A scanning electron microscope survey of rat sciatic nerve tissue regeneration through a silicone rubber multiple lumen nerve cuff utilizing growth stimulant patterns

by

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1. INTRODUCTION

The purpose of this experiment was to conduct a scanning electron microscopy survey of regenerated sciatic nerve tissue of the rat. The tissue was regenerated in eight weeks through a symmetric six lumen silicone rubber nerve guide with a gap length of 15.0 mm.

The multiple lumen system is unique because it allows for more than one experiment to be accomplished in the same environment. In this experiment, half of the lumens were filled with a collagen gel matrix and half of the lumens were filled with a mixture of collagen gel, laminin and fibronectin.

The goals of this experiment were to:

- determine whether peripheral nerves can regenerate axons across a large (15 mm) gap in a short (8 weeks) time period for small diameter conduits (0.5 mm) in the presence of either collagen or collagen, fibronectin and laminin growth promoting gels
- characterize the shapes and sizes of regenerated nerve tissue with respect to point-to-point variations and with respect to differences in fillings of specific lumens in the gap region of the nerve guide
- compare the general overall structure of the nerve fibers grown in the muliple lumens to the controls
- evaluate the multiple lumen device specifics and characteristics; possible improvements; conformation characteristics of tissue to surface and influence on cable shape.

2. LITERATURE REVIEW

2.1 Background

2.1.1 Anatomy of the mammalian peripheral nerve

The peripheral nervous system is composed of all of the neural tissue outside of the central nervous system. The peripheral nervous system uses bundles of nerve fibers called axons to deliver sensory information to the central nervous system and convey motor commands to peripheral tissue.

The peripheral nerve is composed of blood vessels, connective tissues, axons and Schwann cells. It is surrounded by a connective tissue sheath called the epineurium which consists of dense collagen fibers. Arteries and veins penetrate the epineurium and branch within the perineurium. The perineurium, a thick fibrous connective tissue which also contains flattened epithelial-like cells, subdivides the nerve into bundles of axons called fascicles. Each axon, the central process of the nerve fiber, is encompassed by the delicate connective tissue fibers making up the endoneurium. Capillaries entering the endoneurium from the perineurium supply the axons with sustenance (Martini, 1995).

In adult peripheral nerve tissue, most axons are enveloped by sheath cells called Schwann cells. The cytoplasm of the covering Schwann cell wraps around the axon uniting layers of membranes of the sheath cell. This forms a lipoprotein complex called myelin. Myelin acts like an insulator for the axon and decreases conduction time of nerve impulses (Junqueira et al., 1983).

The sciatic nerve is a major nerve originating in the sacral plexus. It passes posterior to the femur into the biceps femoris muscle. The sciatic nerve divides into the tibial and peroneal nerves. The sciatic nerve has both motor and sensory functions. Injury to the sciatic nerve causes paralysis of the flexion and extension of the toes; abduction and adduction of the toes; rotation inward and adduction of the foot; plantar flexion and lowering of the ball of the foot; paralysis of dorsiflexion and adduction of foot; paralysis of rotation of the ball of the foot outward and of the raising of the toes; and also anesthesia in cutaneous distribution (Thomas, 1993).

2.1.2 Nerve degeneration and regeneration

When an axon is transected, degeneration occurs and can be followed by a reparative phase. The proximal segment of the axon initially degenerates close to the wound then starts to grow after debris is removed by macrophages. This occurs because the proximal segment maintains its continuity with the neuron cell body, hence, regeneration frequently occurs. The distal portion of the axon which is separated from the neuron cell body degenerates totally. The distal portion of the axon and its myelin sheath are consumed by tissue macrophages but the connective tissue remains (Junqueira et al., 1983).

While these changes are occurring, Schwann cells proliferate within the remaining connective tissue creating rows of Schwann cells which act as guides to sprouting proximal regenerating axons. The regenerating axon's growth follows the Schwann cell columns and eventually reaches an effector organ. When there is a

gap between the proximal and distal segments, the regenerated axons may form a bulbous mass called a neuroma.

Regeneration is only functionally effective when the axons find the columns of Schwann cells directed to the correct location. Therefore, a nerve guide, such as the multiple lumen nerve guide, is necessary for regeneration to occur across gaps of greater than one centimeter.

2.2 Peripheral Nerve Repair Techniques

Peripheral nerves of adult mammals have the capability to regenerate functional connections after injury if they have the proper complex humoral, extracellular and cellular environment. The most common peripheral repair techniques are autografts and entubulization.

Autografts can be used in nerve injuries that result in a loss of tissue which cannot be closed by an end-to-end repair. The autograft acts as a biocompatible conduit for axonal regeneration. A disadvantage to autografts is limited supplies of tissue. Second surgeries, donor-site morbidity, and axonal end organ mismatching are also problems (Keeley et al., 1993; Ide et al., 1983).

The entubulization repair method has a long history. This method varies depending upon the biodegradable and non-biodegradable materials used, the length of the gap region, the filling in the gap region, and the number of lumens in the gap region.

Biodegradable materials such as collagen, poly-D,L-lactates, and polyglycolic acid have been used to make entubulization repairs. Nerve guides constructed of polyglycolic acid and poly-D, L-lactates lose their strength after approximately two months of implantation (den Dunnen et al., 1996). The period can be sufficient for axons to cross a 10 mm gap in the rat sciatic nerve. During degradation, the biomaterial tends to swell and can deform which may have a harmful effect on nerve regeneration (Henry et al., 1985; den Dunnen et al., 1996).

The most common non-biodegradable material used for entubulization repair is silicone rubber. Medical grade silicone rubber (polydimethyl siloxane) was developed by Dow Corning. It has good biocompatibility, workability, cost and effectiveness, and it is available in liquid and solid forms. A disadvantage of silicone rubber nerve guides is that they remain in the body and can cause a chronic foreign-body reaction. A second surgery to remove the nerve guide after regeneration occurs would be required to prevent secondary nerve injury due to compression (den Dunnen et al., 1996).

The regeneration of peripheral nerve tissue across a short gap length of 10 mm or less using the entubulization repair method has been successful. Numerous studies have succeeded in regenerating nerves across short gaps using biodegradable nerve guides (da Silva et al., 1985; Madison et al., 1985; Tong et al., 1994; and den Dunnen et al., 1995, 1996) and non-biodegradable nerve guides (Jenq and Coggeshall, 1984, 1985, 1987; Williams et al., 1983; Danielsen et al., 1988; Le Beau et al., 1988; and Daniel, 1991).

The regeneration of peripheral nerve tissue across a gap length of greater than 10 mm using the entubulization repair method has been successful when chemical stimulants are utilized. Successful studies regenerated nerves across long gaps using non-biodegradable silicone rubber nerve guides (Lundborg et al., 1981, 1982; Jenq and Coggeshall, 1986; Madison et al., 1988; Woolley et al., 1990; and Bailey et al., 1993).

Laminin (0.50 mg/ml) and fibronectin (0.50 mg/ml) are glycoproteins that have been used in combination to promote axonal growth across long gaps (Woolley et al., 1990 and Bailey et al., 1993). Collagen gel has been used to create a support matrix for migrating axons using 2.4 mg/ml collagen and 2.4 mg/ml laminin (Madison et al., 1988) or 0.25 mg/ml collagen and 7 mg/ml laminin (Valentini et al., 1987).

Many studies have been performed using single lumen nerve guides. The multiple lumen nerve guide was used successfully to cross a 5 mm gap (Daniel, 1991).

2.3 Chemicals Influencing Peripheral Nerve Regeneration

Fibronectin and laminin are two major glycoproteins associated with basal laminae and are important in axonal growth. Laminin is a stimulatory agent and substrate for axonal elongation. Fibronectin promotes neuritic outgrowth by influencing the organization and/or assembly of the cytoskeletal elements within the growing neurite tip (Bailey et al., 1993).

The sequence of events for peripheral nerve regeneration in simple, unfilled entubulizational repairs follows:

- An acellular matrix accumulates within the chamber prior to cellular growth.
- At seven days, the matrix contains fibronectin but little or no laminin.
- After 14 days, the matrix exhibits cellular growth and both fibronectin and laminin are evident. The cellular influx is associated with the accumulation of neurite-promoting factors.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Silicone rubber

The silicone rubber tubing used to construct the multiple lumen and outer lumen parts of the nerve guide was Dow Corning 602-135 (0.508 mm inner diameter, 0.9398 mm outer diameter) and 602-305 (1.98 mm inner diameter and 3.175 mm outer diameter) (Midland, MI), respectively. Dow Corning 819 SILASTIC® Medical Adhesive Silicone Type A (Midland, MI) was used to glue and form the lumen assembly into a cylindrical shape.

3.1.2 Collagen, fibronectin and laminin

In this experiment, three of the six lumens of the nerve guide were filled with collagen gel. The other three lumens were filled will a gel mixture of collagen, fibronectin and laminin.

VITROGEN 100® (Collagen Corporation, Palo Alto, CA) is bovine dermal collagen suitable for cell culture and biochemical applications. It is 99.9% pure collagen composed of 95-98% type I collagen with the remainder being type III collagen. The VITROGEN 100® has a concentration of about 3.0 mg of collagen per ml of 0.012 N HCl (pH = 2.0) and has to be neutralized in order to gel.

Laminin (1 mg of laminin/ml of saline, #L2020, Sigma, St. Louis, MO) is produced from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma.

Fibronectin (1 mg of powder, #F0635, Sigma, St. Louis, MO) is produced from rat plasma and lyophilized from 0.05 M Tris buffered saline.

