locations were of interest because they
gave information about the wound healing response in the presence and absence of the
collagen-GAG matrix. The Fisher's exact test was performed to determine the level of
significance for the frequency of myofibroblasts and regenerating axons.

2.6.4 Transmission Electron Microscopy

Tissue samples from the six week study, were post-fixed in 1% osmium tetroxide
and embedded in Epon. Ultrathin sections, 65 nm in thickness, were cut using an
ultramicrotome and stained with uranyl acetate in preparation for transmission electron
microscopy (TEM). Tissue sections were selected for TEM using the
immunohistochemical results as a guide for where myofibroblasts might be located. The
sections selected for TEM study came from the cross sections of the tube surface (Figure
2.16). The sections were viewed on a JEOL transmission electron microscope (Model
JEM-100S, JEOL, Tokyo, Japan) The sections were studied for the ultrastructural features
of the myofibroblast: bundles of actin filaments (7-10 nm in diameter), Pinocytotic
vesicles, indented nuclei, and well developed rough endoplasmic reticulum and Golgi
apparatus.

**Figure 2.16** Schematic defining the location of the tube surface and the defect site.
Longitudinal sections are defined as parallel to the nerve axis and cross sections are normal
to the nerve axis.
CHAPTER 3: RESULTS

3.1 GENERAL RESULTS

The surgical procedure was performed on 82 rats, with 70 surviving the entire length of the study. Of the 12 animals that died or were sacrificed early, 3 died during or immediately following the surgery. The deaths were probably due to complications with the anesthetic. Eight animals died in conjunction with an animal identification problem. Initially, the animals were marked with a numbered, metal ear tag for identification. Some of the animals removed the tags, and it became necessary to use an ear punch to create a triangular notch in the right ear of one rat per cage. Animals which already had surgery, 18 total, ranging from 1 to 7 weeks post-operative, were re-anesthetized to administer the ear punch. Six of those animals died due to effects of the anesthetic and the stress of a second procedure. Two animals were sacrificed intentionally because both animals were from the same cage and had removed the ear tags; therefore, it was impossible to identify them. The final death, an animal implanted with an empty porous collagen device, occurred at 12 weeks post operative for no known reason. This animal was not replaced; however, all of the other 11 animals that died were replaced by another rat treated with the same device.

The general condition of the animals was good during the length of the experiment. The animals adjusted well to the nerve lesion and showed no severe signs of discomfort. The animals were able to move around the cage and get to food and water easily, even in the early weeks following surgery. There were no signs of lost appetite or lack of grooming at any time during the experiment. One exception was that the toenails on the experimental foot were not trimmed by the rat. This is a common result of sciatic nerve transection.\footnote{17}

A major concern during this study was the occurrence of autotomy. The rats were monitored daily for any signs of autotomous behavior. During the entire experiment, only
one animal expressed any degree of self-mutilation. At 6 weeks post-operative, the rat was observed to lack one toenail. The toe itself was not damaged; therefore, this incident was rated zero on the autotomy scale (See Table 2.2), consistent with the absence of autotomy. The animal was immediately examined by the surgeon and was monitored daily. The animal never displayed any additional self-mutilation. It is conceivable that the toenail was removed accidentally rather than being removed as an act of autotomy. Throughout the entire experiment, all animals (n=70) scored zero on the scale, indicating no autotomous behavior. This result confirmed the report that the Lewis strain of rats had no inclination toward self-mutilation\textsuperscript{12}; and therefore, was a good choice for this study.

3.2 Functional Results

3.2.1 Gait Analysis

Footprints of each animal were obtained and measured at week 1, 2, and every 2 weeks thereafter until 30 weeks. Three footprint measurements were taken both for normal and experimental feet: print length, toe spread and intermediate toe spread (See Figure 2.11 for a graphical description of terms). Those six measurements were combined into a weighted sciatic functional index (SFI) (See section 2.4.1). Selected plots are shown below to describe trends within the data; however, not all data is represented. The remaining data, for all experimental groups, is given graphically in Appendix C for reference.

Print length was measured as the linear distance from the heel to the third toe. The normal print length for the Lewis rat was 27.3 ± 0.1 mm (n=71). When the sciatic nerve was injured, the average print length increased to 36.8 ± 0.2 mm (n=66) at 1 week post-operative for all experimental groups, excluding the sham-operated control. The small standard error of the mean (± 0.2 mm) at 1 week indicated that, regardless of treatment type, the initial deficiency in print length was the same for all animals. The sham-operated group (n=5) had a print length of 29.2 ± 0.4 mm at 1 week post-operative which differed
slightly from normal. By 4 weeks, the print length for the sham group decreased back to normal values and remained at these levels through 30 weeks. This indicated that in the first few weeks after surgery, the print length was affected by muscle trauma. Figure 3.1 is a plot of print length as a function of time for the empty (Se) and matrix-filled (Sm) silicone tube groups. Over the course of 30 weeks, the print length improved very little for both groups, Se and Sm. At 30 weeks, the print length for the Se group (n=9) was 38.3 ± 0.6 mm which was an increase from the initial measurement at 1 week. The Sm group (n=9) measured 34.9 ± 0.9 mm at 30 weeks which was not a large improvement, but was the smallest print length of all the experimental groups. Both groups showed fluctuation in the print length over time, but no sustained trend of improvement. The print length data for the other experimental groups was similar to the Se and Sm groups, with no differences due to the presence of the matrix or the tube type. The inhibited repair prosthesis group (n=3) had

**Figure 3.1** Print length measurement as a function of time, from 1 to 30 weeks post-operative, for the empty and matrix-filled silicone tube groups (n=9).
a print length of $39.0 \pm 0.6$ mm at 30 weeks which was the largest print length of all groups. None of the experimental groups approached the normal print length.

The second footprint parameter, toe spread, was measured as the linear distance between the first and fifth toes. Normal toe spread was $20.2 \pm 0.2$ mm ($n=71$) as measured pre-operatively; however, the normal toe spread increased to $22.5 \pm 0.1$ mm at 30 weeks. The increase in normal toe spread was probably due to the growth of the animals. The sham-operated group ($n=5$) had a toe spread of $22.0 \pm 0.5$ mm at 1 week post-operative and decreased to within normal range, $20.7 \pm 0.7$ mm, by week 4. After injury to the sciatic nerve, the toe spread decreased dramatically to an average value of $6.1 \pm 0.1$ mm at week 1 for all groups ($n=66$). This distance was basically equivalent to the width of the foot with all the toes pressed together, or the minimum possible toe spread. Figure 3.2 is a plot of the toe spread measurement as a function of time for the empty (Se)
(n=9) and matrix-filled (Sm) (n=9) silicone tube groups. Both groups increased steadily from 1 - 12 weeks, with a toe spread at 12 weeks of 8.8 mm for both groups. From weeks 12 -20, the toe spread remained relatively constant, appearing to have reached a plateau. After week 20, however, the groups began to diverge. The toe spread for the Sm group began to increase to a final value of 10.2 ± 0.4 mm at 30 weeks. For the Se group, the toe spread decreased to a 30 week value of 8.1 ± 0.3 mm. The Se group was the only group that followed this pattern, increasing to a peak value, and then decreasing. The rest of the experimental groups followed two basic patterns. In the first, the toe spread increased, reached a plateau, and then increased again. The autograft and the matrix-filled silicone tube group followed this path. In the second pattern, the toe spread increased steadily until 18 weeks, and then remained relatively constant the remainder of the time. The remaining groups, empty and matrix filled porous collagen and empty and matrix-filled non porous collagen followed this path. In both patterns, the toe spread never decreased dramatically with time and the final toe spread was approximately 10 mm in each of these groups. The inhibited repair (IR) group improved very little over time; and therefore, never developed a clear pattern. The final toe spread for the IR group (n=3) was 7.7 ± 0.3 mm. None of the experimental groups recovered to more than 50% of the normal toe spread.

The third footprint parameter measured was the intermediate toe spread which is defined as the linear distance between the second and fourth toes. Normal (n=71) intermediate toe spread was 10.3 ± 0.1 mm pre-operative and increased to 12.0 ± 0.1 mm by 30 weeks. The intermediate toe spread of the sham operated group (n=5) never differed from normal, which suggested that muscle trauma had no effect on this parameter. Post-operatively, the intermediate toe spread decreased to 3.1 ± 0.1 mm for all experimental groups (n=66) at 1 week. Again, this was the minimum distance between the toes. Data for the empty (Se) and matrix-filled (Sm) silicone tube groups are shown in Figure 3.3 as a function of time. The patterns seen for these groups, and the rest of the experimental groups, were similar to the toe spread data. For all groups, there was a greater standard
FIGURE 3.3 Intermediate toe spread measurement as a function of time, from 1 to 30 weeks post-operative, for the empty and matrix-filled silicone tube groups (n=9).

error of the mean and an unsteady and inconsistent curve. The intermediate toe spread increased to a final value of $5.0 \pm 0.2$ mm for the Se group (n=9) and $5.4 \pm 0.2$ mm for the Sm group (n=9). In the remaining groups, with the exception of the IR group, the final intermediate toe spread was between 5.3 and 5.7 mm. The intermediate toe spread for the IR group (n=3) was $4.3 \pm 0.1$ mm. These long term, 30 week, values were less than 50% of the normal intermediate toe spread.

The six footprint measurements were combined into a weighted equation to give a single value which indicated the degree of functional recovery, the sciatic functional index (SFI) (See section 2.4.1 for details). Normal rats, walked pre-operatively, gave an SFI value of $-8.9 \pm 0.7$ with a sample size of 71 animals. The SFI of the sham operated group (n=5) fell slightly, to $-10.4 \pm 2.1$, at 1 week but recovered at 2 weeks and remained comparable to normal thereafter until sacrifice. One week after surgery, the SFI dipped to
-110.2 ± 0.5 for all experimental animals (n=66), regardless of prosthesis group. Figure 3.4 shows the SFI as a function of time for the empty (Se) and matrix-filled (Sm) silicone tube groups. The pattern that each group followed was similar to the toe spread plot. The similar pattern is a direct product of the weighted equation and the fact that the toe spread is the most heavily weighted variable. The Se group (n=9) reached a final SFI value of -100.3 ± 2.2 which was an overall improvement of 9.9 index points from week 1. At 30 weeks, the Sm group (n=9) reached a value of -83.9 ± 3.2 which was an improvement of 26.3 index points from week 1. The remaining experimental groups all performed very similar to the Sm group, with each group improving between 23 and 27 index points. The exception was the inhibited repair group (n=3) which improved only 8.4 index points between weeks 1 and 30.

A major experimental problem encountered in making the footprint measurements

![Figure 3.4](image-url)  
**Figure 3.4** Sciatic functional index (SFI) as a function of time, from 1 to 30 weeks post-operative, for the empty and matrix-filled silicone tube groups (n=9).
was the development of toe contractures by a portion of the animals. Once a contracture formed, the toes curled under and the print length could not be measured as usual (See Figure 2.11). The modified method for evaluating contracture footprints was discussed in section 2.4.1. Of the 65 animals, 53 developed toe contractures which represented 82% of the population. All of the toe contractures developed between 6 and 18 weeks. Animals which developed a toe contracture remained that way until sacrifice; therefore, the contractures were considered permanent. Figure 3.5 a & b are photographs of two experimental feet: with no contracture and with contracture. Figure 3.6 shows the percent of animals with toe contractures as a function of time in each experimental group. The plot begins at 6 weeks, since that was the earliest appearance of contracture, and ends at 18 weeks, since there was no change in the plot after that time. The sham operated group, inhibited repair group and normal are missing from the plot because none of those animals developed toe contractures. The autograft group (n=9) developed contractures earliest with 50% of the animals having toe contractures by 6 weeks, and 100% of the animals by 10

**Figure 3.5** Photographs of experimental feet at 30 weeks post-operative. (a) Operated foot with no toe contracture. Notice the length of the ungroomed toenails. (b) Operated foot with toe contracture. This was the permanent position of the foot.
FIGURE 3.6 Contracture formation as a function of time, from 6-18 weeks post-operative.

weeks. The empty (n=8) and matrix-filled (n=9) porous collagen tube groups had at least 50% of the animals with contractures by 9 weeks and 100% by 16 weeks. The empty (n=9) and matrix-filled (n=9) non porous collagen tube groups had 50% of the animals with contractures at 7 weeks, and reached 100% by 12 and 18 weeks, respectively. The matrix-filled silicone (n=9) tube group reached 50% by 7 weeks, but never reached 100% as 1 of the animals never developed a contracture. Only 1 of 9, 11%, empty silicone tube group animals developed a toe contracture.

3.2.2 OTHER PERFORMANCE ANALYSIS TECHNIQUES

SENSORY FUNCTIONAL TEST

The sensory functional test, or pinch test, indicated that animals in every implant group, except the empty silicone tube group, recovered some degree of nociceptive function at 30 weeks. The normal foot of each animal was tested in the three pinch
locations before testing the experimental foot to establish the type of positive response
given by the animal. In some cases (3 animals), the animal gave no response to the pinch
on the normal foot. If this occurred, the experimental foot was not tested because no
baseline response was established on the normal foot. This result indicated that a false
negative response, when an animal should have responded positively but did not, occurred
10% of the time, 3 out of 31 normal animals tested. In addition, the test was not performed
on some of the first animals to be sacrificed (12 animals). This reduced the number of
animals in some of the prosthesis groups. The medial toe pinch location was an internal
control for this test. The medial toe is served by the saphenous nerve; and therefore,
should generate a response to deep pain regardless of the degree of sciatic regeneration.
100% of the animals, from all groups (n=29), gave positive responses to the medial toe
pinch for deep pain. This result validated the internal control.