3.2 Methods

3.2.1 Multiple lumen nerve cuff

3.2.1.1 Cuff fabrication The initial step in fabrication of the multiple lumen nerve guide was to construct the multiple lumen assembly. Seven pieces of silicone rubber tubing (Dow Corning 602-135) of 0.508 mm inner diameter and 0.9398 mm outer diameter were cut to a length of 25.0 mm. A small amount of silicone adhesive was applied to the outside surface of each piece of tubing. The seven pieces of tubing were then placed into a circular geometry and then rolled to form a tightly packed cylindrical grouping.

Each of the lumens at the proximal end of the nerve guide were color coded. The red, green and yellow color coded lumens were designed for a mixture of collagen, fibronectin and laminin. The blue, purple and orange color coded lumens were designated for collagen. Viewing the assembly from the distal end looking proximally, the colors were applied clockwise in the following order: red, blue, green, orange, yellow and purple.

Next, both ends of the color coded assembly were dipped into silicone adhesive to plug the ends of the lumens. The lumens were temporarily plugged so that when the assembly was inserted into the adhesive filled polyethylene tube, the

adhesive would not fill the lumens. The assembly was rolled to remove the excess adhesive from the sides of the tubing. This prevented the outer diameter of the assembly from increasing. The assembly was allowed to dry overnight.

A piece of polyethylene tubing of 2.92 mm inner diameter and 3.73 mm outer diameter (#7471, Clay Adams, Parsippany, NJ) was cut to a 30.0 mm length. Approximately 2.0 cc of Silicone Rubber Type A (Dow Chemical Corporation, Midland, MI) adhesive was placed into a syringe. The adhesive was then injected into the polyethylene tubing. The multiple lumen assembly with plugged ends was then slowly pushed into the filled tube. This gap assembly unit was allowed to dry for approximately four days. Since the vulcanization time of Silicone Rubber Medical Adhesive Type A depends upon the thickness of the adhesive layer and the relative humidity of the atmosphere, drying time varied from four days to two weeks. The assembly appeared translucent when completely dry.

The final step in fabrication of the multiple lumen nerve guide was to insert this multiple lumen assembly into a silicone rubber outer cuff. First, the multiple lumen assembly was removed from the polyethylene tube by bending the tubing to loosen the dried silicone rubber adhesive, which held the seven tubes together, from the inner wall of the polyethylene tube. A metal rod was used to push the loosened assembly from the polyethylene tube. Second, the adhesive plug on the proximal end of the assembly was removed to open the lumens. The assembly was trimmed so that the color coding was 2.0 mm from the proximal end. The distal end of the assembly was also trimmed to open the lumens. The completed multiple lumen

assembly had a length of 15.0 mm. The center lumen was then plugged with silicone rubber adhesive; this left the six outer lumens open. Third, a 25.0 mm piece of silicone rubber tubing (Dow Corning 602-305) of 1.98 mm inner diameter and 3.175 mm outer diameter was cut. This larger tubing was submerged in xylene (UN1307, Lot # 920348, Fisher Chemical, Fair Lawn, NJ) for 20 minutes. The xylene swelled the tubing so the 15.0 mm multiple lumen gap assembly could be inserted. After insertion, the unit was allowed to dry for 12 hours in a ventilated hood. The unit was cleaned by submerging it in boiling sodium bicarbonate solution and then rinsing it in distilled water and air drying it. The complete silicone rubber multiple lumen nerve guides were stored in a covered dish.

3.2.1.2 Cuff specifications The multiple lumen nerve guide had an outside diameter of 3.52 mm. The total length of the cuff was 21.0 mm with a multiple lumen portion of 15.0 mm and 3.0 mm each for the proximal and distal stump insertions. The inner diameter of each lumen was 0.508 mm with approximately 0.432 mm between lumens (Figure 3.1).

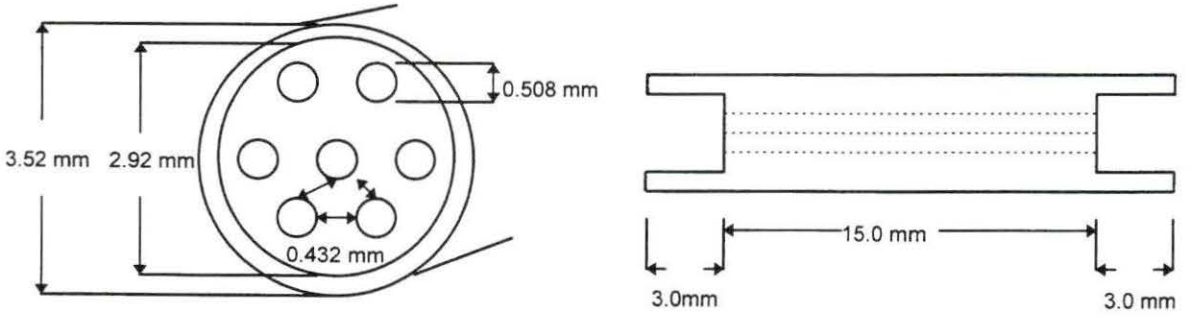

Figure 3.1 - Cross sectional and longitudinal view of the multiple lumen nerve nerve guide.

3.2.2 In vivo experimentation

Ten adult male Sprague-Dawley rats, four months old, were utilized for the microstructural studies (Table 3.1). The right sciatic nerve was isolated and severed. A silicone rubber six lumen nerve guide with a 15.0 mm gap length containing alternating lumens filled with a collagen gel (orange, blue and purple lumen color code) and with a mixture of collagen, fibronectin and laminin as a gel (red, green and yellow lumen color code) was implanted. After eight weeks of implantation, the right sciatic nerve with the nerve guide and the left sciatic nerve were surgically removed. The rats were euthanized after the retrieval surgery.

Table 3.1 - Rat information - prior to surgery

Animal Number	Weight (kg)	Implant
Rat 3	0.422	Multiple lumen cuff
Rat 4	0.405	Multiple lumen cuff
Rat 5	0.434	Multiple lumen cuff
Rat 6	0.435	Multiple lumen cuff
Rat 7	0.406	Multiple lumen cuff
Rat 8	0.423	Multiple lumen cuff
Rat 9	0.421	Multiple lumen cuff
Rat 10	0.415	Multiple lumen cuff
Rat 11	0.426	Multiple lumen cuff
Rat 12	0.420	Multiple lumen cuff

3.2.3 Collagen, fibronectin and laminin

A volume of 8.0 ml of Vitrogen 100® collagen was mixed with 1 ml of 10x phosphate buffered saline solution (0.2 M Na_2HPO_4; 1.3 M NaCl; pH = 7.4). This collagen solution was neutralized by adding 1.0 ml of 0.1 M NaOH and mixing. The pH of this mixture was adjusted to 7.4±0.2 by adding drops of 0.1 M HCl or 0.1 M NaOH while monitoring the solution by phenol red solution (pH 6.8 = yellow; pH 8.2 = red). The pH was adjusted to neutralize the collagen so that it would gel. This solution contained 2.4 mg/ml collagen. It was mixed with saline (1:1 volumetric addition) to give 1.2 mg/ml of collagen for the collagen lumen filling. After adding this liquid to three of the lumens of the six lumen nerve guide and subsequently heating the unit in an oven to 37°C for 15 minutes just prior to surgery, the gel was established in the lumens.

Approximately 9 μl of the collagen, laminin and fibronectin mixture was needed to fill each of the red, yellow and green lumens of the guide. This second type of lumen filling was obtained by mixing the chilled Vitrogen 100® collagen with fibronectin and with laminin. A 2:1:1 volumetric addition was used. Thus, 2 ml of collagen was mixed with 1 ml of laminin and with 1 ml of fibronectin. The mixture contained a concentration of 0.25 mg/ml of laminin, a concentration of 0.25 mg/ml of fibronectin and a concentration of 1.2 mg/ml of collagen. This material also gelled when the cuff assembly was placed in the oven at 37°C for 15 minutes.

Two hours prior to surgery, the empty multiple lumen nerve guides were sterilized by immersing them in 70% ethanol. The ethanol evaporated from the lumens prior to filling them with the collagen and collagen, fibronectin and laminin.

While the animal was being shaved and the skin was being cleaned for surgery, the multiple lumen nerve guide was filled with the collagen and the collagen, fibronectin and laminin mixture. The filled guide was placed in the 37°C oven for 15 minutes for gelation to occur. The filled guide was retrieved from the oven at the time that the right sciatic nerve was being severed. The guide was then immediately implanted.

3.2.4 Surgical protocol

The rats were anesthetized with a 2:1 volumetric mixture of Ketamine (Ketaset®, 100 mg/ml, Fort Dodge Lab Incorporated, Fort Dodge, IA) and Xylazine (Rompun®, 20 mg/ml, Miles Incorporated, Shawnee Mission, KS). One and one-half ml/kg body weight were administered as the anesthesia by an intraperitoneal injection into the lower left quadrant of the abdomen. This provided approximately 60 minutes of anesthesia (425 g rat). The rat was continuously observed during the procedure to ensure that he remained anesthetized, and an additional 1.5 ml/kg was administered if the animal began to awaken.

The rat was in dorsal recumbancy on a stainless steel tray. Using animal hair clippers, the hair was removed from the ventral surface. The cranial extent of hair removal was the diaphragm. The caudial extent was the base of the tail. The hair was also removed from the medial surface of the right thigh and right leg. The rat

was secured to the tray by stretching the fore limbs forward cranially and taping them to the tray. Both hind limbs were taped to the tray with the right limb stretched caudally. The skin was cleaned, three times, with sponges saturated with 70% ethanol and with Nolvasan® solution (Fort Dodge Laboratory, Fort Dodge, IA). Nolvasan® is a 4% chlorhexidine diacetate solution. It was used as a disinfectant. Beginning at the incision site and working toward the periphery, the skin was scrubbed in a circular motion. The sponges were discarded each time after reaching the periphery.

The rat and tray were transferred to the surgery table. The entire rat, except the head, was covered with one half of a sterile Steri-Drape® (3M Medical, St. Paul, MN) and aseptic conditions were maintained.

Using a size 15 sterile rib-back carbon steel blade (Bard-Parker, Rutherford, NJ), a 2.0 cm skin incision was made. The incision followed the cranial edge of the biceps femoris muscle parallel from just cranial to the patella toward the midline. Using blunt dissection, the gracilis was gently spread to expose the deeper muscle layers. The fascia separating the adductor magnus and the semimembraneous medially was separated. A Weitlaner self-retaining retractor held the incision open. This exposed the sciatic nerve on the biceps femoris. The nerve was detached from the biceps femoris, using a pair of curved Mayo scissors, 20.0 mm proximal to the bifurcation of the common peroneal and the tibial nerve branches.

For the right sciatic nerve, the proximal nerve stump was inserted into the guide by passing a 3/8 inch circular needle with a 9-0 nylon black monofilament

ophthalmic suture (Ethicon Incorporated, Somerville, NJ) from the outside of the guide through the wall of the guide near the red color coded lumen of the gap assembly within the nerve guide. The proximal stump was gently held with iris tissue forceps and the needle was inserted through the epineurium 2.0 to 3.0 mm from the cut surface of the nerve. The needle was then passed through the inside wall of the guide near the original insertion point and was gently pulled. The nerve stump slipped into the outer lumen of the guide. The stump was secured with a square knot placed over the outer wall of the guide. No direct tension was placed on the nerve since the knot was on the outer wall of the cuff. The same procedure was used for the distal stump, except the suture was placed near the orange color coded lumen entrance. By placing the suture for the proximal stump at the red color coded lumen orientation and the suture for the distal stump at the orange color coded lumen orientation, compression of the lumens of the nerve guide was not expected to occur. The distal nerve section outside of the guide was placed in an "S-pattern" to prevent crimping and compression (Bailey et al., 1993). No tissue was excised from the severed nerve.

The nerve guide was placed upon the biceps femoris and the retractor was removed. The adductor magnus and semimembraneous were maneuvered into their original positions. A simple continuous suture pattern was used to close the gracilis with 4-0 Chromic Gut (#570-31, Davis & Geck, Danbury, CT) suture material. The skin was closed with 2-0 Dermalon® (nylon, black monofilament, #1644-51, Davis & Geck, Montreal, Canada) using a simple interrupted pattern. Bitter Orange

(#67002, ARC Lab, Atlanta, GA) was applied to the incision, the right hind-limb, and footpad to help prevent self-mutilation.

The rats were returned to the animal care facility room after they were awake and alert. The environment consisted of 12 hour light cycles, 22°C temperature control and 45% humidity. The cages contained enough sawdust to decrease contact of the paralyzed limb with the plastic cage floor. Bitter orange was applied to the incision site twice daily for seven days post-implantation. Bitter orange was applied to the right hind-limb and footpad as needed when self-mutilation occurred. A post-operative analgesic containing 12.0 mg codeine and 120.0 mg of acetaminophen per 5.0 ml of water was used. This was administered at a rate of 1.0 ml post-operative analgesic per 100.0 ml of drinking water. This treatment was administered for seven days to all of the animals. Animals that exhibited self-mutilation were continued on this until the wound healed. The skin sutures were removed 10 days after surgery.

3.2.5 Histological procedures

3.2.5.1 Fixation Prior to sacrifice, the right sciatic nerve was isolated and removed from the anesthetized rat. The nerve tissue with the nerve guide was submerged into 20.0 ml of 10% buffered formalin. A 25.0 mm segment of the left sciatic nerve was removed and submerged in 10% buffered formalin. All of the nerve tissue was held in formalin for 24 hours.

3.2.5.2 Tissue dissection After 24 hours of fixation, the nerve tissue was dissected in a ventilated hood and then placed into vials containing 10% buffered formalin. The tissue was held for 28 days and then dehydrated.

The dissection procedure began by removing the encapsulating tissue from the outside of the cuff using a scalpel blade.

For the right sciatic nerve with the multiple lumen nerve guide, the 9-0 nylon suture was gently removed from the distal end of the guide using a scalpel. Sampling proceeded from the distal end toward the proximal end of the cuff. The segments were taken by cutting through the nerve guide with a razor blade.

First, 2.5 mm of distal stump (Figure 3.2; 18.5-21.0 mm position) was removed. An 8-0 blue monofilament polyprolene suture (Ethicon Incorporated, Somerville, NJ) was placed in the silicone tubing at the distal-most side of the sample designating the red color coded lumen. Measuring from 17.5 to 18.5 mm,

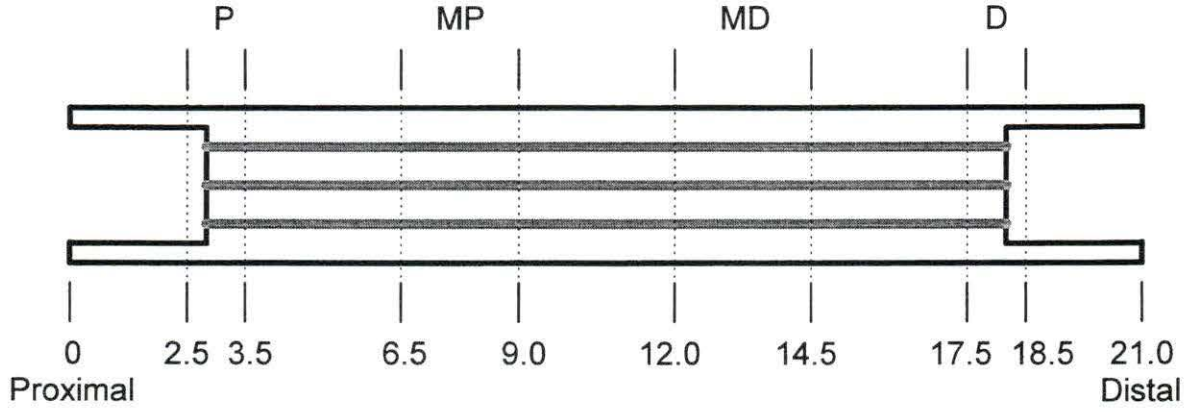

Figure 3.2 - Section designations and scale (mm): P = Proximal; MP = Middle Proximal; MD = Middle Distal; D = Distal.

0.5 mm of distal stump and 0.5 mm of the gap region was taken for the Distal sample (Figure 3.2). The nerve tissue with the intact cuff was placed in a vial of formalin.

The gap region of the cuff from 14.5 to 17.5 mm was removed. An 8-0 blue polyprolene suture was placed in the silicone tubing at the distal-most side of the sample designating the red color coded lumen location. The Middle Distal sample from 12.0 to 14.5 mm was taken. The segment of cuff was held with a bulldog clamp while a 0.5 mm diameter metal wire was inserted into the red color coded lumen to propel the tissue from the lumen (Figure 3.3). Since the wire was the same diameter as the lumen, the wire acted as a plunger. The red color coded

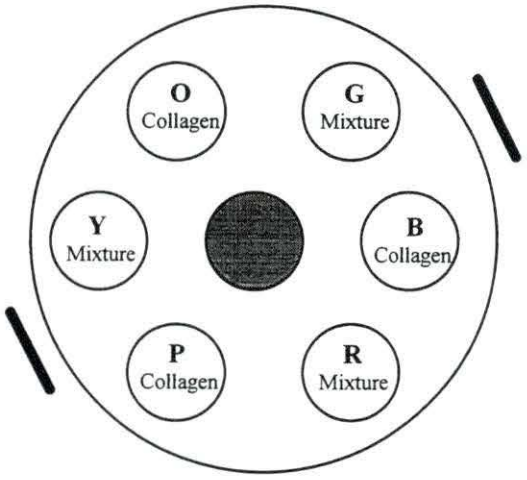

Figure 3.3 - Cross-sectional view of multiple lumen cuff from the distal end looking proximally (R = Red; P = Purple; Y = Yellow; O = Orange; G = Green; B = Blue). Bars represent bulldog clamp position.

lumen and orange color coded lumen tissues were removed from the segment and placed in vials of formalin (labeled Middle Distal Red and Middle Distal Orange, respectively). The 2.5 mm tissue samples with the blue, green, purple and yellow color codings were also placed in labeled vials of formalin.

Next, the 9.0 to 12.0 mm section was removed and the blue polyprolene suture was placed near the red color coded lumen. The segment from 6.5 to 9.0 mm was taken using the same procedure (Middle Proximal Red, Middle Proximal Orange, and Middle Proximal samples).

Prior to the removal of the color coded section of the guide from 3.5 to 6.5 mm, a red ink dot was placed on the red color coded lumen. An 8-0 blue polyprolene suture was placed in the silicone tubing at the distal-most side of the sample designating the red color coded lumen. The Proximal section from 2.5 to 3.5 mm was removed and the nerve tissue and guide were placed into a labeled vial of formalin.

The green, yellow, blue and purple strands were removed from the nerve guide and stored in 10% buffered formalin to be processed at a later date.

Prior to, during, and after each dissection, the instruments and cutting surface were cleaned.

3.2.5.3 Dehydration

All of the dissected tissue in the glass vials of 10% buffered formalin were taken to the histopathology laboratory. All of the tissues were placed onto tissue paper and put into plastic cartridges for the automated dehydration process. The

samples in the cartridges were held in 10% buffered formalin until all of the samples were ready for dehydration.