Both the cutaneous and deep lateral toe regions are served only by the sciatic nerve;
and therefore, a positive response was interpreted as the reconnection of axons with
functional sensory endpoints. Figure 3.7 indicates the percent of animals with a positive
response at each pinch location at 30 weeks post-operative. In the empty silicone (Se) tube
group (n=4), none of the animals responded to the deep or cutaneous lateral toe pinch.
Conversely, in the matrix-filled silicone (Sm) tube group (n=4), 100% of the animals
responded positively at both pinch locations. In the collagen tube groups, the distinction
between empty and matrix-filled tubes was less clear. 67% of the empty porous collagen
(PCe) tube group (n=3) responded to the deep lateral toe pinch, while only 33% of the
matrix-filled porous collagen (PCm) tube group (n=3) responded positively. In both
groups, PCe and PCm, 67% responded to the cutaneous lateral toe pinch. In the empty
non porous collagen (NPce) tube group (n=4) and the matrix filled non porous collagen
(NPcm) tube group (n=5), 50% and 80% responded to the deep lateral toe pinch,
respectively, and 75% and 60% respectively, responded to the cutaneous lateral toe pinch.
The inhibited repair group (n=3) had one positive response to the deep lateral toe pinch.
FIGURE 3.7 Percent of animals with positive responses to the toe pinch of the lateral toe for deep pain and the lateral toe for cutaneous pain at 30 weeks post-operative. Positive responses included vocalization and withdrawal of the foot.

This result indicated there was a 33% chance of a false positive response to deep pain (n=3) and no chance of a false positive to cutaneous pain (n=3), since the regeneration was impeded in this group. Overall, it appeared that the animals were more responsive to the cutaneous rather than the deep pain.

CIRCUMFERENTIAL MUSCLE MASS

At 30 weeks, the muscle mass was measured at two locations: the level of the gastrocnemius (20 mm from the heel) and the level of the thigh (30 mm from the heel).

This analysis technique was instituted after some animals had been sacrificed; therefore, the number of animals in each prosthesis group was different and in some cases the sample size was very small. Figure 3.8 shows the circumferential muscle measurement for each prosthesis group at the level of the gastrocnemius. The normal gastrocnemius
circumference, measured on the uninjured leg of each animal, was $57.2 \pm 0.8$ mm with a sample size of 34. The average measurement for the sham operated animals ($n=5$) was $58.6 \pm 1.0$ mm, which was statistically equivalent to normal ($p=0.44$) using the student’s t-test. Two way analysis of variance (ANOVA) revealed that there was no significant effect of the matrix ($p>0.25$) or tube type ($p>0.25$) on muscle circumference at the level of the gastrocnemius. In addition, there was no significant interaction between the tube and matrix ($p>0.25$). The muscle circumference of the autograft ($n=4$), $53.0 \pm 1.4$ mm, was significantly different than the empty non porous collagen ($n=4$), $47.0 \pm 2.0$ mm ($p=0.05$) tube group. All other tube groups were equivalent to the autograft. The empty silicone group ($n=2$), $41.0 \pm 7.0$ mm, was statistically equivalent to the inhibited repair group.

**Figure 3.8** Muscle circumference, measured at 30 weeks post-operative, at the level of the gastrocnemius (20 mm from the heel of the rat) for each prosthesis group. Error bars represent the standard error of the mean and the sample size is indicated on the graph.
(n=3), 33.7 ± 1.2 mm (p=0.27). None of the experimental groups were statistically equivalent to normal.

At the level of the thigh, the normal circumferential measurement was 67.9 ± 0.6 mm. Figure 3.9 shows the measurements for the experimental groups at the level of the thigh. The sham operated group (n=5) was statistically equivalent (p=0.43) to normal with an average for the sham group of 68.8 ± 0.2 mm. Two way ANOVA showed no significant effects of tube type (p>0.25), or matrix (p>0.25); however, the tube and matrix had a significant interaction (p<0.01). The t-test revealed a significant difference between the empty (n=4), 57.5 ± 1.8 mm, and matrix-filled (n=4), 62.3 ± 0.6 mm, non porous collagen tubes (p=0.04). The empty porous collagen (n=3), 64.0 ± 1.0 mm, and autograft (n=4), 67.3 ± 1.8 mm, groups were statistically equivalent to normal.

**Figure 3.9** Muscle circumference, measured at 30 weeks, at the level of the thigh (30 mm from the heel of the rat) for each prosthesis group. Error bars represent the standard error of the mean and the sample size for each group is given.
(p=0.11 and p=0.9 respectively). Again, the empty silicone (n=2), 51.5 ± 5.5 mm, and inhibited repair (n=3), 53.3 ± 0.9 mm, groups were the same (p=0.70).

HEEL ULCERATIONS

At sacrifice, each animal’s experimental foot was evaluated for the presence of heel ulcerations which are common in studies of peripheral nerve injury\(^\text{17}\). Using the scoring criteria shown in Table 2.3, the operated foot was evaluated and scored. 9 out of 49 animals, 18%, had an ulcer score of one or greater. Three of those animals had small ulcers (score = 1) in which the heel was slightly red, but not swollen. The remaining six animals had more serious ulcers (score = 2) in which the heel was red and swollen, but did not appear to effect gait or cause pain. None of the animals had severe ulcers (score = 3).

Figure 3.10 shows the operated foot of an animal with a level 2 ulcer. The empty non porous collagen tube group (n=6) had the highest frequency of ulcers, 50%, with one animal that had a level 1 ulcer and two animals with level 2 ulcers. In the empty porous collagen group (n=5), 40% of the animals had level 1 ulcers. The matrix-filled porous collagen (n=6) and matrix-filled silicone (n=6) tube groups each had two animals with level 2 ulcers. No ulcer deformities were apparent in the other experimental groups, including the autograft.

**Figure 3.10** Photograph of an operated foot with a heel ulceration (score = 2). Note the swelling of the heel. The animal also has ungroomed toenails on the middle toes.
3.3 Morphological Results

3.3.1 Gross Morphology

Gross observation of the explanted nerves showed that for the empty porous collagen tube (n=5), matrix-filled porous collagen tube (n=6), empty non porous collagen tube (n=6) and matrix-filled non porous collagen tube (n=6) reconnection with the distal stump was achieved in 100% of the animals. In these groups, the tissue in every case filled the entire diameter of the tube. The tissue appeared to have adhered to the tubes, which were left attached to the tissue for histological analysis. In the matrix-filled silicone tube group (n=6), reconnection occurred in 83% of the animals; however, the tissue inside the tube only filled approximately half the cross sectional area and the remainder was void space (Figure 3.11a). In the one animal in which reconnection did not occur, there was evidence of infection. Outside the tube was a thick, yellow capsule which was hard to the touch. The inside of the tube was filled with a yellow, cottage cheese-like substance which dissolved in the embedding solvents. In the empty silicone tube group (n=6), reconnection occurred 50% of the time. The tissue in the gap, whenever present, was a very thin cable that filled less than 10% of the tube cross section, with the remainder of the tube being fluid filled (Figure 3.11b). One of the empty silicone tube prostheses, however, had a substantial tissue cable that filled approximately 25% of the tube cross section. The silicone tubes were not attached to the nerve tissue. The tubes slid off the tissue easily and were removed prior to embedding. The inhibited repair prostheses (n=3) had no tissue within the gap and the tubes were fluid filled. Another gross observation at 30 weeks post-operative was that the collagen tubes, both porous and non porous, were not degraded. The tubes appeared grossly to be about the same size as they were at implantation. The porous collagen tubes retained their original shape (Figure 3.12a) but the non porous collagen tubes collapsed and took on an elliptical shape (Figure 3.12b). In both cases, the tissue within the tube was difficult to see because the tubes were opaque.
FIGURE 3.11 Gross morphology of the silicone tube groups. (a) Matrix-filled silicone tube implant after 30 weeks of implantation. Note the thick tissue cable within the tube. The proximal nerve stump is on the left and the distal nerve branches are visible on the right end of the tube. (b) Empty silicone tube implant after 30 weeks of implantation. The tissue cable is very thin and the remainder of space is filled with fluid. The distal stump (on the right) is surrounded by fat making the branches difficult to see.

FIGURE 3.12 Gross morphology of the collagen tube groups after 30 weeks of implantation. (a) Porous collagen tube implant. The tube is approximately the same size and shape as before implantation. The proximal nerve stump is on the left side of the photograph. (b) Non porous collagen tube implant. The tube deformed during implantation into an elliptical shape. Originally, the tube was round. The tissue cable is visible traveling through the center of the tube.
3.3.2 Qualitative Histology

Hematoxylin & Eosin staining revealed very little cellular activity within the regenerated tissue and around the tube edges. The cells present within the defect site were primarily Schwann cells which were associated with specific axons. The presence of the Schwann cells indicated that myelination was actively occurring at 30 weeks post-operative. The collagen tubes appeared to be intact with very little cellular activity in and around them. Few active cells had migrated into the collagen tubes; however, fat cells were often found in the outer pores of the porous collagen tubes.

Masson's Trichrome staining confirmed the presence of collagen within the defect site. The collagen appeared loosely packed surrounding the axons and was much more prominent than in normal nerve tissue. In many cases, the axons were bundled into small groups of 10-100 axons, which have been termed minifascicles. The axon bundles were usually surrounded by a ring of collagen tissue. The tissue appeared well vascularized. In the collagen tubes, the trichrome stain confirmed that the tissue had adhered to the tubes.

3.3.3 Quantitative Histology

Tube and Tissue Area

The cross sectional areas occupied by the tube and the regenerated tissue were measured using light microscopy, and were found to be coupled in the collagen tube groups. The shape and size of the porous collagen tubes appeared unchanged at the gross level; however, investigation at the light microscope level confirmed that the originally circular cross sections were elliptical after 30 weeks of implantation. The pre-implantation tube outer diameter was 3.0 mm. After 30 weeks, the tube had a major axis of $3.07 \pm 0.01$ mm and a minor axis of $2.41 \pm 0.02$ mm (n=11). The cross sectional area occupied by the porous collagen tube was $5.30 \text{ mm}^2$ before implantation and fell to $4.49 \pm 0.06 \text{ mm}^2$ at 30 weeks post-operative, for the empty and matrix-filled groups combined (n=11). The area of the porous collagen tube was reduced by 15% over the course of 30 weeks and appeared
unaffected by the presence of the collagen-GAG matrix. Figure 3.13 is a plot of the pre- and post-implantation tube areas for each of the tube types. The lumen of the porous collagen tubes was completely tissue filled in every case, both empty (n=5) and matrix-filled (n=6). The pre-implantation lumen area was $1.77 \text{ mm}^2$ and at 30 weeks, the empty porous collagen tube group had a tissue area of $1.35 \pm 0.10 \text{ mm}^2$ (n=5), while the matrix-filled porous collagen tube groups had a tissue area of $1.28 \pm 0.07 \text{ mm}^2$ (n=6). These areas were statistically similar (p=0.5), and were equivalent to 24% and 28% reductions in the lumen area, respectively. Figure 3.14 shows the regenerated tissue area for each prosthesis group at 30 weeks post-operative. A detailed schematic of each tube before implantation was presented in Figure 2.4. A similar schematic, using the same scale, is shown in Figure 3.15 for the tissue filled tubes at 30 weeks, to highlight the changes in the

![Figure 3.13](image-url)

**Figure 3.13** Pre- and post-implantation (30 weeks) tube areas for each of the tube types. The values for the empty and matrix-filled groups, in each tube type, were averaged since there was no effect of the matrix on tube area.
FIGURE 3.14 Regenerated tissue area for each prosthesis group at 30 weeks.

FIGURE 3.15 Detailed schematic of the tube and tissue dimensions after 30 weeks of implantation. The tissue size was averaged for the empty and matrix-filled collagen tube devices, which were similar, to obtain a representative area. Figure 2.4 can be referenced for the pre-implantation dimensions. (a) Porous collagen tube filled completely with regenerated tissue. (b) Non porous collagen tube filled completely with tissue. (c) Silicone tube with tissue shown for both empty and matrix-filled devices, since the two were significantly different.
shape of the tube and the corresponding shapes of the tissue for each group.