The tray of cartridges was removed from the formalin and allowed to drip for approximately one minute. The tray was then placed in the Modular Vacuum Processor (MVPI) for automated dehydration. The nerve tissue was dehydrated in graded ethanols in the following order and duration: 70% ethanol for one hour; 80% ethanol for one hour; 95% ethanol for one hour; 100% ethanol for one hour; 100% ethanol for one hour; and finally, 100% ethanol for one hour.

All of the nerve tissue was placed into labeled vials containing 100% ethanol.

3.2.6 Scanning electron microscopy

After fixation and dehydration were completed, some nerve tissue was freeze fractured and some tissue was left with the "wet cut" surface obtained during tissue dissection. All of the tissue was then critical point dried, mounted on aluminum stubs, and sputter coated. The tissue was then ready for viewing with the JEOL JSM-5800 Low Vacuum Scanning Electron Microscope.

The scanning electron microscope (SEM) images were taken using an accelerating voltage of 10 kV, a 200 μm diameter aperture and a vacuum level of 10^{-5} Torr.

3.2.6.1 Freeze fracturing The freeze fracturing of the tissue using liquid nitrogen was performed to expose the interior of the cable structure for viewing by

the SEM. This process creates a smooth cut surface allowing fine structural cross sectional details to be viewed.

The left sciatic nerve (control) of some of the rats was freeze fractured as follows: First, the necessary tools were prepared. A styrofoam reservoir was filled with liquid nitrogen and a metal block was immersed. The back of a single edged razor blade was secured in the grips of a self-locking clamp to be used as a fracturing tool. The secured razor and the tips of a pair of forceps were also submerged in the nitrogen so that the tissue could be handled without thawing.

While the tools were cooling, the ethanol dehydrated tissue was placed into a petri dish containing 100% ethanol. A 2.0 cm piece of parafilm sheet was rolled into a cylinder. The cylinder was submerged in the petri dish with the nerve tissue. The tissue was floated into the cylinder using a wooden rod. The parafilm cylinder was crimped at both ends using the back of a single edged razor blade. The nerve was now enclosed in a pillow shaped-pouch of ethanol.

The enclosed nerve was dropped into the liquid nitrogen reservoir. When the pillow was completely frozen, the pillow was placed on the frozen metal block. The fracturing tool was oriented above the pillow so that a cross sectional fracture could be made in the tissue. A quick blow was made to the pillow and the fragments were removed from the block with the forceps and placing into the a labeled vial of 100% ethanol.

The regenerated strands from the gap region of the right sciatic nerve of the nerve guide were too small to be fractured using the above procedure. The tiny strands were not visible in the frozen parafilm pillow.

A regenerated strand was fractured by placing the strand and a drop of 100% ethanol in an aluminum dish. The dish was treated to a shallow immersion in liquid nitrogen. An extra drop of ethanol was placed over the frozen specimen in the dish ensuring that the strand was completely covered by frozen ethanol. The dish was placed on the frozen metal block and the fracturing tool was used to fracture the strand. The fragments were returned to a vial containing 100% ethanol.

3.2.6.2 Critical point drying All of the nerve tissue was completely dehydrated using the Denton Critical Point Dryer, DCP-1. This critical point method uses carbon dioxide as the pressurized transitional liquid.

First, the specimen chamber was cooled by submerging the lower three-fourths of the chamber in a beaker of 15°C water. While the chamber was cooling, each piece of tissue was placed into a small porous Teflon container with a tight lid. The containers were submerged in 100% ethanol in order to prevent the tissue from drying. Six of the containers were placed into a wire mesh basket and placed into the critical point drier. The chamber was filled with 100% ethanol and then the chamber lid was secured. The inlet value for the CO_2 was opened and the chamber filled with liquid CO_2. The pressure in the chamber increased to 900 psi. The chamber exhaust valve was slightly opened and the chamber was vented until

frozen CO_2 was expelled from the exhaust outlet. The exhaust outlet was closed for five minutes.

After five minutes, the exhaust valve was slightly opened and the chamber was flushed for two minutes. The five minute hold and two minute flush cycles were repeated seven times.

At the end of the seventh flush, the exhaust valve and CO_2 inlet valve were closed. The chamber was immersed in a beaker of 55°C water until the pressure in the chamber increased to 1650 psi which brought the specimens through critical point. Finally, the exhaust valve was opened slightly to vent the chamber for 10 minutes.

The specimens were removed from the chamber and stored in a desiccator over a half pound of anhydrous $CaSO_4$ drying agent Drierite® (W. A. Hammond Drierite Company, Xenia, OH) to prevent rehydration.

3.2.6.3 Mounting All of the nerve tissue was mounted by means of conductive tape and adhesive to properly ground the specimens. Grounding aids in the flow of electrons off the specimen during viewing of the tissue with the SEM. Improper mounting can lead to excessive charging, thermal damage and distorted images.

Aluminum tape with adhesive on one side was placed on the top of aluminum stubs adhesive side down. Silver paint was applied to the edges of the tape to provide extra contact between the aluminum tape and the aluminum stub. The critical point dried tissue was mounted to the stub with a small smear of silver paint.

The tissue were mounted vertically so the cross sectional view was on top. The pieces of tissue ranged in height from about 0.25 to 2.0 mm. The mounted tissue was stored in a desiccator with Drierite®.

3.2.6.4 Sputter coating Most biological specimens must be coated with a conductive metal to prevent the buildup of high voltage static charges which will degrade the quality of the SEM image. The mounted tissue was sputter coated in the SEM Coating Unit E5100 Series II (Polaron Instruments Incorporated, Watford Hertfordshire, UK) with a current of 30 mA and voltage of 2.4 kV. This system used argon as the chamber purging gas. A 200 Å coating of 80% palladium and 20% gold was applied.

Sputter coating completed the tissue processing. The tissue was stored in a desiccator with Drierite® to prevent rehydration which could crack the sputter coating.

3.2.6.5 Images All of the images were printed as 3.5 inch x 2.75 inch Sony black and white printouts. The software used by the JEOL system was Picture Publisher 5.0 and ARC58. Some of the images were stored on a 230 Megabyte 3.5 inch Verbatim Rewritable Optical Disk (Verbatim Corporation, Charlotte, NC) in a bitmap (bmp) format of approximately 300,000 bytes of information. The remaining images were scanned from the Sony prints and saved on 3.5 inch floppy disks with approximately 200,000 bytes of information. An HP Desk Scan IIc scanner was

used at the black and white photo setting and set to acquire 200 vertical dots per inch and 200 horizontal dots per inch from the Sony print.

All of the images were computer enhanced using Corel 4, Corel PHOTO-PAINT!. The sharpness, noise removal, brightness, contrast, intensity, and gamma adjustments were used. The sharpness selection increases distinction between neighboring pixels thus enhancing the edges and bringing out the detail. The noise removal softens the edges and reduces the random speckles in an image created by the scanning process. The brightness changes lightness or darkness of an image and contrast adjusts the range between the light and the dark regions. The intensity emphasizes the brighter features. The gamma adjustment lightens the dark areas and subdues the bright regions. The images were printed with an HP Laser Jet 4 at 600 dots per inch.

4. RESULTS

The results of the characterization of the peripheral nerve tissue, scanning electron microscopy preparation methods and assembly aspects of the silicone rubber multiple lumen nerve guide of the experiment will be presented.

4.1 Tissue Characteristics

4.1.1 Left sciatic nerve - control

4.1.1.1 Control size and fascicle pattern The sciatic nerve fascicle pattern and number of fascicles varied from animal to animal. The number of fascicles ranged (Figures 4.1-5) from one to four (Table 4.1). For example, Rat 3 had two fascicles, Rat 9 had four fascicles, and Rat 7 had three fascicles. Also, the number of fascicles for Rat 12 changed from location to location with three fascicles in one location and two fascicles in another (Figures 4.3 and 4.4). The pattern in which the fascicles were arranged changed from rat to rat. For some of the controls, the bundles were organized in a circular pattern (Figure 4.5), and for some, the bundles were organized in an elliptical shape (Figures 4.1-3). The diameter of the fascicles from the controls varied from 510 μm by 700 μm (short axis by long axis) to 1390 μm by 1520 μm (Table 4.1).

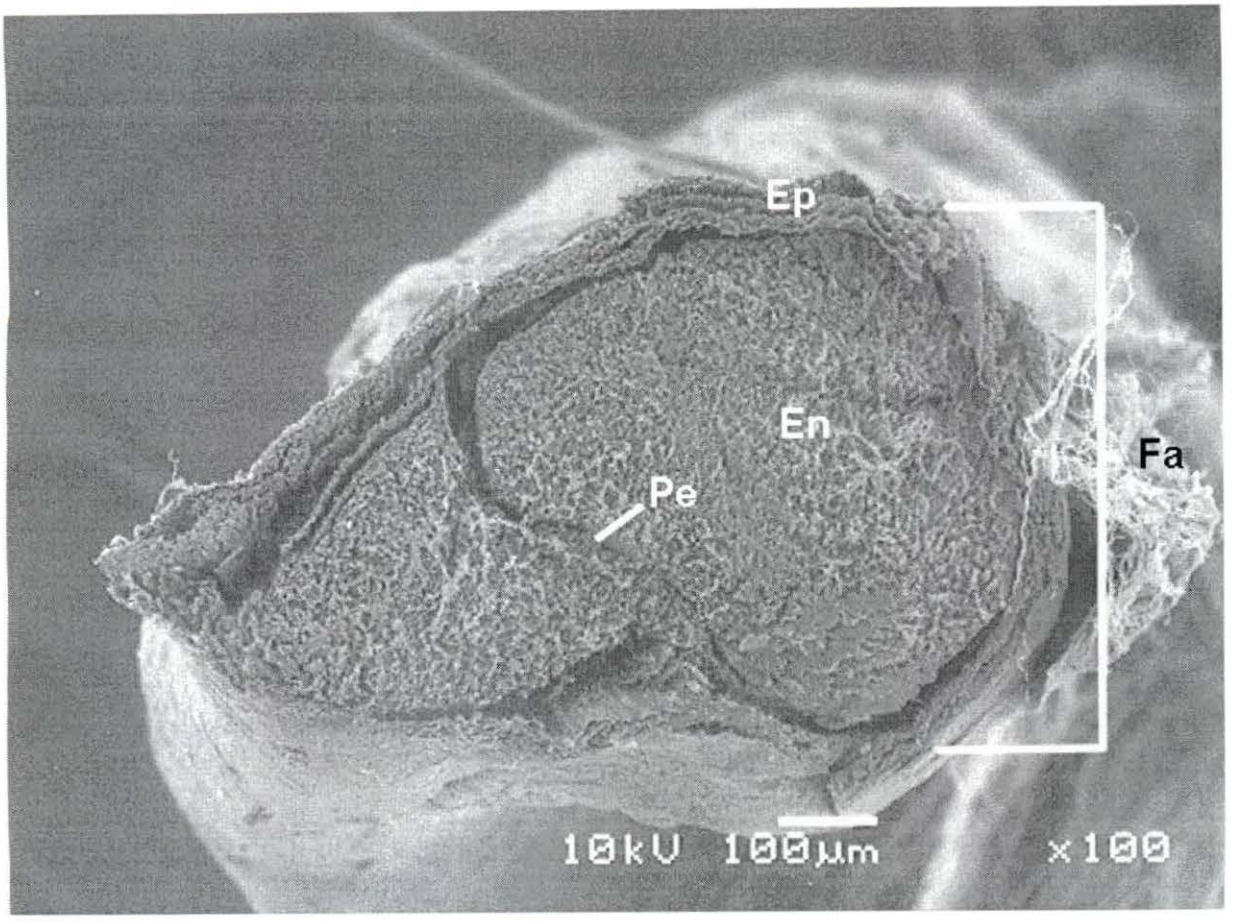

Figure 4.1 - Rat 3 Control cross section with two fascicles in a noncircular pattern. Epineurium (Ep), perineurium (Pe), endoneurium (En), fascicles (Fa). Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

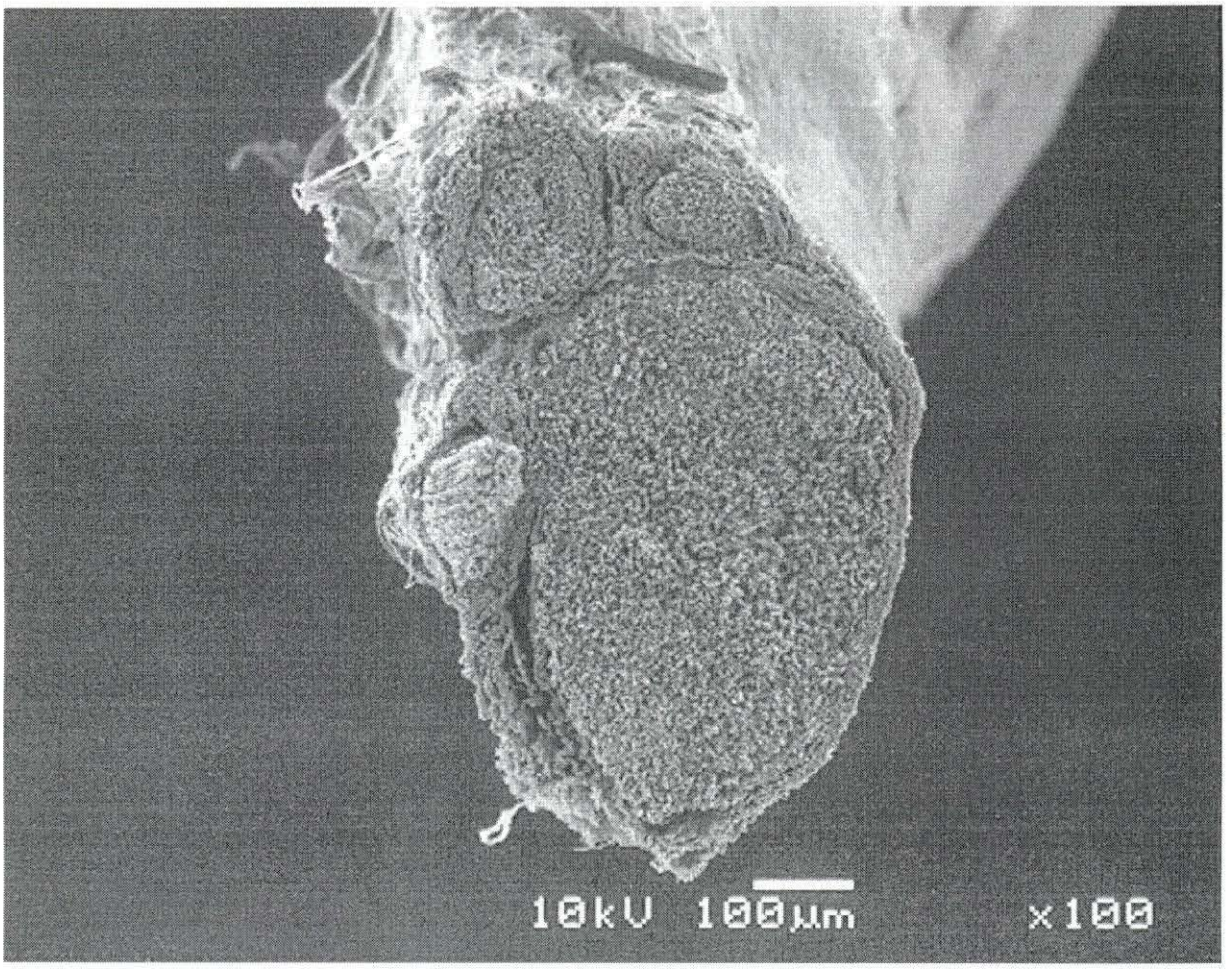

Figure 4.2 - Rat 9 Control cross section with four fascicles in a noncircular pattern. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

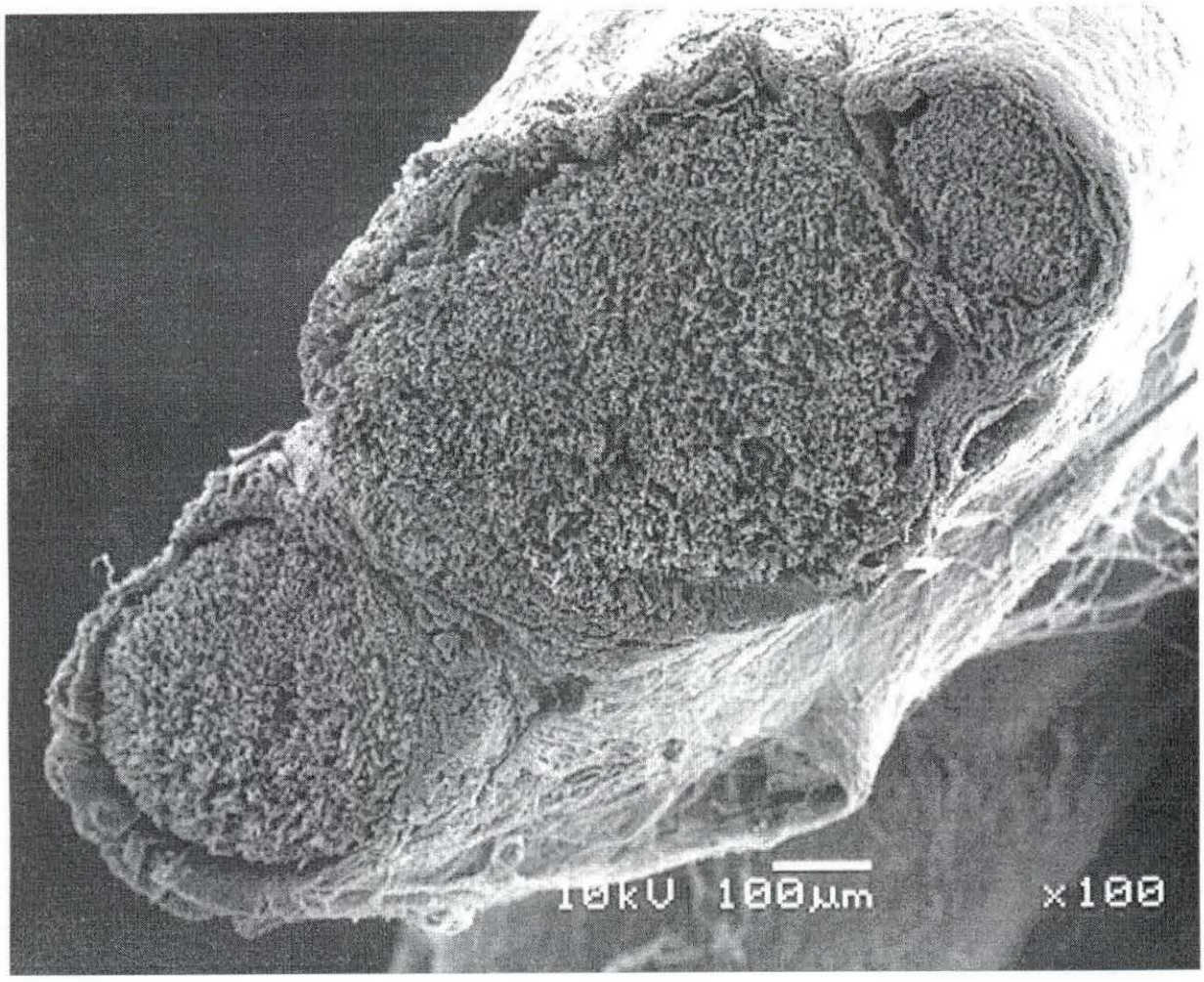

Figure 4.3 - Rat 12 Control cross section with three fascicles in a noncircular pattern (compare with Figure 4.4). Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