The non porous collagen tube deformed drastically over the course of 30 weeks. The cross section of the tube became elliptical with a major axis of 2.98 ± 0.01 mm and a minor axis of 1.18 ± 0.02 mm (n=12). The tube was originally circular with an outer diameter of 1.8 mm. Before implantation, the cross-sectional area occupied by the non porous collagen tube was 0.78 mm² with a total area (the area calculated by using the tube’s outer diameter) of 2.54 mm². At 30 weeks, the tube’s cross-sectional area increased by 220% to an average of 2.48 ± 0.05 mm², for the empty and matrix-filled cases (n=12) (Figure 3.13). The total area was 2.77 ± 0.05 mm² at 30 weeks which was an increase of only 9% over the pre-implantation value. This indicated that most of the tube expansion was inward, toward the center, reducing the lumen area of the tube from the pre-implantation value of 1.77 mm². At 30 weeks, the empty tubes (n=6) had a lumen area of 0.25 ± 0.03 mm², while the matrix-filled tubes (n=6) had an area of 0.34 ± 0.04 mm². These areas were statistically similar (p=0.11). In both cases, the tissue entirely filled the tube; therefore, the lumen area and the tissue area were equivalent (Figure 3.14). The lumen areas at 30 weeks represented an 86% and 81% decrease, respectively, in the area available for the regeneration of a nerve cable (Figure 3.15 b).

The inert and highly flexible nature of the silicone tube prevented any deformation of the tube at 30 weeks. The cross-sectional area occupied by the silicone tube was 1.32 mm², before and after implantation, and the lumen area remained constant at 1.70 mm² over the length of the study (Figure 3.13). As noted from the gross morphology, the tissue in the silicone tubes did not fill the entire lumen of the tube. For the empty silicone tube group (n=6), the tissue area was 0.09 ± 0.07 mm², which filled approximately 5% of the lumen area. The matrix-filled silicone tubes (n=5) had a regenerated tissue area of 0.39 ± 0.02 mm², which filled 23% of the lumen area (Figure 3.14). The student’s t-test revealed a significant difference in tissue area between the empty and matrix-filled silicone tube groups (p=0.003). In both cases, the tissue cable was circular in cross section and had...
diameters of 0.19 ± 0.05 mm for the empty group and 0.70 ± 0.01 mm for the matrix-filled group (Figure 3.15 c).

Two-way analysis of variance (ANOVA) indicated a significant effect of tube type (p<0.005) and the presence of the matrix (p<0.005) on the tissue area. The interaction between the tube and matrix was not significant (p>0.25). The porous collagen tubes had a significantly larger tissue area than the silicone (p<0.001) and the non porous collagen (p<0.001) tubes, which were similar (p=0.27). As expected, the tissue area for the autograft (n=6), 0.51 ± 0.03 mm², was comparable to normal (n=6), 0.49 ± 0.02 mm² (p=0.55).

**Density of Myelinated Axons**

The density of myelinated axons within the regenerated nerve cables was dependent on the amount of tissue area. In general, prosthesis groups with large tissue areas had lower axon densities, while groups with small tissue areas had much higher axon densities. Figure 3.16 is a plot of the axon density for each prosthesis group. Normal nerve (n=6) had an axon density of 10530 ± 547 axons/mm². Within the tubular groups, two-way ANOVA revealed a significant effect of the tube type (p<0.005) and a significant effect of the matrix (p<0.01). The interaction between the tube and matrix was also significant (p<0.005). The empty silicone tube group (n=6) had an axon density of 7020 ± 6479 axons/mm², which was significantly different (p=0.008) from the matrix-filled silicone tube (n=5) axon density, 31257 ± 1244 axons/mm². The empty (n=5), 8222 ± 2051 axons/mm², and matrix-filled (n=6), 13053 ± 1343 axons/mm², porous collagen tube axon densities were equivalent (p=0.07). The empty (n=6) and matrix-filled (n=6) non porous collagen tube axon densities were also statistically similar (p=0.24), 31848 ± 1813 and 28451 ± 2021 axons/mm², respectively. The autograft group (n=6) had an axon density of 28097 ± 3159 axons/mm², which was similar to the empty (p=0.33) and matrix-filled (p=0.93) non porous collagen tubes and the matrix-filled silicone tube (p=0.41).
TOTAL NUMBER OF MYELINATED AXONS PER NERVE

For each animal, the axon density was multiplied by the tissue area to obtain the total number of myelinated axons per nerve. Figure 3.17 shows the total number of myelinated axons for each prosthesis group. The porous collagen with matrix prosthesis group (n=6) had the most axons per nerve, 16689 ± 2080 axons/nerve, which was more than three times the normal (n=6), 5075 ± 199 axons/nerve. Two-way ANOVA indicated that both the matrix (p<0.005), and the tube type (p<0.005), had significant effects on the total number of regenerated axons. The interaction term was not significant (p>0.25). The empty silicone tube group (n=6) had 2683 ± 2611 axons/nerve, which was significantly different (p=0.01) than the matrix-filled silicone tube group (n=5), 12044 ± 616 axons/nerve. The matrix-filled porous collagen prosthesis group had a larger mean than the empty porous collagen group (n=5), 11165 ± 3188 axons/nerve; however, the difference was not significant (p=0.17). The empty (n=6), 7930 ± 1100 axons/nerve, and matrix-
FIGURE 3.17 Total number of myelinated axons per nerve at 30 weeks post-operative.

filled (n=6), 9297 ± 580 axons/nerve, non porous collagen prosthesis groups were statistically similar (p=0.30). Of the matrix-filled prosthesis groups, the filled non porous collagen tube was statistically different from the filled silicone (p=0.01) and the filled porous collagen (p=0.007) tube groups. All of the empty tube groups were statistically similar (Se vs. PCe, p=0.07; Se vs. NPCe, p=0.09; PCe vs. NPCe, p=0.33). The autograft (n=6) had 13957 ± 1408 axons/nerve which was equivalent to the empty porous collagen (p=0.42) and the matrix-filled porous collagen (p=0.30) and silicone (p=0.28) tube groups.

**MYELINATED AXON DIAMETER DISTRIBUTION**

An important aspect of regenerating peripheral nerves is the size of the axons. Large diameter axons, 6 μm in diameter or greater, are desirable because they are responsible for motor function and are more difficult to regenerate. The myelinated axon diameter distribution of normal nerve was uniform from 3 μm to 12 μm. There were no
myelinated axons smaller than 1 μm and few at the 2 μm level. Figure 3.18 shows the normal distribution with the regenerated distributions of the empty (n=6) and matrix-filled (n=6) non porous collagen tubes. The mode for each regenerated nerve distribution, including all prosthesis groups, was 3 μm. The matrix-filled non porous collagen prosthesis group had more axons in the large diameter region (≥ 6 μm) than the empty group. The matrix-filled porous collagen and silicone tubes also had larger axons than the empty tubes (See Appendix C for additional plots). Figure 3.19 shows representative photographs of normal and regenerated nerve which highlight the diameter distribution differences.

A simpler way to compare the regeneration of large diameter axons, was to sum the number of axons greater than 6 μm, to obtain one number for each prosthesis group. The number of large diameter axons, greater than 6 μm, is shown in Figure 3.20. Analysis of

![Graph](image-url)

**Figure 3.18** Myelinated axon diameter distribution for the empty and matrix-filled non porous collagen tube groups at 30 weeks. The normal distribution is shown for reference.
FIGURE 3.19 Photographs of peripheral nerve sections. Stained with 1% osmium tetroxide and toluidine blue and viewed at 100x magnification. Scale bar = 10 μm. (a) Normal nerve (b) Regenerated nerve (Matrix-filled non porous collagen prosthesis). Note the large axons, approaching 10 μm in diameter. Many small diameter axons are present compared to the normal. Also, the Schwann cells are visible in the regenerated case, indicating active myelination.
variance (two factor) indicated a significant effect of tube type (p<0.05) and the presence of the collagen-GAG matrix (p<0.005) on the total number of large, myelinated axons. The matrix-filled silicone tube group (n=5) had 999 ± 130 axons ≥ 6 μm/nerve. This was significantly different (p<0.001) than the empty silicone tube (n=6) which had only 35 ± 35 axons ≥ 6μm/nerve. Of the tubular implants, the matrix-filled porous collagen tube group (n=6) had the most large diameter axons, 1303 ± 305 axons ≥ 6 μm/nerve; however, that was only 38% of normal (n=6), 3468 ± 76 axons ≥ 6 μm/nerve. The empty porous collagen tube (n=5) had 695 ± 195 axons ≥ 6 μm/nerve, which was only 53% of the number in the matrix-filled case. The difference, however, was not significant (p=0.14). The empty (n=6) and matrix-filled (n=6) non porous collagen tube groups had 690 ± 259 and 1093 ± 191 axons ≥ 6 μm/nerve, respectively. The mean difference was not significant (p=0.24). All of the matrix-filled tubes were statistically equivalent with respect

**Figure 3.20** Number of myelinated axons greater than or equal to 6 μm for each prosthesis group at 30 weeks post-operative.
to the number of large diameter axons (Sm vs. PCm, p=0.42; Sm vs. NPCm, p=0.71; PCm vs. NPCm, p=0.57). The empty porous collagen and empty non porous collagen tube groups were significantly different from the empty silicone tube group (Se vs. PCe, p=0.005; Se vs. NPe, p=0.03), but were equivalent to each other (p=0.99). The autograft group (n=6) had 1821 ± 221 axons ≥ 6 μm/nerve which was 53% of the normal number of large axons. The autograft was equivalent to the matrix-filled porous collagen tube group (p=0.20).

Another method of comparing prosthesis groups, was to calculate the mean axon diameter for each animal. The problem with this technique was that the mode of each distribution was so strong, at 3 μm, that the groups were difficult to differentiate using this method. Figure 3.21 shows the mean axon diameter for each prosthesis group and normal. The empty silicone tube group (n=2) had a mean axon diameter of 2.28 ± 0.13 μm, which was statistically different (p=0.001) than the matrix-filled silicone tube group (n=5), 3.04 ±

![Figure 3.21](image)

**Figure 3.21** Mean axon diameter for each prosthesis group at 30 weeks post-operative.
0.05 μm. All other groups, including the autograft, had a mean axon diameter of approximately 3 μm, and the empty silicone tube group was the only group that was different.

3.4 DISCOVERY OF CONTRACTILE CELLS

3.4.1 TISSUE WITHIN THE DEFECT SITE

Tissue within the defect site was studied in both longitudinal and cross section (See Figure 2.16). The immunohistochemical procedure stained specifically in the regenerated tissue for the α - smooth muscle actin present in myofibroblasts. Normal nerve displayed no myofibroblasts in either orientation, longitudinal or cross section. In regenerated tissue, myofibroblasts were found within the defect site oriented parallel to the nerve axis as shown schematically in Figure 3.22. The defect site was defined as the regenerated

![Diagram](image-url)

**Figure 3.22** Schematic of a longitudinal section which shows the orientation of myofibroblasts within the defect site. The myofibroblasts were found in the regenerating tissue with the cell axes elongated parallel to the nerve axis as shown. Myofibroblasts adjacent to the tube surface (See Figure 3.27) are shown, cut in cross-section, for reference. Not to scale.
tissue inside the tube, excluding the tissue touching the tube surface which will be addressed separately in the next section. Figure 3.23a is a light micrograph showing the myofibroblasts cut in cross section. In this particular orientation, the nerve axis is perpendicular to the plane of the graph. In some cases, the nucleus of the myofibroblast is visible; however, in most of the cells, only the tail portion of the cell is in the plane. Figure 3.23b is a longitudinal section of the defect site showing the myofibroblasts parallel to the nerve axis. Tissue was analyzed qualitatively for the presence or absence of myofibroblasts. Myofibroblasts were detected, parallel to the nerve axis, within tissue regenerated through silicone, porous collagen and non porous collagen tubes. There was no difference qualitatively between the empty and matrix-filled tubes with respect to the

\[\text{FIGURE 3.23} \alpha\text{-smooth muscle actin immunohistochemical stain of regenerated nerve at six weeks post-operative. The reddish-brown color identifies the presence of } \alpha\text{-actin within the cells. (a) Cross sectional view of the defect site. The blood vessel (V) serves as a positive control for the actin stain. In some myofibroblasts, the nucleus is visible (1) and in others, the nucleus appears to be in a different plane (2). The myofibroblasts are aligned normal to this section. (b) Longitudinal section of the defect site. The myofibroblasts (M) are highly aligned along the nerve axis (arrow indicates axis direction). Scale bar = 16 \mu m.}\]
presence of myofibroblasts. When present, myofibroblasts appeared more frequently in the distal stump as opposed to more proximal sections (Figure 3.24). In nerves that had axonal elongation according to quantitative histology, 93% of the distal sections had myofibroblasts present (n=15). The tissue was considered to have axonal elongation, or regeneration, if the total number of myelinated axons per nerve was greater than zero. In the same nerves, no myofibroblasts were detected in any of the proximal sections (n=8). Regenerated nerves that had no axons showed no myofibroblasts within the defect site, neither proximal nor distal (n=3) (Table 3.1). Using Fisher’s exact test, there was a significant correlation between regeneration of axons and the presence of myofibroblasts (p=0.005) in the distal stump.