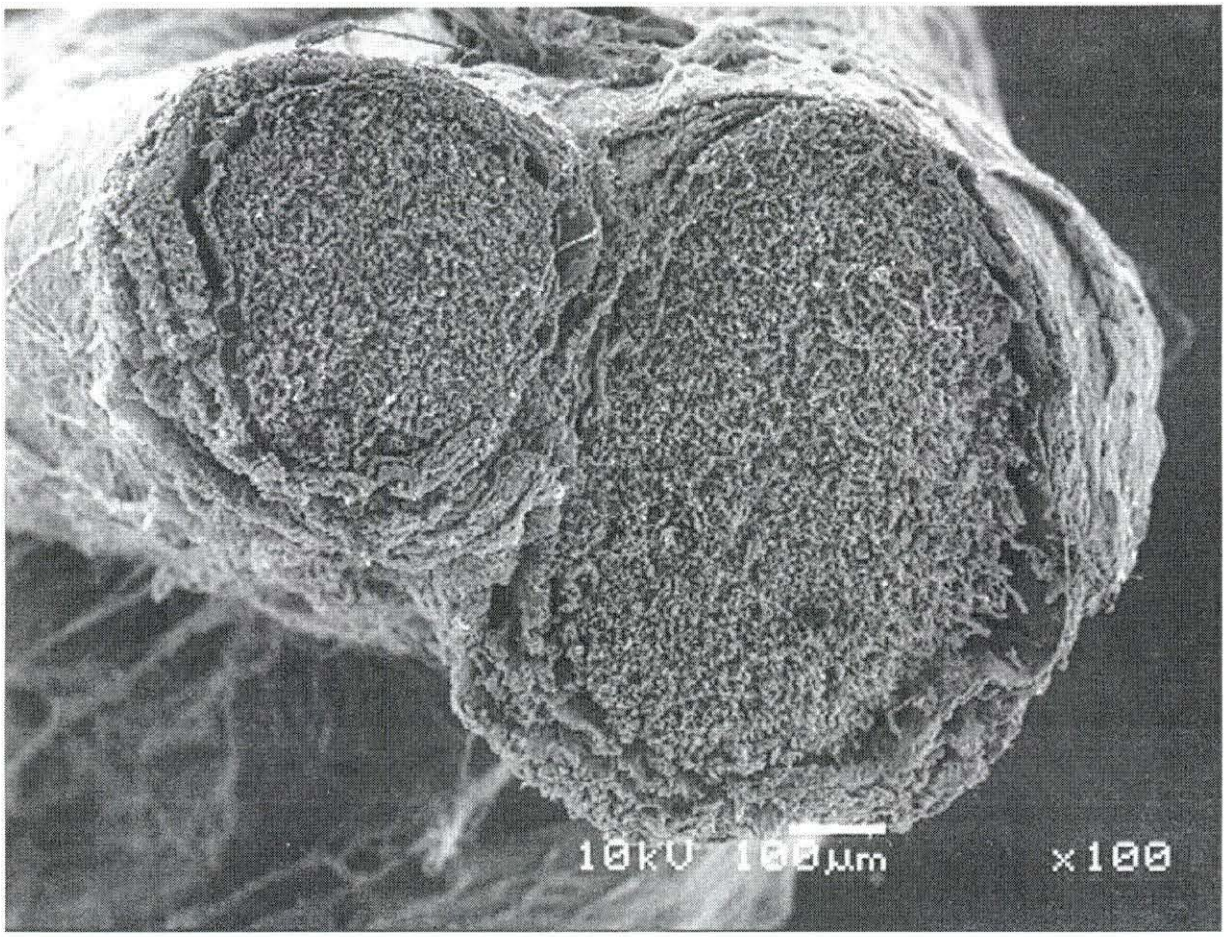

Figure 4.4 - Rat 12 Control cross section with two fascicles. Fascicle number varied from location to location in the same animal (compare with Figure 4.3). Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

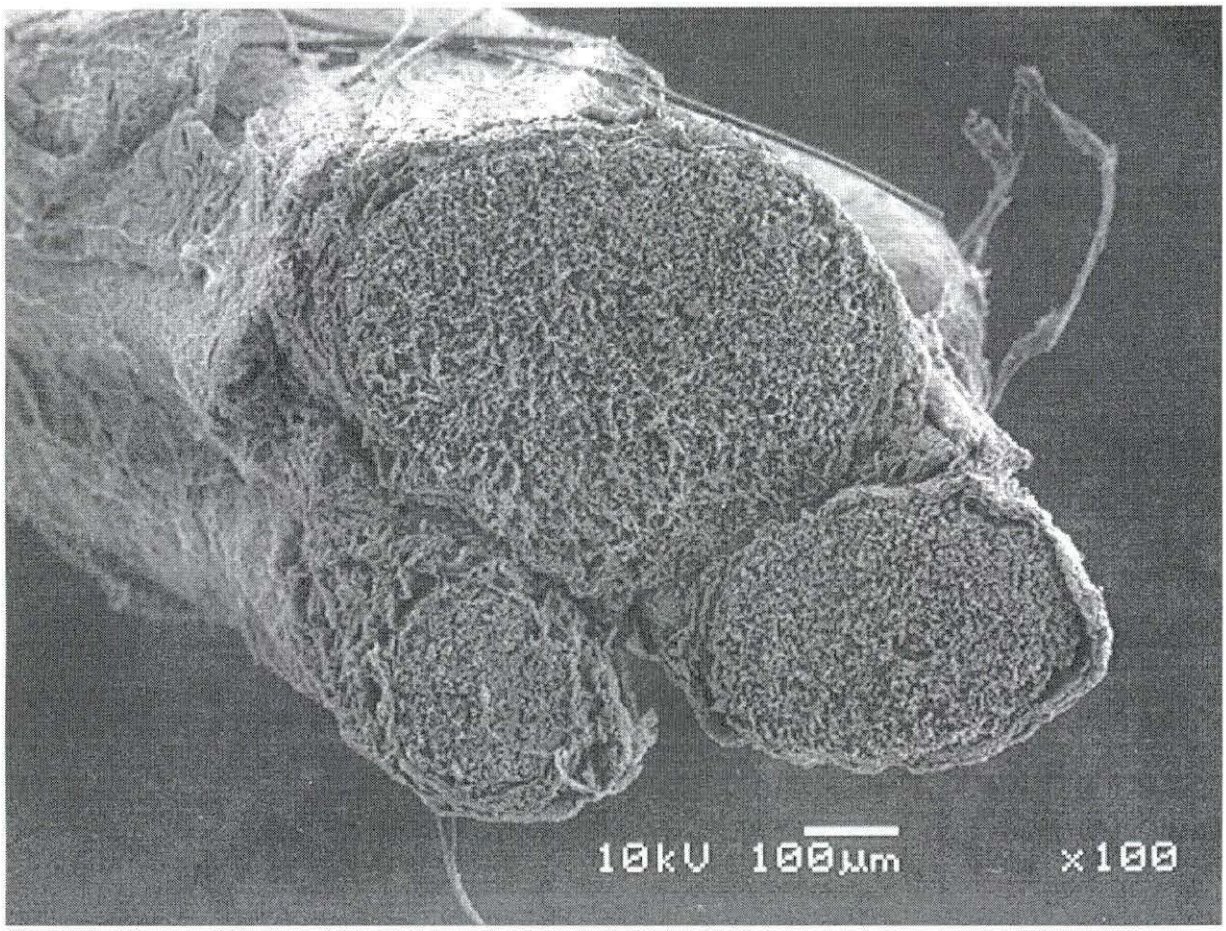

Figure 4.5 - Rat 7 Control cross section with three fascicles in a circular pattern. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

Table 4.1 - Control and strand sizes and features

Animal Number	**Control:** Size (μm)	Features	**Gap region (collagen):** Position	Size (μm)	Features	Other	**Gap region (collagen, fibronectin, laminin):** Position	Size (μm)	Features	Other
Rat 3	550x750	2 fascicles								
Rat 4	580x870	2 fascicles	MDO	22x23		Tissue				
Rat 5	850x950	3 fascicles	MPO	48x53	2μm rim*	Tissue	MDR	40x46		Tissue
Rat 6	1120x1560	2 fascicles	MDO			Tissue				
Rat 7	760x890	3 fascicles	MPO	460x560		Plates**	MPY	320x320	2.6μm rim	Hollow***
			MDO	240x330		Plates	MPG			Plates
			MPP	270x330	2.6μm rim	Plates	MPR	330x410		Plates
			MDP	240x290	13μm rim	Plates	MDY	260x330	2.6μm rim	Plates
			MPB	280x320		Plates	MDG	300x300		Plates
			MDB	270x350		Plates	MDR	330x450		Plates