**Figure 3.24** Simple schematic defining proximal and distal segments of the defect site.

<table>
<thead>
<tr>
<th></th>
<th>Proximal Portion of the Defect Site</th>
<th>Distal Portion of the Defect Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerves with Regeneration (Total number of axons &gt; 0)</td>
<td>0/8</td>
<td>14/15</td>
</tr>
<tr>
<td>Nerves without Regeneration (Total number of axons = 0)</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

**Table 3.1** Myofibroblast data, at 6 weeks post-operative, for the proximal and distal nerve segments. In the proximal region, no myofibroblasts were found, regardless of the extent of regeneration. In the distal region, myofibroblasts were present in 14 of 15 nerves that had regeneration. When the nerve did not regenerate, there were no myofibroblasts.
3.4.2 TISSUE ADJACENT TO THE TUBE

The tissue adjacent to the tube surface, both inside and outside, was studied using a cross sectional view. The tissue considered to be adjacent to the tube was directly apposed to the tube, either on the inner or outer tube surface. The α-smooth muscle actin stain identified myofibroblasts on both the inside and outside tube surfaces of the prostheses using light microscopy. The tube surfaces are defined schematically in Figure 3.25. Of the silicone tube prostheses, 100% had a myofibroblast layer on the inside tube surface (n=7), and 100% had a similar layer on the outside tube surface (n=3) (Figure 3.26 a & b). In both cases, the myofibroblasts appeared to form a concentric shell about several cells thickness. Of the collagen tube prostheses, both porous and non porous, 14% had myofibroblasts on the inside tube surface (n=7); however, no myofibroblasts were present on the outside tube surface (n=7) (Table 3.2). Myofibroblasts were significantly more likely to be present on the inside (p=0.002) and outside (p=0.008) tube surfaces of the silicone tubes than the collagen tubes. In addition, the myofibroblasts adjacent to the collagen tube edge did not appear cell continuous and were only one cell thick where

![Figure 3.25](image)

**Figure 3.25** Simple schematic defining the inside and outside tube surfaces. The definition of proximal and distal tube sections is also shown.
FIGURE 3.26 α-smooth muscle actin immunohistochemical stain of tissue around the surfaces of a silicone tube prosthesis at six weeks post-operative. The reddish-brown color identifies the presence of α-actin within the cells. (a) Inside tube surface. Myofibroblasts (M) have formed an actin rich capsule along the tube surface. The capsule is several cells thick and the cells are elongated circumferentially around the nerve. (b) Outside tube surface. The myofibroblasts (M) are highly aligned circumferentially and are cell continuous along the tube surface. Scale bar = 16 μm.

The density of myofibroblasts on the tube surfaces was independent of the location along the tube, proximal or distal.

Transmission electron microscopy on the tissue adjacent to the tube confirmed the immunohistochemical findings that myofibroblasts were abundant on the inner and outer surfaces of the silicone tubes and were elongated and arranged circumferentially around the tube, as shown schematically in Figure 3.27. The myofibroblast layer was several cells thick and the ultrastructural features of the myofibroblast were easily identified in every cell along the tube surface (Figure 3.28). Actin microfilaments were visible and bundled very densely, pinocytotic vesicles were present near the cell membrane, the rough endoplasmic reticulum was well developed, and the cell-matrix connection (fibronexus) was visible (Figure 3.29).
### TABLE 3.2
Myofibroblast data, at 6 weeks post-operative, for the proximal and distal tube surfaces, both inside and outside. The silicone tube had myofibroblasts present at the inner and outer tube surfaces in 100% of the samples studied. In the collagen tubes, only 1 sample had myofibroblasts present at the tube surface. The location along the tube edge, proximal or distal, had no effect on the appearance of myofibroblasts.

<table>
<thead>
<tr>
<th>Tube Type</th>
<th>Inside Tube Surface</th>
<th>Distal Portion of the Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Tubes with Myofibroblasts</td>
<td>Silicone</td>
</tr>
<tr>
<td></td>
<td>3/3</td>
<td>0/2</td>
</tr>
<tr>
<td>Outside Tube Surface</td>
<td>Number of Tubes with Myofibroblasts</td>
<td>2/2</td>
</tr>
</tbody>
</table>

**FIGURE 3.27** Schematic of a cross section which shows the orientation of myofibroblasts around the tube edges. The cell axes were elongated circumferentially around the tube as shown, on both the inside and outside tube surfaces. The nerve axis is perpendicular to the plane of the graph. Myofibroblasts found within the defect site (See Figure 3.22) are shown, cut in cross-section, for reference. Not to scale.
FIGURE 3.28  Transmission electron micrograph showing the myofibroblasts on the inside edge (E) of a silicone tube prosthesis. The dense actin bundles (B) can be seen in every cell and show that the layer is several cells thick. Pinocytic vesicles (P) are also visible. Magnification: 30,000x. Scale bar = 0.67 μm.
FIGURE 3.29 Transmission electron micrograph showing a myofibroblast on the inside edge of a silicone tube prosthesis. This micrograph shows the fibronexus (F) which mechanically links the cell to the matrix. The actin bundles (B) are densely packed throughout the perimeter of the myofibroblast. The cell has well developed rough endoplasmic reticulum (ER). Pinocytotic vesicles (P) are also visible.

Magnification: 60,000x. Scale bar = 0.33 μm.
CHAPTER 4: DISCUSSION

4.1 FUNCTIONAL EVALUATION

4.1.1 GAIT ANALYSIS

One of the problems involved in assessing the improvement of the footprint parameters, was the growth of the animals over the course of the study. The values of print length, toe spread, and intermediate toe spread, on the normal foot increased approximately 2 mm each over the course of 30 weeks. Other investigators have reported similar increases in the normal footprint parameters after 24 weeks\(^3\). The experimental footprint parameters should have grown at the same rate as the normal; therefore, the footprint measurements would need to be adjusted. For example, the experimental toe spread improved from 6 mm to 10 mm for most groups (See Figure 3.2), a total increase in toe spread over 30 weeks of 4 mm. The growth rate for the toe spread, however, was 2 mm, resulting in a net increase in experimental toe spread of only 2 mm (4 mm - 2 mm). This makes comparison of individual footprint parameters difficult and care must be taken to accurately interpret the improvement. The SFI is based on the difference between normal and experimental footprint values at each time interval (Normal - Experimental) (See Section 2.4.1); therefore, the growth of the animal should cancel in the SFI equation. The problem with using the SFI to compare experimental groups is that it can obscure changes in individual footprint parameters\(^3\). Because the SFI is a weighted composite index, it can also enhance subtle differences that are undetected by looking only at the individual footprint measurements\(^3,32\).

Another major problem was the formation of chronic toe contractures. Toe contractures have been attributed to the poor regeneration of the peroneal nerve\(^5\). The peroneal nerve serves the extensor muscles of the foot, while the tibial nerve serves the flexor muscles. Electrophysiological studies; however, have shown that the response of
the peroneal nerve to stimulation was equivalent to the tibial nerve response after tubulation repair. This suggests that a natural imbalance in the antagonist muscle strength (flexor muscles being stronger than extensor muscles) is responsible for the formation of toe contractures. The toe contractures developed between 6 and 18 weeks and once formed, remained for the duration of the experiment. The contractures made measurement of the toe spread and intermediate toe spread difficult, and rendered the conventional method of print length measurement useless. A technique for measuring the print length in the presence of a contracture was developed (See Section 2.4.1) so that the gait analysis could continue for the length of the experiment. Section 4.3.1 discusses in more detail the reasons for contracture development and their clinical significance.

One concern with the accuracy of the gait analysis method was that the gait began improving as early as 2 weeks post-operative for all experimental groups (See Figures 3.2-3.4). At that time, axons had barely begun to enter the gap and would have made no functional connections. This suggested that early improvement in the footprint measurements, from weeks 2-6, must be due to an adaptation by the animal to the injury, rather than a return of function. The SFI improved a total of approximately 25 index points over 30 weeks for the experimental groups. In the first six weeks post-operative, the SFI improved 10 index points. This improvement can not be credited to the regeneration of a functioning nerve cable.

Electrophysiological studies have shown that for empty and collagen-GAG matrix-filled silicone tubes, and a gap length in the sciatic nerve of 10 mm, a silent period exists for the first 12-17 weeks post-operative. During that period, no electrical signals could be carried by the regenerating nerve cable. After the silent period, the electrophysiological measurements began to improve and plateaued between 25 and 30 weeks. The current study indicated that the gait pattern recovered in an opposite fashion. In general, gait improved for the first 12 weeks post-operative and then reached a plateau. In a simple model, recovery of normal electrophysiological function and normal gait are expected to
correlate at least roughly over time. The lack of correlation suggests the need for a more complex interpretation of the data from the two procedures. It is unlikely that the coordination and muscle reinnervation necessary for improving gait could appear weeks before an electrical impulse can be carried by the nerve cable.

The gait analysis technique produced disappointing results that were generally not sensitive enough to differentiate between experimental groups. Many investigators have successfully used the sciatic functional index (SFI) to differentiate between an experimental group and a negative control, or ‘no repair’ group^{31,32,53}. In the current study, the inhibited repair group served as a negative control and was distinctly different from the repair groups, verifying that the SFI can distinguish between a negative control and a repair group. The technique was not sensitive enough; however, to distinguish between any of the repair groups. One exception was the empty silicone tube group which performed similarly to the inhibited repair group and could be distinguished from the other experimental groups.

Although, gait analysis is a nominally simple, noninvasive technique for assessing functional recovery, the results obtained did not warrant further use of the method. Electrophysiological measurements provide an accurate description of the signal carrying ability of the regenerated nerve trunk and, in some cases, can be used to determine whether axons have made functional connections. Electrophysiology cannot, however, determine whether motor axons have connected to their original target muscle, creating a functioning motor unit that receives the appropriate signals from the brain. The recovery of gait is a complex behavioral correlate. It is not expected that gait relates simply to electrophysiological recovery of a single nerve trunk among the many which contribute to its expression. New functional methods must be developed which accurately measure functional return, and are sensitive enough to distinguish between repair groups.
4.1.2 **Sensory Analysis**

The sensory functional test, or pinch test, showed that animals in every group had some degree of sensory recovery. Several locations on the foot were pinched, using forceps, and the animal was monitored for a positive response to the pinch. Positive responses included vocalization and withdrawal of the foot. The medial toe, which is served by the saphenous nerve, provided a convincing internal control for the pinch test. The overall performance of the matrix-filled silicone tube group was superior to all other prosthesis groups (See Figure 3.7). Because the test was not quantitative, it was difficult to make comparisons between the groups. In addition, the errors involved with this test made interpretation of the results difficult. False negative responses occurred 10% of the time and false positives occurred 16% of the time. A large portion of the error was probably due to animal discomfort with handling by the investigator. Since the rats were handled infrequently, they were very frightened and distressed during the pinch test. The response given by each animal was possibly altered due to an uncontrolled extent of fear. For example, an animal may respond positively, with vocalization or withdrawal, not to the pinch, but to the fear of being held in an unfamiliar area. This type of sensory test is subjective and has a lack of sensitivity\(^2\). The results should be interpreted with care.

In the future, handling of the rats by the investigator in the experimental area may reduce the animal's fear and may eliminate some of the errors. Handling the animals daily in the experimental testing area, prior to beginning the experiment, has been shown to reduce the amount of false responses\(^6\). Testing of the animals at earlier time points, and more often, would also give more interesting results. This would allow study of the time course of sensory recovery.

4.1.3 **Muscle Mass Measurement**

The extent of muscle tone after sciatic nerve transection is of value in assessing the return of motor function. The muscle tone was studied by making a circumferential
measurement of the leg at the level of the gastrocnemius and the level of the thigh. Uncontrolled errors occurred due to the variable extent of fat and connective tissue at the measurement location\textsuperscript{25}. Despite the possible errors, the circumferential measurement was chosen because it resembles a noninvasive, clinical technique.

The results of this study indicated that the circumferential muscle mass measurement was able to distinguish experimental groups with very poor regeneration (empty silicone and inhibited repair) from the remaining experimental groups at both the level of the gastrocnemius and the thigh. Only the empty non porous collagen tube was significantly different from the autograft (p=0.05) at the level of the gastrocnemius. The measurement at the thigh; however, was able to distinguish more clearly between the experimental groups. The matrix-filled non porous collagen tube had a significantly larger muscle circumference at the level of the thigh than the empty non porous collagen tube group (p=0.04), and two experimental groups, the empty porous collagen and the autograft, were equivalent to normal.

The muscle mass measurement was a simple, noninvasive technique which was able to detect small differences between experimental groups. Measurement of the muscle mass at several time points throughout a study may give an interesting picture of functional return. Because measurements were only taken at sacrifice, 30 weeks, it was impossible to tell how the muscle tone was changing with time. Tracking the muscle atrophy, and subsequent muscle rebuilding, may differentiate more clearly between groups and give an idea of when the muscle groups become reinnervated.

4.2 MORPHOLOGICAL EVALUATION

4.2.1 DEFORMATION AND DEGRADATION OF THE COLLAGEN TUBES

The collagen tubes, both porous and non porous, deformed into tubes with elliptical cross section, over the course of 30 weeks (See Figure 3.15). The deformation was probably caused by pressure applied to the tube by the surrounding tissues. The sciatic
nerve lies in an intermuscular septum; however, the diameter of the implanted tubes was greater than the diameter of the nerve. When the muscle tissue was closed around the repaired nerve, the tube may have deformed into the available space. Furthermore, the proliferative response to the surgical procedure could have led to increased demand for space at the site of implantation. Total cross sectional area (based on the outer diameter of the tube), of the porous collagen tubes reduced 18%, while the non porous collagen tubes increased by 9%.

The discovery that the tubes were not degraded at 30 weeks indicated that the \textit{in vivo} life of the tubes was much longer than anticipated. Another study, which employed a nominally identical porous collagen tube (from the same manufacturer), indicated that the tube had been completely degraded at 3.5 years post-operative in a primate model\textsuperscript{4}. The porous collagen tubes; therefore, must resorb in the period between 30 weeks and 3 years. Hematoxylin & Eosin staining revealed very few inflammatory cells at the tube surfaces at

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Cross-sectional schematic of the porous collagen tube before implantation (left) and after 30 weeks of implantation in the rat sciatic nerve (right). The shape of the tube became elliptical and the cross-sectional area of the tube reduced by 15%. Drawing is to scale with scale bar as shown.}
\end{figure}
30 weeks and suggested that the tubes were not being actively degraded. At 30 weeks, the cross sectional area of the porous collagen tubes had been reduced by 15% as shown schematically in Figure 4.1. It is unclear, however, whether the reduction in area was due to degradation of the tube. Another possible explanation for the reduction in area, was that the pores within the tube absorbed some of the pressure from the surrounding tissue, and the pores became condensed. Therefore, the area occupied by void space (pores) decreased and the area occupied by the collagen fibers remained constant. The degradation of the porous collagen tube was, therefore, probably less than 15% and it is conceivable that negligible degradation occurred during the 30 week period.

The cross sectional area of the non porous collagen tubes increased by 220% from the pre-implantation value as shown schematically in Figure 4.2. Tube material was not created in vivo, but the existing tube material expanded. The expansion of the tube was

![Figure 4.2](image)

**Figure 4.2** Cross-sectional schematic of the non porous collagen tube before implantation (left) and after 30 weeks of implantation in the rat sciatic nerve (right). The shape of the tube became elliptical and the cross-sectional area of the tube increased by 220%. The lumen area of the tube was completely tissue filled at 30 weeks, but was reduced by 83% from its pre-implantation value. The total area of the tube, based on the outer diameter, increased by only 9%. Drawing is to scale with scale bar as shown.
probably caused by a combination of factors: body temperature, hydration, pressure and possibly degradation. Axons were not present within the tube material, indicating that the tube expansion occurred early, prior to the elongation of axons. The tube expanded primarily inward toward the lumen of the tube, since the total area (calculated from the outer diameter of the tube) increased by only 9%, while the lumen area (calculated from the inner diameter of the tube) decreased by 83%. The substantial decrease in the lumen area was critical because the space available for a nerve cable to regenerate through became very small.

The advantage of using type I collagen tubes is that the material can be resorbed by a variety of cell types in vivo: fibroblasts, macrophages, and polymorphonuclear leukocytes. In addition, the resorption rate of the collagen tubes can be controlled by varying the amount of chemical crosslinking, or the density and thickness of the collagen membrane. An optimum in vivo life for a tubular prosthesis has not been studied; however, degradation of axons, demyelination and other abnormalities have been reported after 1 year of implantation with the silicone tube. The changes in the regenerated nerves may be a result of constrictive forces applied by the tube in the late stages of regeneration. If this is the case, an ideal tube would be degraded before one year of implantation.

The primary role of the tube is to provide guidance for the nerve and to serve as a chamber for the delivery of substrate materials. At 30 weeks, tissue had completely crossed the gap in every collagen tube implant. Other investigators have reported that tissue, including axons, reached the distal stump by 6 weeks post-operative. In addition, the collagen-GAG matrix substrate was degraded by 6 weeks post-operative. These results suggest that a more rapidly degrading collagen tube could still provide the guidance necessary for axonal regrowth.

Fabrication of a family of collagen tubes which vary according to pore size and degradation rate would lead to a study which could result in optimization of the degradation rate of the tube. Complete resorption of the tube between 6 and 30 weeks would reduce the
possibility of degenerative changes due to the presence of the tube. In vitro collagenase degradation studies could be used to predict the half-life of implanted tubes. In vitro thermal stability assays can also be used to determine an approximate in vivo life of the tubes. Permeability has been shown to positively effect the regeneration of axons; however, the pore size which maximizes regeneration is unknown. Varying the permeability of the tubes would give insight into the optimum pore size.

4.2.2 Quantitative Morphology

Replacement of the Silicone Tube with a Collagen Tube

One objective of this study was to determine whether the silicone tube could be replaced with a resorbable, collagen tube and achieve similar regeneration. All of the empty tube groups, silicone, porous collagen, and non porous collagen, were equivalent with respect to the total number of myelinated axons per regenerated nerve (See Figure 3.17). The empty porous and non porous collagen tubes, however, had significantly more large diameter axons (> 6 μm) than the empty silicone tube group (p=0.005 & p=0.03) (See Figure 3.20). In the matrix-filled groups, the silicone and porous collagen tubes were equivalent, while the non porous tubes had significantly less axons per nerve (p=0.01 & p=0.007). All three matrix-filled groups had an equivalent number of large diameter axons. The porous collagen tube performed equivalently or better than the silicone tube morphologically, in both the empty and matrix-filled cases. The non porous collagen tube performed equivalently or better than the silicone tube in three of the categories, but had fewer axons per nerve in the matrix-filled case. These results suggested that the silicone tube can be replaced by the porous collagen tube, in both the empty and matrix-filled cases, while achieving similar regeneration of axons.

Effect of Filling Tubes with Collagen-GAG Matrix

Another objective was to determine if the collagen-GAG matrix improved the regeneration of axons based on histological assessment. Overall, the analysis of variance
indicated a significant effect of the presence of matrix on the total number of myelinated axons per nerve (p<0.005) and on the number of large diameter axons (p<0.005). The matrix-filled silicone tube (n=5) was superior to the empty silicone tube (n=6) with respect to number of axons (p=0.01) and the number of large diameter axons (p<0.001).

Although the mean of the matrix-filled collagen tubes (n=6) was higher in all cases than the empty tubes (n=5 for PC, n=6 for NPC), there were no significant differences between the empty and matrix-filled collagen tubes (p>0.15 in each case). Filling with the collagen-GAG matrix significantly improved the performance of the silicone tube in all morphological categories. In the collagen tubes, both porous and non porous, the matrix increased the number of axons and large diameter axons over the empty tubes; however, the increase was not significant.

**COMPARISON WITH THE AUTOGRAFT STANDARD**

A third objective was to compare the experimental groups with the clinical standard, the autograft. With respect to the total number of axons per nerve, the empty porous collagen, matrix-filled porous collagen, and matrix-filled silicone tube groups were similar to the autograft. The matrix-filled porous collagen tube group was the only experimental group that was equivalent to the autograft based on the number of large diameter axons. The matrix-filled porous collagen tube; therefore, performed similarly to the autograft in all histological categories using the student’s t-test, indicating that a completely resorbable prosthesis could replace the current clinical standard.

**EFFECT OF TIME ON REGENERATION**

Previous results, at 6 weeks post-operative, were compared to the current results, at 30 weeks post-operative, to examine the improvement of morphology over time. Prostheses of identical origin were used in each study, allowing the direct comparison of groups at two time points. Figure 4.3 shows the total number of myelinated axons per nerve for each prosthesis at 6 and 30 weeks. Most of the prostheses improved significantly over time with the exception of the empty silicone (p=0.5), matrix-filled non porous
collagen ($p=0.61$), and autograft ($p=0.67$) groups. In a previous study, it was observed that during the first 12 weeks of regeneration, the number of axons increased rapidly; after that time, the number of axons continued to rise slowly over time, and then finally, decreased to within the normal range by 2 years post-operative$^{45}$. These findings may explain the large standard errors at 6 weeks, since, at that time the number of axons was changing at a rapid pace. Large animal to animal variations should be expected during the early stages of regeneration. The number of large diameter axons at 6 and 30 weeks is shown in Figure 4.4 for each prosthesis group. Again, for most groups, the number of large diameter axons increased over time. The exceptions were the empty ($p=0.14$) and matrix-filled ($p=0.15$) non porous collagen tube groups, and the empty silicone ($p=0.52$) tube group. The large diameter data for the empty and matrix-filled porous collagen tube groups are plotted as a function of time in Figure 4.5. Improvement over time is clearly
visible in this plot. At 6 weeks, the two groups were indistinguishable, but at 30 weeks the two were beginning to diverge. The matrix-filled porous collagen tube had 38% of the normal number of large axons, while the empty porous collagen tube had only 20% of normal at 30 weeks.

**Rank Ordering of the Various Prostheses**

The final objective of the morphometric analysis was to rank order the family of prostheses. Based on the overall histological performance, the matrix-filled porous collagen tube appeared to be the superior choice for axonal regeneration. It performed equivalently or better to the matrix-filled silicone tube in all categories, indicating that the silicone tube can be adequately replaced by the porous collagen tube. In addition, it was the only prosthesis to perform as well as the autograft in all histological categories. This was perhaps the most important finding, since the autograft is the current clinical standard.

**Figure 4.4** Total number of large (≥ 6 µm), myelinated axons per nerve at 6 and 30 weeks post-operative for all prosthesis groups.
Table 4.1 summarizes these findings. The second best prosthesis was the matrix-filled non porous collagen tube. Although it had significantly fewer axons per nerve than the matrix-filled silicone and the autograft groups, it had an equivalent number of large diameter axons to the matrix-filled silicone tube group. In addition, the nerve trunk generated by this implant qualitatively, was similar to normal nerve. The axons were very dense within the regenerated nerve. Another positive result for this group was that the animal to animal variation was very small at 30 weeks. The results for this implant group were very consistent, which can be seen from the small error bars (Figures 4.3 & 4.4). The disadvantage of the matrix-filled non porous collagen implant was the collapse of the tube. Because the lumen area decreased by as much as 83%, the available area for axons was

![Graph showing total number of large axons for empty and matrix-filled porous collagen tube implants as a function of time. Normal is shown for reference.](image-url)
TABLE 4.1 Table summarizing the histological results in the matrix-filled silicone, matrix-filled porous collagen and autograft groups at 30 weeks post-operative.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Number of Axons</th>
<th>Total Number of Large Diameter (≥ 6 μm) Axons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix-Filled Silicone (n=5)</td>
<td>12044 ± 616</td>
<td>999 ± 130</td>
</tr>
<tr>
<td>Matrix-Filled Porous Collagen (n=6)</td>
<td>16689 ± 2080</td>
<td>1303 ± 305</td>
</tr>
<tr>
<td>Autograft (n=6)</td>
<td>13957 ± 1408</td>
<td>1821 ± 221</td>
</tr>
</tbody>
</table>

very small compared to that for the porous collagen and silicone tube groups. This limited the total number of axons. The remaining groups performed similarly, with the exception of the empty silicone tube (n=6) which had 67% of the animals with the total number of regenerated axons equal to zero. The inhibited repair group had no tissue, as expected, and proved to be an adequate negative control.

4.3 FUNCTIONAL AND MORPHOLOGICAL RELATIONSHIPS

4.3.1 EFFECT OF MORPHOLOGY ON CONTRACTURE FORMATION

The formation of chronic contractures is a common problem in the clinical situation after peripheral nerve injury. Contractures can be attributed to partial muscle paralysis or muscle imbalance\(^7\). In the clinic, contractures can be avoided by using physical therapy methods; however, in animal studies this is not feasible.

Use of the gait analysis technique is impaired by the presence of toe contractures\(^17\); which in the current study amounted to 82% of the population (See Figure 3.6). Several investigators reported problems associated with the development of contractures. By 14 weeks, 35% of rats repaired with direct suture had developed contractures\(^53\) and by 24 months, 100% of rats repaired with direct suture had developed contractures\(^17\). In some studies, employing a silicone tube bridging a 1-cm gap, a 1-cm autograft\(^64\) and no repair\(^7\),
contractures were not reported. These studies indicate that the development of a toe contracture was dependent on the type of repair or implant.

Our findings also suggest that contracture development was implant dependent (See Figure 3.6); however, there was a much stronger correlation found with morphometric results, regardless of implant type. Of the animals that did not develop a toe contracture (n=9), the mean number of myelinated axons per nerve was 40 ± 40 axons/nerve, with only one of the animals having any axons. Animals which developed a contracture (n=35) had an average of 11972 ± 800 axons/nerve, which is shown graphically in Figure 4.6. Fisher’s exact test\(^3\) was performed based on the data given in Table 4.2. The test revealed a highly significant correlation (p<0.001) between regeneration of more than 500 axons

![Figure 4.6](image-url)

**Figure 4.6** Total number of myelinated axons at 30 weeks for the animals that had contracture and the animals that did not develop a contracture. Error bars represent standard error of the mean.
and the development of a toe contracture. In addition, observations recorded at each walking session, revealed that in 7 cases, the animal spread the toes visibly one session (2 weeks) prior to the formation of a contracture. Spreading of the toes was not observed at other times during the study. These results suggest that the development of toe contractures may be related to the reinnervation of distal muscles since, animals with axonal regeneration developed the condition and, in addition, certain animals displayed functional return just prior to contracture development. Similar findings were obtained in an electrophysiological study, in which animals with successful regeneration, based on the return of compound muscle action potentials, formed toe contractures\textsuperscript{14}. These findings support the theory that a natural imbalance in the antagonist muscle strength (flexor muscles being stronger than extensor muscles) is responsible for the formation of toe contractures.