Note:
MPO = Middle Proximal Orange color coding (collagen filled) - 3.5 to 6.0 mm into the gap region from the proximal end of the cuff
MDO = Middle Distal Orange color coding (collagen filled) - 9.0 to 11.5 mm into the gap region from the proximal end of the cuff
MPR = Middle Proximal Red color coding (collagen, fibronectin & laminin filled) - 3.5 to 6.0 mm into the gap from the proximal end of the cuff
MDR = Middle Distal Red color coding (collagen, fibronectin & laminin filled) - 9.0 to 11.5 mm into the gap from the proximal end of the cuff
MPP = Middle Proximal Purple color coding (collagen filled) - 3.5 to 6.0 mm into the gap region from the proximal end of the cuff
MDP = Middle Distal Purple color coding (collagen filled) - 9.0 to 11.5 mm into the gap region from the proximal end of the cuff
MPG = Middle Proximal Green color coding (collagen, fibronectin & laminin filled) - 3.5 to 6.0 mm into the gap from the proximal end of the cuff
MDG = Middle Distal Green color coding (collagen, fibronectin & laminin filled) - 9.0 to 11.5 mm into the gap from the proximal end of the cuff
MPB = Middle Proximal Blue color coding (collagen filled) - 3.5 to 6.0 mm into the gap region from the proximal end of the cuff
MDB = Middle Distal Blue color coding (collagen filled) - 9.0 to 11.5 mm into the gap region from the proximal end of the cuff
MPY = Middle Proximal Yellow color coding (collagen, fibronectin & laminin filled) - 3.5 to 6.0 mm into the gap from the proximal end of the cuff
MDY = Middle Distal Yellow color coding (collagen, fibronectin & laminin filled) - 9.0 to 11.5 mm into the gap from the proximal end of the cuff
* Rim means there is an increase in the density of the tissue at the edge region of the strand.
** Plates are plate shaped crystals.
*** Hollow means there is no tissue or crystals in the central region.
**** Needles are needle shaped crystals.

Table 4.1 - continued

Animal Number	**Control:** Size (μm)	Features	**Gap region (collagen):** Position	Size (μm)	Features	Other	**Gap region (collagen, fibronectin, laminin):** Position	Size (μm)	Features	Other
Rat 8	1390x1520	2 fascicles	MPO	210x290	24μm rim	Tissue & Plates				
			MPP	260x330		Tissue				
			MPB	210x340		Plates				
			MDP	270x350		Tissue				
			MDB	fragment		Needles****				
Rat 9	510x700	4 fascicles	MPO	100x100	5.8μm rim	Tissue				
Rat 10	540x1390	1 fascicle	MPO	98x160		Tissue	MPR	79x100	8μm rim	Tissue
			MDO	110x160	3.5μm rim	Tissue				
Rat 11	540x1040	3 fascicles	MDO	96x110	4μm rim	Tissue	MPR	320x380		Plates
			MPP	340x360		Plates	MDR	250x280		Plates
			MDP	270x330		Plates	MPY	280x360		Plates
							MDY	220x370		Plates
Rat 12	590x1210	3 fascicles								

4.1.1.2 Control microstructure The major microstructural elements of interest for the left control sciatic nerve were the epineurium, perinerium, endonerium and axons. The epinerium was densely packed, consisting of coarse fibrils (~2 μm diameter) of collagen connective tissue which sheathed the entire nerve (Figure 4.1). The perinerium was the sheath of connective tissue surrounding the individual fascicles and the endonerium was the less dense connective tissue surrounding each nerve fiber (Figure 4.1).

A representative control grouping of axons in a fascicle is shown in Figure 4.6. The structure of an individual axon and its myelin sheath surrounded by endonerium was examined. In these images, the myelin sheath contained irregularly shaped pits caused by sample preparation (Figure 4.7).

4.1.1.3 Wet cut and freeze fractured controls After fixation in 10% buffered formalin and dehydration in graded ethanols, there were observable differences between specimen segments that were wet cut and specimen segments that were freeze fractured in liquid nitrogen. The cross sectional view of the Rat 5 Control wet cut specimen has elongated features due to drawing of the tissue whereas the freeze fractured specimen has a well-defined relatively flat surface with few sectioning distortions (Figures 4.8 and 4.9, respectively). Also, the wet cut epineural tissue appears to have been compressed compared to the freeze fractured epineurial tissue with little distortion of the tissue (Figure 4.10 and 4.11, respectively).

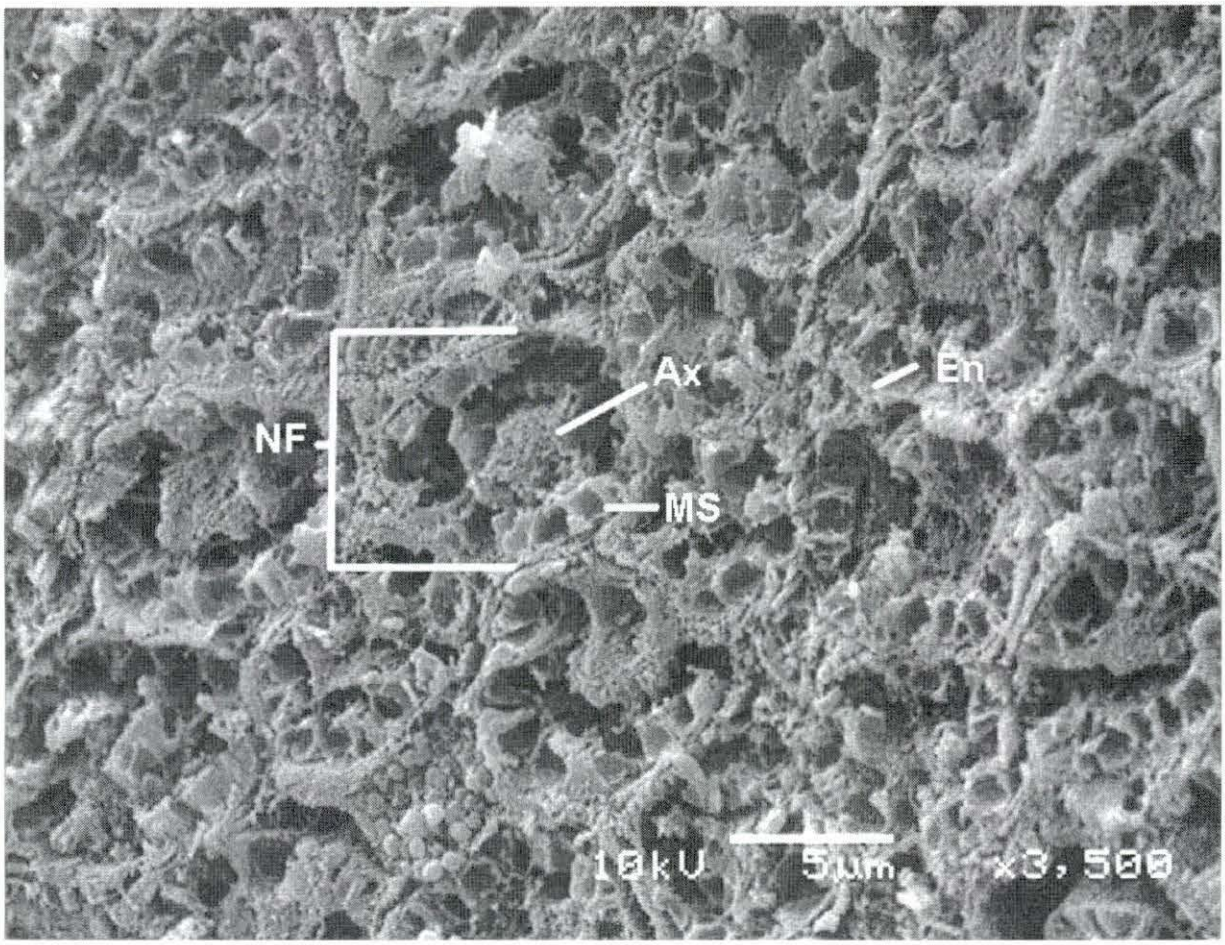

Figure 4.6 - Rat 4 Control endoneurium region with myelinated nerve fibers (NF). The myelin sheath (MS) with pits (caused by exposure to ethanol dehydration) that surround the individual axons (Ax). 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold.