The time period in which the contractures developed also supported the theory that contractures were related to reinnervation. All of the contractures developed between 6 and 18 weeks post-operative. An electrophysiological study recorded the first responses to distal stimuli at 12-17 weeks post-operative for silicone tubes filled with several types of collagen-GAG matrices\textsuperscript{15}. This indicated that at 12 weeks, enough axons had reached the distal muscle targets to generate a measurable signal, and that all animals had a recognizable signal no later than 17 weeks. This post-operative period in the previous study\textsuperscript{15} is similar to the 18 week period in which all animals developed contractures in the current study. It has been shown that, in an empty silicone tube with a 10-mm gap, axons reach the distal

<table>
<thead>
<tr>
<th>Greater than 500 axons at 30 weeks</th>
<th>Animals With No Contracture</th>
<th>Animals With Contracture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0/9</td>
<td>35/35</td>
</tr>
</tbody>
</table>

Table 4.2 Number of animals based on contracture development and total number of axons per nerve at 30 weeks, regardless of prosthesis group.
stump after 5-6 weeks\textsuperscript{78}. This result suggests that it is feasible for the muscles to have some innervation, in the more proximal muscle groups, by 6 weeks post-operative.

Comparison of the total number of axons at 30 weeks post-operative and the week in which the animal developed the toe contracture revealed a correlation. Figure 4.7 shows the number of axons per nerve versus the week in which the contracture formed. Each data point represents one animal, regardless of experimental group. Linear regression produced a coefficient of determination ($R^2$) value of 0.6181, which indicated that the linear relationship explains 62% of the experimental variability. The corresponding correlation coefficient ($R$) was 0.79 for the relationship. Although the data do not fit a linear model, the plot shows a trend toward the earlier development of contracture when an animal had a larger number of axons. A similar, but weaker, relationship existed with the number of large, myelinated axons (Figure 4.8).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.7.png}
\caption{Total number of myelinated axons at 30 weeks post-operative as a function of the time when the contracture developed.}
\end{figure}
The results of the current study make a convincing argument that the formation of toe contractures after sciatic nerve transection and repair was caused by reinnervation of the distal target muscles, and was not due to the formation of scar tissue, poor regeneration, or muscle paralysis. The flexion contracture may be formed by the reinnervation of the naturally stronger flexor muscles, while the antagonist extensor muscle group either innervates more slowly or less completely causing a muscle imbalance. It was also shown that the time when the contracture formed was loosely related to the total number of myelinated axons per nerve.

4.3.2 Effect of Morphology on Gait

One objective of the current study was to compare the functional (gait) and morphological measurements. Based on Figure 4.9 it appears that the sciatic functional index (SFI) was independent of the total number of myelinated axons per nerve. The data
shown represent the sciatic functional index and the axons per nerve for each animal, regardless of prosthesis group. The normal SFI and axon count data are included on the plot. The individual footprint measurements (print length, toe spread, and intermediate toe spread) also had no correlation to the number of axons per nerve (See Appendix C for additional plots). Other investigators have been unable to correlate results from the gait analysis to the number of axons per nerve. Shenaq, et al64, bridged a 1-cm gap in the sciatic nerve with a collagen tube, a silicone tube, and an autograft and found no correlation between the SFI measurements and the axon counts at 17 weeks. The results of the Shenaq study were very similar to the results of the current study, with SFI values between -90 and -95, and axon counts between 8500 and 12000 axons per nerve (taken at 17 weeks); however, no toe contractures were reported.
There are two possible explanations why functional (gait) and morphological (axons per nerve) results do not correlate. The first is that, although many axons regenerated, they did not connect to the appropriate distal endpoints, which made them functionally useless. There is some evidence that axons have preference to their original distal endpoints\textsuperscript{62} and that motor axons can preferentially reinnervate the appropriate nerve branches more successfully than sensory axons\textsuperscript{11}. Once the axon reaches the endpoint; however, it is unclear how successful the axon is at re-forming a functional synapse.

Three events are required for the axon to become functional after injury: (1) the axon must survive transection and regenerate across the defect site, (2) it must reach an appropriate target organ, and (3) it must make a functional synapse\textsuperscript{47}. In this study, the first event has clearly occurred because the axons were observed and counted in the mid-section of the defect. In a previous study\textsuperscript{15}, which employed a collagen-GAG matrix-filled silicone tube, the compound muscle action potential amplitude recovered to within normal range by 40 weeks post-operative. These results indicated that the muscle was reinnervated and synaptic reconnection had occurred (satisfying (2) and (3))\textsuperscript{15}. The axons which reinnervated the muscle, however, may not be the appropriate axons. For example, sensory axons may have innervated muscle end points forming a functional, but useless, connection. This inappropriate connection of axons may have caused the functional scores to be poor. The second possible explanation is that the SFI technique is not appropriate for use in nerve transection studies. The method was developed by deMedinaceli to study primarily crush injuries and the transection with no repair was used as a negative control\textsuperscript{18}. Other investigators have had some success using the SFI technique with transection and direct suture repair procedures\textsuperscript{31,32,53}, however, in those studies, the direct suture procedure was being compared to a ‘no repair’ control. In studies where a gap existed between nerve stumps\textsuperscript{29,64}, no differences between groups, including the negative control, could be detected. These results, along with the results of the current study, suggest that the SFI technique is not sensitive enough to distinguish between repair groups.
Although the total number of axons per nerve did not correlate with the SFI, it appeared that the number of large diameter axons did correlate. Figure 4.10 shows the number of large (≥ 6 μm), myelinated fibers compared to the SFI, both measured at 30 weeks post-operative. Each data point represents one animal, including all prosthesis groups and normal. The linear regression line is shown on the plot and has a coefficient of determination \( R^2 \) of 0.7167, indicating that the linear relationship explains 72% of the experimental variability. The corresponding correlation coefficient \( R \) is 0.85. The normal data appear in the upper, right corner of the plot and help to assist in interpretation of the data. Similar plots for the print length, toe spread and intermediate toe spread can be found in Appendix C. Two factors support the relationship between large diameter axons and the SFI. The first, is that large diameter axons are known to be responsible for controlling motor function and have the highest conduction velocities\(^7\). The second

\[
y = 34.617x + 39.997 \\
R^2 = 0.7167
\]

**Figure 4.10** Total number of myelinated axons greater than 6 μm plotted against the sciatic functional index (SFI), both measured at 30 weeks post-operative.
possible explanation, is that large diameter axons have probably reached a target organ (step (2) above) and made a functional connection (step (3) above). This is evident because of the axon diameter. Regenerating axons grow until they reach and connect to an endpoint. Once the axon makes a functional connection, it begins to increase in diameter. This suggests that large diameter axons have reached and connected to an endpoint. It is still unknown, however, whether the axons have connected to an appropriate target organ. The axons which have not connected to an endpoint will eventually degenerate. This makes it likely that, after a longer time period, the number of axons would decrease and correlate with the SFI.

4.3.3 Effect of Morphology on Muscle Mass

The histological evaluation of peripheral nerves after injury was intended to determine the extent of peripheral nerve regeneration; however, it was also necessary to compare that data directly to a functional measurement. Muscle mass has been observed, qualitatively, to correlate with electrophysiological results. In the current study, muscle mass was measured quantitatively by recording the circumference of the experimental limb in two locations: the level of the gastrocnemius and the level of the thigh. The muscle mass circumference, in both locations, did not correlate with the total number of myelinated axons per nerve (See Appendix C for plots). Similar to the gait analysis, the number of large diameter (≥ 6 μm), myelinated axons seemed to have a weak correlation with the muscle mass data. Figure 4.11 shows the number of large diameter axons plotted against the muscle mass circumference at the level of the gastrocnemius. The linear regression line is shown and represents 55% of the experimental variability ($R^2 = 0.5546$, $R = 0.74$). A similar relationship existed with the muscle mass at the level of the thigh with the regression line explaining 48% of the experimental variability ($R^2 = 0.4778$, $R = 0.69$) (See Appendix C for plot). Overall, the correlation between morphology and muscle mass was very weak.
FIGURE 4.11 Total number of myelinated axons ≥ 6 μm plotted against the muscle circumference at the level of the gastrocnemius (20 mm from heel). Both measurements were made at 30 weeks post-operative.

4.4 ROLE OF CONTRACTILE CELLS

Myofibroblasts were found in two distinct locations in the implants harvested at 6 weeks post-operative: within the defect site and adjacent to the tube surface. The finding of myofibroblasts in one location was independent of the other. Within the defect site myofibroblasts were aligned parallel to the nerve axis (See Figure 3.22). These cells are discussed in section 4.4.1. Adjacent to the tube surface, myofibroblasts were aligned circumferentially around the tube, on both the inner and outer surfaces (See Figure 3.27). Tissue inside the tube, but directly apposed to the tube, was considered adjacent to the tube surface, and not a part of the defect site. These cells are discussed in section 4.4.2.
4.4.1 WITHIN THE DEFECT SITE

The identification of myofibroblasts within the defect site represents the first report of the cells in healing peripheral nerve. Unlike the dermal wound healing model, the presence of the collagen-GAG matrix did not affect the number of myofibroblasts within the wound bed based on qualitative observation. Tube type also had no effect on the presence of the myofibroblasts within the defect site.

The histological findings of the current study indicated that there was a statistically significant relationship (p=0.005) between the presence of myofibroblasts and a total number of axons greater than zero in the peripheral nerve at 6 weeks post-operative. In addition, myofibroblasts were found exclusively in the distal segment of the healing peripheral nerve and were elongated parallel to the nerve axis (See Figure 3.22). These findings, combined with the known effects of mechanical forces on nerve cells in vitro (See Section 1.6), suggested that the myofibroblasts may play a role in the elongation of axons. Figure 4.12 is a schematic of a proposed role of the myofibroblast in peripheral nerve wound healing. In the proposed mechanism, myofibroblasts provide tensile forces

![Figure 4.12](image_url)

**Figure 4.12** Schematic showing the proposed mechanism for axonal regeneration involving myofibroblasts. Not to scale.
which are applied to the extracellular matrix via the fibronexus. The advancing axon tips are attached to the matrix through adhesion molecules. As the myofibroblasts pull on the matrix, tension is applied to the axons. Through this proposed mechanism, the myofibroblasts provide ‘towed growth’ of the axons.

Each of the histological results is consistent with this proposed mechanism. First, myofibroblasts were only found in the defect site when axonal elongation had occurred. This suggested the presence of the cells was related to the elongation of axons and not the formation of scar tissue, since scar tissue would be equally or more prevalent in cases where no regeneration of axons had occurred. Also, the orientation of the cell axis, parallel to the nerve axis, was conducive to pulling the axons toward the distal stump. Finally, the cells were located distal to the mid-line of the tube and into the distal stump (See Figure 3.24), an observation which correlated with the approximate location of the advancing axons. Williams reported that axons crossed a 10-mm gap, ensheathed in a silicone tube, within 5-6 weeks of injury. In the six week samples in the current study, axons were found entering the distal stump. Myofibroblasts were identified at this location, but not at more proximal sites.

Additional studies are necessary to test the proposed mechanism. A study which incorporates varying sacrifice times, from 1 to 6 weeks post-operative, would provide samples which follow the spatial-temporal progress of the axons and the myofibroblasts. The co-localization of myofibroblasts and axons within the defect site at locations along the tube length would help verify that the two are coupled. In addition, transmission electron microscopy is necessary, within the defect site, to confirm that myofibroblasts are present by identifying the ultrastructural features.

4.4.2 APPOSED TO THE TUBE EDGES

Myofibroblasts have been found to be responsible for tissue contraction around certain silicone implants; for example, breast implants, but have not been previously
reported on the surface of silicone tubes bridging nerve defects. The contractile capsule, found on both the inner and outer tube surfaces, was present at six weeks post-operatively. The capsules adjacent to the silicone tubes, both on the inner and outer surfaces, were several cells thick and completely cell continuous around the entire tube. These results were found using light microscopy and the cell type confirmed using transmission electron microscopy on cells located at the tube surface. Only one collagen tube had myofibroblasts at the tube edge, and the cells were randomly dispersed and not cell continuous. This finding supports using degradable collagen tubes which were less likely to develop a contractile capsule.
APPENDIX A: PROTOCOLS

A.1 COLLAGEN-GLYCOSAMINOGLYCAN 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.

11. Reblend remaining solution at 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, reblend 15 minutes on LOW speed, 4°C, before using if stored more than four weeks).
A.2 COLLAGEN-GAG MATRIX MANUFACTURING 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). Squirt 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. Be sure to 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. Dearate 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 (Becton Dickinson model 5604, Becton Dickinson Co., Rutherford, NH). Expel air bubbles. Attach a 25 gauge needle (Becton Dickinson 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 about 3-5 mm of needle is beyond the silicone plug.

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. 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.
9. Attach the large gear to either motor on the freezing apparatus. Tape four prepared PVC jackets to the PVC hanger using colored tape. Place the hanger on the gear train and 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. 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 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. 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 the 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.

**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) and let it cool until it reaches -45°C.

3. When the shelf reaches -20°C, insert the product.

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 **NERVE PROSTHESIS ASSEMBLY AND STERILIZATION PROTOCOL**

**One to two days before graft preparation:**

1. **Sterilize the necessary implements:**

   **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 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 through 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. Done the same as with the matrix.

   **Sterilize Liquids Using ZapCaps and autoclaved bottles:**

   - 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 prostheses
   - 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 tubes, 1 for matrix, 1 for the pen)

3. Put on latex gloves, a cap, a mask and a clean, disposable lab coat. Wipe bench, metal frames and ring stand with 70% ethanol.

4. Using sterile techniques, set up sterile field with Teflon pad. Open all tool packs and pour onto sterile field, including prosthesis, 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, store in 70% ethanol and rinse in two rinses of sterile saline solution immediately before surgery). Carefully open Whirl-pak bags and stand on metal frames in hood.

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

6. Trim prosthesis tubes to 20mm using scalpel (19mm 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 10mm segments to be inserted into the center of the trimmed tubes.

7. Place prostheses into specimen jars with PBS, close jars tightly, label, place in Whirl-paks, and close bags. Label each prosthesis by type: Se for silicone empty, Sm for silicone with matrix, PCe for porous collagen empty, PCM for porous collagen with matrix, NPCe for non porous collagen empty, and NPCm for non porous collagen with matrix.
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 meal 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
   1 bottle pentobarbital (Nembutal Sodium Solution), 50mg/ml
   10-0 sutures
   4-0 sutures
   1 ml syringes
   sterile pen

4. Ready other non-sterile items:

   surgical board
   4 rubber bands
   rat ear punching tool
   numbered ear tags
   microsurgery glasses
   hair clippers
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, the surgical area should be arranged 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, the animal should be shaved 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. The animal is placed on the surgical board in the prone position with the arms and legs secured to the board using rubber bands. The legs should be in 30° abduction. A piece of gauze is placed under the appropriate thigh to elevate the leg slightly.

7. The shaved portion of the animal is cleaned 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. A hole is cut in the sterile draping small enough so that only the leg is exposed. Place the draping over the animal.

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

9. Using the surgical scissors, the muscles are separated until the sciatic nerve is visible. The muscle is then carefully cut back along the skin incision line exposing the sciatic nerve.

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

11. **For a tubular repair:**
   The nerve is transected midway between the proximal nerve trunk and the distal bifurcation using microscissors. The prosthesis is measured and a mark placed 5 mm in on each end. The tube is placed in the gap and the proximal nerve stump placed 5 mm into the tube end as marked. The nerve is secured in place by using two 10-0 sutures which travel through the epineurium and then through the tube. The sutures are tied with four single knots. The distal nerve end is placed 5 mm into the other end of the tube and secured in the same manner.

   **For an inhibited repair device:**
   The procedure is the same as for a tubular repair except the distal end is not inserted into the tube, it is left resting on the underlying tissue. The 10 mm gap is measured from the proximal nerve stump to the inside edge of the silicone plug.
**For an autograft:**
A 10 mm segment in the middle of the nerve is marked off using the sterile pen. At the proximal end, 10-0 pre-sutures are placed on either side of the mark. The nerve is transected at the marked point using microscissors, being very careful not to cut through the pre-sutures. Once transected, the pre-sutures are pulled tightly and tied off using four single knots. Another 10-0 suture is placed at the proximal end of the autograft to secure the nerve. The same procedure is then followed at the distal nerve end.

**For a sham operation:**
Nothing is done during this step. The nerve is left undamaged.

12. The paper clip retractors are removed. The muscle is closed using three 4-0 sutures. The skin is closed using two 4-0 sutures and three skin staples.

13. The animals are placed back in the cage and observed frequently until they are awake.
A.5 WALKING TRACK PAPER DYEING PROTOCOL

SOLUTIONS:

0.5% Solution Bromphenol Blue in Absolute Acetone

Bromphenol Blue: Sulfone Form Sigma #: B 0126

Bromphenol Blue 2.0 grams
Absolute Acetone 480 mL

(Amounts can be adjusted as needed for more or less paper)
(2 grams of Bromphenol Blue makes approximately 25 sheets)

PROCEDURE:

1. Turn on drying oven to medium power setting and set the temperature to 125°C.

2. Let oven heat until the temperature reaches 125°C. Place the following items in the oven for approximately 1 hour or until dry:
   - Metal pan (cookie sheet)
   - Metal spatula
   - Metal weighing dish
   - Glass flask: choose the appropriate size
   - Graduated cylinder: choose the appropriate size
   - Stir bar
   - Forceps

3. Remove items and place on a cool surface.

4. Measure out Bromphenol Blue on the dry weighing dish.

5. Measure out absolute acetone in the hood.

6. Add approximately half of the acetone to the flask. Pour in bromphenol blue from the weighing dish.

7. Place stirring plate in the hood and begin to mix the solution. Pour some of the remaining acetone into the weighing dish to remove all the bromphenol blue from the dish. Pour into flask.

8. Add remaining acetone and allow to mix thoroughly.

9. Line the surface of the hood with paper towels to avoid a mess. Place pan in the center of the hood and pour the solution into the pan.

10. Pass paper strips through the solution using the forceps and immediately hang to dry (in the hood!).

11. When dry, after about 5 minutes, place paper in a Ziploc bag for storage.
A.6 PRE-SACRIFICE ANALYSIS PROTOCOLS

Pinch Test

PROCEDURE:

1. The animal was held firmly by the scruff of the neck, allowing the hind legs to hang freely.
2. Using forceps, the inner toe and outer toe were pinched deeply on the normal foot to indicate the type of response the animal would give to pain (vocalization, withdrawal of the foot, or muscle flexion).
3. The skin of the outer toe was pinched on the normal foot and monitored for type of response. With the cutaneous pain, the forceps only pinched the top layer of skin.
4. The experimental foot was then tested in the same manner. Inner toe first, then outer toe and then the cutaneous region. Responses were considered positive if they closely matched the magnitude of the normal response. If a weaker response was detected, it was labeled as a partial response.

Muscle Mass Measurement

PROCEDURE:

1. The animal was weighed and then anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal Sodium Solution, 50 mg/ml). The dosage of anesthetic was 50 mg of solution per kg of animal. This measurement is taken prior to sacrifice.
2. Both legs of the animal were shaved using clippers.
3. On both the normal and experimental leg, a mark was placed at 20 mm and 30 mm proximal to the heel.
4. At each mark, the circumference of the leg was measured using a marked piece of string. The string was pulled tight, but was not pinched or constricting the tissue. The string was then measured linearly using a ruler.
A.7 ANIMAL SACRIFICE AND TISSUE PROCESSING PROTOCOL

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)

SOLUTIONS

Yanoff's Fixative

Stock Solutions

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

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

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

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.
PROCESSING PROCEDURE

1. Explant sciatic nerve and the proximal portions of the tibial and peroneal nerve. Clearly mark the distal portion of the tibial nerve with a suture.

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 according to Figure A.1.

    L= Liquid storage, P=Paraffin embedding, E=Epon embedding.

**Figure A.1** Sectioning diagram for the rat sciatic nerve and distal branches.

7. Place each small nerve section into an individual 1 dram vial containing 70% EtOH. Process each according to the specific embedding protocol that applies.
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 in 100 ml of distilled water

Stock B: 0.2 M HCl (mw 36.46)
    1.7 ml HCl in 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

2% Cacodylate Buffered Glutaraldehyde

8 ml 25% Glutaraldehyde
92ml Cacodylate Buffer

0.2 M Cacodylate Buffered Sucrose Solution

6.846 grams Sucrose (mw 342.3)
100 ml Cacodylate Buffer

1% Osmium Tetroxide
(Cat# 0972A, Polysciences Inc, Warrington, PA)

2 ml 4% Osmium Tetroxide
6 ml Distilled Water

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

1. Soak nerves in 2% cacodylate buffered glutaraldehyde for 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 1.5 to 2 hours at room temperature (in the hood).

5. Dehydrate nerves in EtOH
   - 30% 5 minutes
   - 50% 5 minutes
   - 70% 5 minutes
   - 80% 5 minutes
   - 90% 5 minutes
   - 95% 5 minutes
   - 100% 5 minutes
   - 100% 5 minutes
   - 100% 5 minutes

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 (May require more time).

*During infiltration, the Epon mixture should NOT contain the hardener.
A.8.2 PARAFFIN EMBEDDING PROTOCOL

SOLUTIONS

**Eosin Y**
(Cat# E-511, Fisher Scientific, Fair Lawn, NJ)
1 gram Eosin
1 Liter 70% EtOH

**Paraffin**
(Paraplast Plus, Cat#15159-464, VWR Scientific, Boston, MA)

PROCEDURE

1. Stain nerves in Eosin for 1 minute.

2. Dehydrate nerves in EtOH

<table>
<thead>
<tr>
<th>EtOH Concentration</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70%</td>
<td>5 min</td>
</tr>
<tr>
<td>70%</td>
<td>5 min</td>
</tr>
<tr>
<td>80%</td>
<td>5 min</td>
</tr>
<tr>
<td>90%</td>
<td>5 min</td>
</tr>
<tr>
<td>95%</td>
<td>5 min</td>
</tr>
<tr>
<td>100%</td>
<td>5 min</td>
</tr>
<tr>
<td>100%</td>
<td>5 min</td>
</tr>
</tbody>
</table>

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

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

5. Embed in paraffin.

6. Cool and store in refrigerator.
A.8.3 HEMATOXYLIN AND EOSIN STAINING PROTOCOL

TECHNIQUE: paraffin embedded

SOLUTIONS:

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

**Eosin Y**
(Cat# E-511, Fisher Scientific, Fair Lawn, NJ)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin</td>
<td>1 gram</td>
</tr>
<tr>
<td>EtOH</td>
<td>1 Liter</td>
</tr>
</tbody>
</table>

Weak ammonium hydroxide water

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium hydroxide</td>
<td>2-4 Drops</td>
</tr>
<tr>
<td>Tap water</td>
<td>500 mL</td>
</tr>
</tbody>
</table>

STAINING PROCEDURE:

1. Deparaffinize and hydrate to distilled water
   - xylene 2 minutes 2X
   - 100% EtOH 1 minute 2X
   - 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 2X
   - xylene 2 minutes 2X

9. Mount with mounting medium (Cytoseal 60, Cat# 8310-16, Stephens Scientific).
A.8.4 Masson's Trichrome Staining Protocol

Fixation: Bouin's or 10% buffered neutral formalin
Technique: paraffin embedded, sectioned at 5-10μm

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 Alcohol, 95%

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 Fuchsin Solution**
(Cat# B-6008, Sigma Chemical Co., St. Louis, MO)

- 90.0 ml Biebrich scarlet, aqueous 1%
- 10.0 ml Acid fuchsin, aqueous 1%
- 1.0 ml Glacial acetic acid

**Phosphomolybdic-Phosphotungstic Acid Solution**
(Cat# 2891, Baker Chemical Co., Phillipsburg, NJ)

- 5.0 gm Phosphomolybdic acid
- 5.0 gm Phosphotungstic acid
- 200.0 ml Distilled water

**Aniline Blue Solution**
(Cat# 02570, Polysciences Inc., Warrington, PA)

- 2.5 gm Aniline Blue
- 2.0 ml Glacial acetic acid
- 100.0 ml Distilled water

**1% Glacial Acetic Acid Solution**

- 1.0 ml Glacial acetic acid
- 100.0 ml Distilled water
STAINING PROCEDURE:

1. Deparaffinize and hydrate to distilled water
   - xylene 2 minutes 2X
   - 100% EtOH 1 minute 2X
   - 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, if formalin fixed.

3. Cool and wash in running water until yellow color disappears

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 10 minutes. Save solution.

11. Rinse in distilled water.


13. Rinse in distilled water.

14. Dehydrate and clear
   - 70% EtOH 1 minute
   - 80% EtOH 1 minute
   - 95% EtOH 1 minute
   - 100% EtOH 1 minute 2X
   - xylene 2 minutes 2X

15. Mount with mounting medium (Cytoseal 60, Cat# 8310-16, Stephens Scientific)
A.8.5 Toluidine Blue Staining Protocol

Fixation: 10% buffered neutral formalin. Post-fixed with 1% Osmium Tetroxide.

Technique: Epon Embedded, sectioned at 1 μm.

Solutions:

**Toluidine Blue Solution**
(Cat# BP107-10, Fisher Biotech, Boston, MA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluidine Blue Powder</td>
<td>1 gm</td>
</tr>
<tr>
<td>Sodium Borate Powder</td>
<td>1 gm</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Solution should be filtered and stored in a stopped bottle.

Staining Procedure:

1. Heat slide 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 (Cytoseal 60, Cat# 8310-16, Stephens Scientific).
A.8.6 IMAGE CAPTURE AND ANALYSIS PROTOCOL

COMPUTER SETUP:
1. Go to the Apple menu, under ‘Control Panels’ select ‘MODE32’. Make sure that MODE32 is enabled.
2. Go to the Apple menu, under ‘Control Panels’ select ‘Memory’. 32 Bit Addressing should be on.
4. Restart computer if any changes were made.

MICROSCOPE SETUP:
1. Turn the microscope on (green button on the right side).
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.

VIDEO BOX SETUP:
1. Turn the video box on. 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 accordingly.

IMAGE CAPTURING PROCEDURE:
1. Open the ‘Tsunami’ drive. Open the ‘applications’ folder. Open the July DIGIT.
2. Find the tissue on the microscope at the appropriate magnification.
3. Select “Grab live video” from “View” menu, select “Full size” and “Grab Frames”.
4. Measure the tissue area and use Table A.1 to determine the correct number of images to capture.

<table>
<thead>
<tr>
<th>Fascicle Area Range (mm²)</th>
<th>Number of Images Necessary</th>
<th>Image Area/Total Fascicle Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;0.355</td>
<td>5</td>
<td>≥10%</td>
</tr>
<tr>
<td>0.355&lt;A&lt;0.639</td>
<td>9</td>
<td>10% - 18%</td>
</tr>
<tr>
<td>0.639&lt;A&lt;0.923</td>
<td>13</td>
<td>10% - 14%</td>
</tr>
<tr>
<td>0.923&lt;A&lt;1.207</td>
<td>17</td>
<td>10% - 13%</td>
</tr>
<tr>
<td>1.207&lt;A&lt;1.49</td>
<td>21</td>
<td>10% - 12%</td>
</tr>
<tr>
<td>A&gt;1.49</td>
<td>21</td>
<td>&lt;10%</td>
</tr>
</tbody>
</table>

**Table A.1** Table indicating the number of images for a given section.
6. Follow the following directions to capture the correct number of images from each nerve section. After each image is captured, ‘Save’ the file, ‘Close’ the document, and select a ‘New’ document. Repeat steps 3 and 4 to grab the next image. (Don’t forget to save before closing the image, DIGIT will not warn you that the image has not been saved).

1. Position the nerve so that the long axis of the nerve is on the Y axis.

2. Divide the nerve into 4 quadrants. Count the number of images from the top center to the bottom center (Y), and the number of images from the left center to the right center (X).

3. Begin Imaging. (I find this order to be the easiest.) Refer to Figure 1 to see image placement.

   Image 5. Go to the geometric center of the cross section and capture.
   Image 11. Go to the geometric center of quadrant 1 and capture.
   Image 12. Move up 1/8 Y, and right 1/8 X and capture.
   Image 15. Move 1/4 X to the left and capture.
   Image 14. Move 1/4 X to the right and capture.
   Image 21. Go to the geometric center of quadrant 2 and capture.
   Image 22. Move up 1/8 Y, and right 1/8 X and capture.
   Image 25. Move 1/4 X to the left and capture.
   Image 23. Move down 1/4 Y and capture.
   Image 24. Move 1/4 X to the right and capture.
   Image 31. Go to the geometric center of quadrant 3 and capture.
   Image 32. Move up 1/8 Y, and left 1/8 X and capture.
   Image 35. Move 1/4 X to the right and capture.
   Image 33. Move down 1/4 Y and capture.
   Image 34. Move 1/4 X to the left and capture.
   Image 41. Go to the geometric center of quadrant 4 and capture.
   Image 42. Move up 1/8 Y, and left 1/8 X and capture.
   Image 45. Move 1/4 X to the right and capture.
   Image 43. Move down 1/4 Y and capture.
   Image 44. Move 1/4 X to the left and capture.

*NOTE: If only 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. Make sure the appropriate images are captured. For example, image 15 follows 12 in the suggested order, usually this image will be skipped. Referring to the schematic is helpful.
Figure A.2 Schematic showing the placement of images around the nerve section.

Image Analysis Procedure:

1. Open NIH Image.

2. Open image file and save immediately using the NIH Image format. (When opening a file captured in DIGIT an error will appear that says invalid TIFF format, saving right away will eliminate this message from appearing again with that file).

3. Go to the “Analyze” menu and select “Set Scale”. Select micrometers as the unit of measure. Use the following table to fill in the “Known Distance” and the “Pixels” entries. (This table is calibrated for the microscope at the Brigham). When the scale is set a diamond appears next to the file name on the top bar of the window.

<table>
<thead>
<tr>
<th>Magnification Power</th>
<th>Known Distance</th>
<th>Pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4.15</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>1.66</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>0.83</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>0.415</td>
<td>1</td>
</tr>
<tr>
<td>100</td>
<td>0.166</td>
<td>1</td>
</tr>
</tbody>
</table>
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).

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. "SAVE AS" 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. Print the analyzed image.

13. Choose "Show Results" from the "Analyze" menu. Copy the results and paste them into Excel for analysis.

14. Close the image. **Do not save the changes to your edited file!**
A.8.7 α-Actin Staining Protocol

Fixation: Formalin
Technique: paraffin embedded, sectioned at 5-10mm

Solutions:

0.1% Trypsin
(Sigma Co. # T-8128)
0.01 grams
Trypsin
10 ml
Phosphate Buffered Saline
(Sigma Co. # P-3813)
0.01 grams
10 ml
Phosphate Buffered Saline

3% Hydrogen Peroxide
(Distilled Water)
1 ml
30 % Hydrogen Peroxide
9 ml
Distilled Water

a-Smooth Muscle Actin Immunohistochemical Staining Kit
(Sigma Co. # IMMH-2)
Kit includes:
Acetate Buffer
AEC Chromagen
Mixing Vial
Primary antibody (Mouse Monoclonal anti-a-Smooth Muscle Actin)
(Sigma #: A2547)
5 μl
Primary Antibody
2 ml
Phosphate Buffered Saline
Secondary Antibody (Goat anti-Mouse Immunoglobulin)
(Sigma #: A3682)
20 μl
Secondary Antibody
2 ml
Phosphate Buffered Saline

20% Goat Serum
(Sigma Co. # G-9023)
0.2 ml
Goat Serum
0.8 ml
Phosphate Buffered Saline

Gill's Hematoxylin Solution
(Sigma Co.)

Negative Control - 1% Mouse Serum
(Sigma Co. # M-5905)
0.1 ml
Mouse Serum
10 ml
Phosphate Buffered Saline

One milliliter of solution is the appropriate amount for 2 slides.
The following solutions can be used if a biotinylated antibody is needed. Two steps must be added to the procedure to switch to these antibodies. They are as follows:

9.3 PBS for 3 minutes (2X)
9.6 2 drops of the peroxidase reagent for 30 minutes

**Secondary Antibody (Biotinylated Goat anti-Mouse Immunoglobulin)**

(Sigma #: B0529)

<table>
<thead>
<tr>
<th>量 (μl)</th>
<th>说明</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Secondary Antibody</td>
</tr>
<tr>
<td>2</td>
<td>Phosphate Buffered Saline</td>
</tr>
</tbody>
</table>

**ExtrAvidin Peroxidase Reagent**

(Sigma #: E2886)

<table>
<thead>
<tr>
<th>量 (μl)</th>
<th>说明</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Peroxidase Reagent</td>
</tr>
<tr>
<td>1</td>
<td>Phosphate Buffered Saline</td>
</tr>
</tbody>
</table>

**STAINING PROCEDURE:**

1. Deparaffinize
   - xylene 2 minutes 2X
   - 100% EtOH 1 minute 2X
   - 95% EtOH 1 minute
   - 80% EtOH 1 minute
   - 70% EtOH 1 minute
   - PBS 3 minutes

2. Trypsin solution (0.1 %) for 60 minutes at 37°C.
3. PBS for 3 min
4. 2 drops of 3% H2O2 (Vial 5) for 5 minutes
5. PBS for 3 minutes
6. 20% goat serum (Blocking agent) for 10 minutes. Wipe slides but do NOT wash.
7. 2 drops of the primary antibody (Vial 1) or the negative control for 2 hours at 37°C.
8. PBS for 3 minutes (2X)
9. 2 drops of the secondary antibody for 60 minutes at 37°C.
10. PBS for 3 minutes (2X)
11. Prepare substrate reagent per instructions. Add to mixing vial (Vial 6):
   - 4 ml deionized water
   - 1 drop 3% H2O2
   - 2 drops Acetate Buffer (Vial 4A)
   - 2 drops AEC Chromagen (Vial 4B)
12. 2 drops substrate reagent (Vial 6) for 15 minutes.
13. Rinse in distilled water for 3 minutes
14. Gill's hematoxylin solution for 1 minutes
15. Rinse in distilled water.
16. Wash in running tap water 3 minutes
17. Mount with aqua-mount and coverslip.
APPENDIX B: CALCULATIONS

B.1 T-TEST CALCULATION TO DETERMINE SAMPLE SIZE

Equation for the t-test:

\[
t = \left\{\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(N_i - 1)S_i^2 + (N_j - 1)S_j^2}{N_i + N_j - 2}}}\right\} \left\{\frac{N_i N_j}{N_i + N_j}\right\}^{1/2}
\]

where,

- \(\bar{x}_i\) = mean for group i
- \(N_i\) = sample size for group i
- \(S_i^2\) = variance of group i

Since the test groups will be the same size, \(N_1 = N_2\)

The t-test equation simplifies to:

\[
t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2 + S_2^2}{2} \left(\frac{N}{2}\right)}}
\]

**Sample Calculation:**

Objective: Find an achievable difference between the means that will be significant

Sample size: \(N = 5\), sample size is the same for each group.

Standard deviation: \(S_1 = S_2 = 25\%\)

Degrees of freedom: \(DF = N_1 + N_2 - 2 = 8\)

for DF = 8, \(t = 2.31\) for 95% confidence.
\[
2.31 = \frac{\bar{x}_1 - \bar{x}_2}{\left( \frac{25^2 + 25^2}{2} \right)^{\frac{1}{2}}} (\frac{5}{2})^{\frac{1}{2}}
\]

\[
\bar{x}_1 - \bar{x}_2 = 36\%
\]

This was considered an achievable difference between means; therefore, \(N = 5\) was chosen as the sample size.
APPENDIX C: ADDITIONAL GRAPHS

C.1 GAIT ANALYSIS

![Graph showing print length measurement as a function of time for empty and matrix-filled porous collagen tube groups.]

**Figure C.1** Print length measurement as a function of time for the empty (n=8) and matrix-filled (n=9) porous collagen tube groups.
FIGURE C. 2 Print length measurement as a function of time for the empty (n=9) and matrix-filled (n=9) non porous collagen tube groups.

FIGURE C. 3 Print length measurement as a function of time for the inhibited repair (n=3) and autograft (n=9) groups.
**Figure C.4** Toe spread measurement as a function of time for the empty (n=8) and matrix-filled (n=9) porous collagen tube groups.

**Figure C.5** Toe spread measurement as a function of time for the empty (n=9) and matrix-filled (n=9) non porous collagen tube groups.
**FIGURE C.6** Toe spread measurement as a function of time for the inhibited repair (n=3) and autograft (n=9) groups.

**FIGURE C.7** Intermediate toe spread measurement as a function of time for the empty (n=8) and matrix-filled (n=9) porous collagen tube groups.
**FIGURE C.8** Intermediate toe spread measurement as a function of time for the empty (n=9) and matrix-filled (n=9) non porous collagen tube groups.

**FIGURE C.9** Intermediate toe spread measurement as a function of time for the inhibited repair (n=3) and autograft (n=9) groups.
Figure C.10  Sciatic functional index (SFI) as a function of time for the empty (n=8) and matrix-filled (n=9) porous collagen tube groups.

Figure C.11  Sciatic functional index (SFI) as a function of time for the empty (n=9) and matrix-filled (n=9) non porous collagen tube groups.
FIGURE C.12 Sciatic functional index (SFI) as a function of time for the inhibited repair (n=3) and autograft (n=9) groups.
C.2 **MYELINATED AXON DIAMETER DISTRIBUTIONS**

**FIGURE C.13** Myelinated axon diameter distribution for the empty and matrix-filled silicone tube groups at 30 weeks. The normal distribution is shown for reference.
FIGURE C.14 Myelinated axon diameter distribution for the empty and matrix-filled porous collagen tube groups at 30 weeks. The normal distribution is shown for reference.

FIGURE C.15 Myelinated axon diameter distribution for the autograft group at 30 weeks. The normal distribution is shown for reference.
C.3 Effects of Morphology on Gait

Figure C.16: Total number of myelinated axons plotted against the print length measurement. Both measured at 30 weeks post-operative.
**Figure C.17** Total number of myelinated axons plotted against the toe spread measurement. Both measured at 30 weeks post-operative.

**Figure C.18** Total number of myelinated axons plotted against the intermediate toe spread measurement. Both measured at 30 weeks post-operative.
**Figure C.19** Total number of myelinated axons greater than 6 μm plotted against the print length measurement. Both measured at 30 weeks post-operative.

**Figure C.20** Total number of myelinated axons greater than 6 μm plotted against the toe spread measurement. Both measured at 30 weeks post-operative.
**Figure C.21** Total number of myelinated axons greater than 6 \( \mu \text{m} \) plotted against the intermediate toe spread measurement. Both measured at 30 weeks post-operative.
C.4 **EFFECTS OF MORPHOLOGY ON MUSCLE MASS**

**Figure C.22** Total number of myelinated axons plotted against the muscle circumference at the level of the gastrocnemius (20 mm from heel), measured at 30 weeks post-operative.
**Figure C.23** Total number of myelinated axons plotted against the muscle circumference at the level of the thigh (30 mm from heel), measured at 30 weeks post-operative.

**Figure C.24** Total number of myelinated axons ≥ 6 μm plotted against the muscle circumference at the level of the thigh (30 mm from heel), measured at 30 weeks.
REFERENCES


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