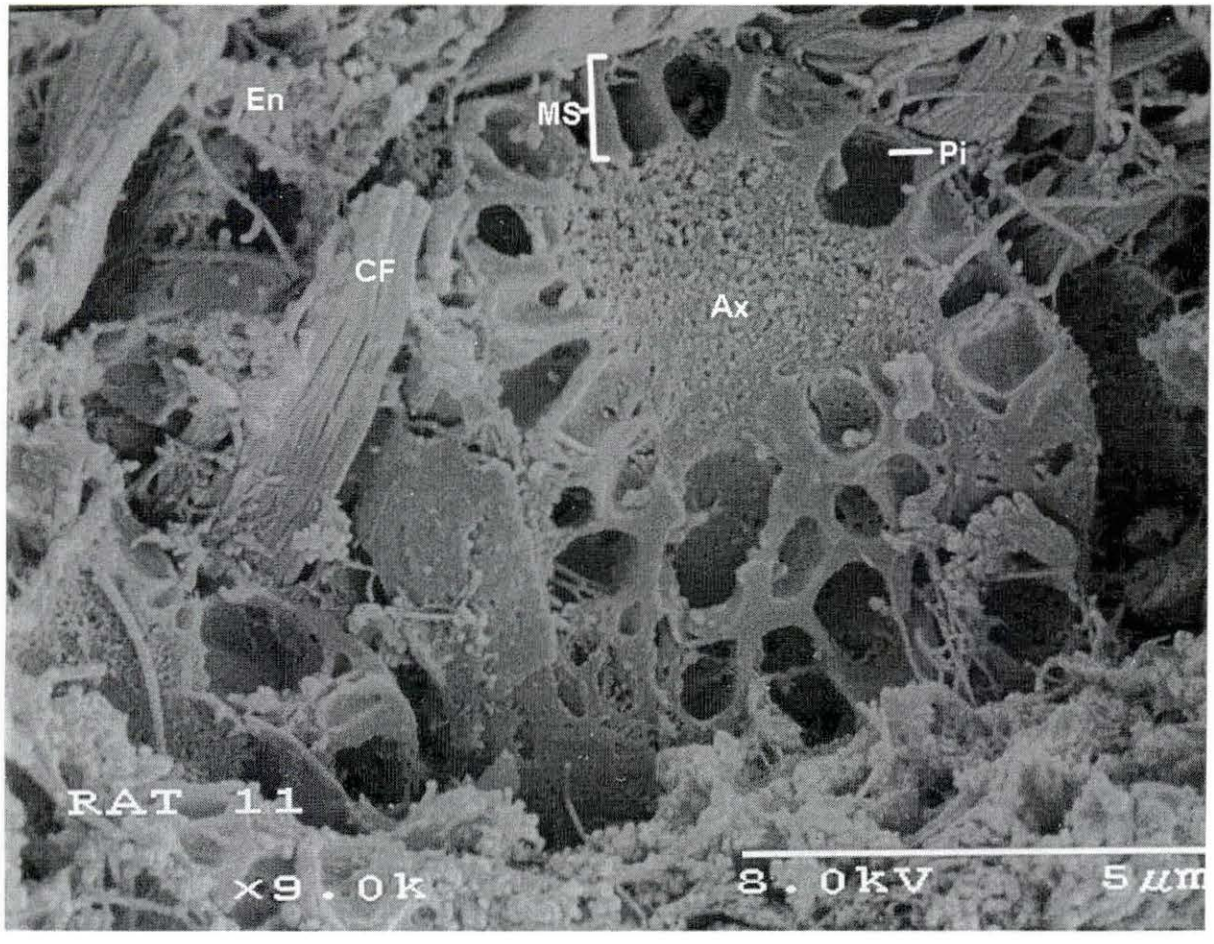

Figure 4.7 - Rat 11 Control - myelinated axon (Ax) with irregular pits (Pi) in the myelin surrounded by endoneurium (En). Collagen fibrils (CF). 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold.

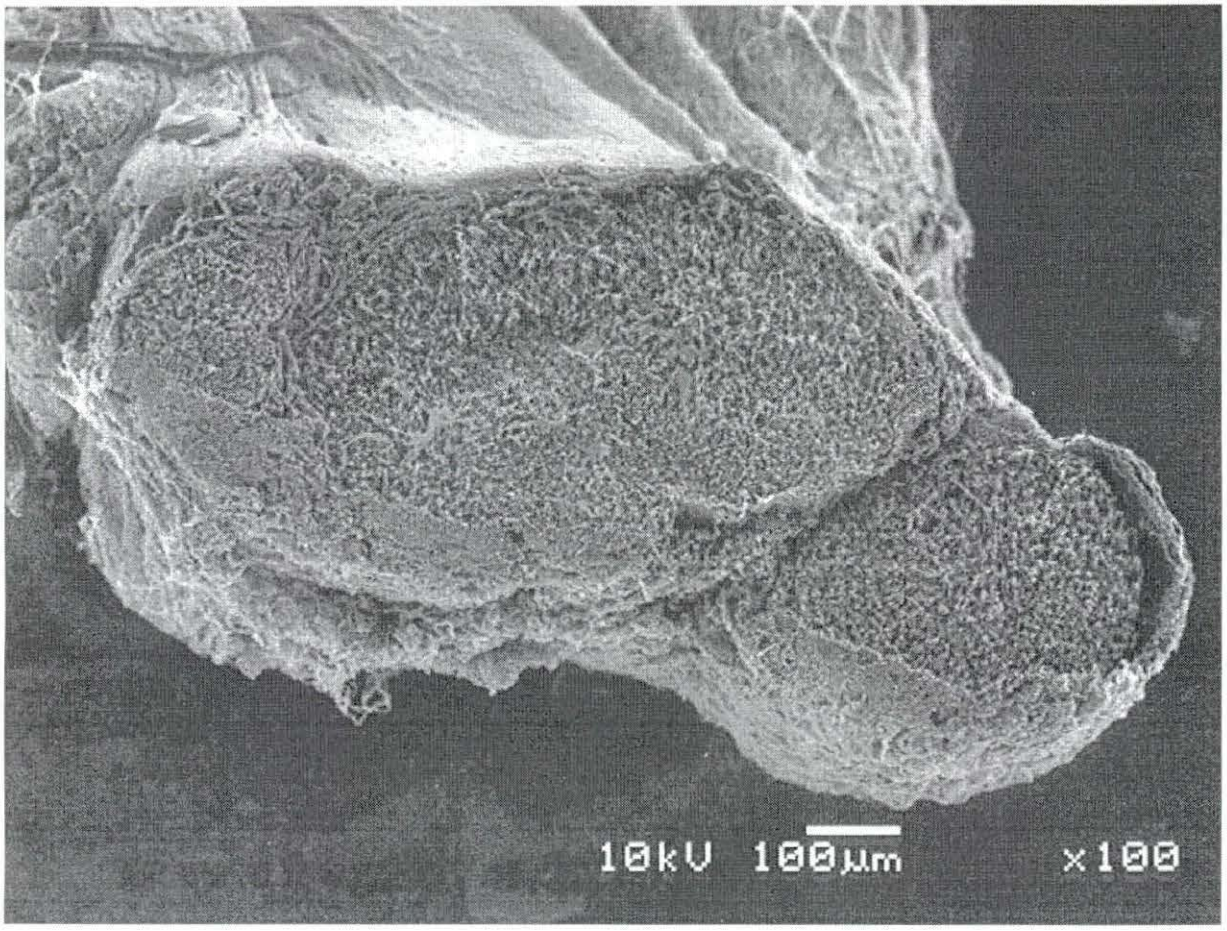

Figure 4.8 - Rat 5 Control cross section - wet cut sample with drawing effect. Also see Figures 4.10 and 4.12. Compare Figure 4.8 with Figure 4.9. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

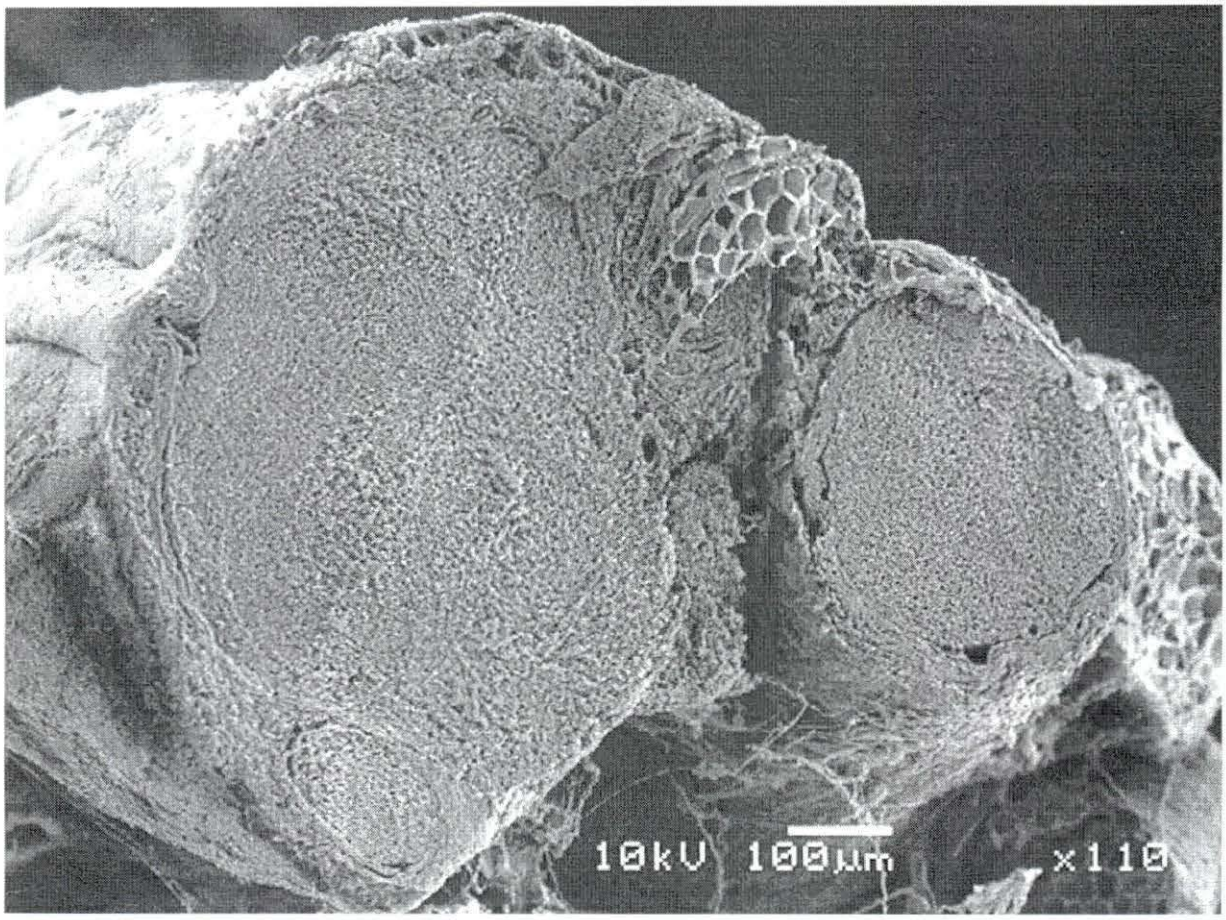

Figure 4.9 - Rat 5 Control cross section - freeze fractured with well-defined features. Also see Figures 4.11 and 4.13. Compare Figures 4.8 and 4.9. 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold.

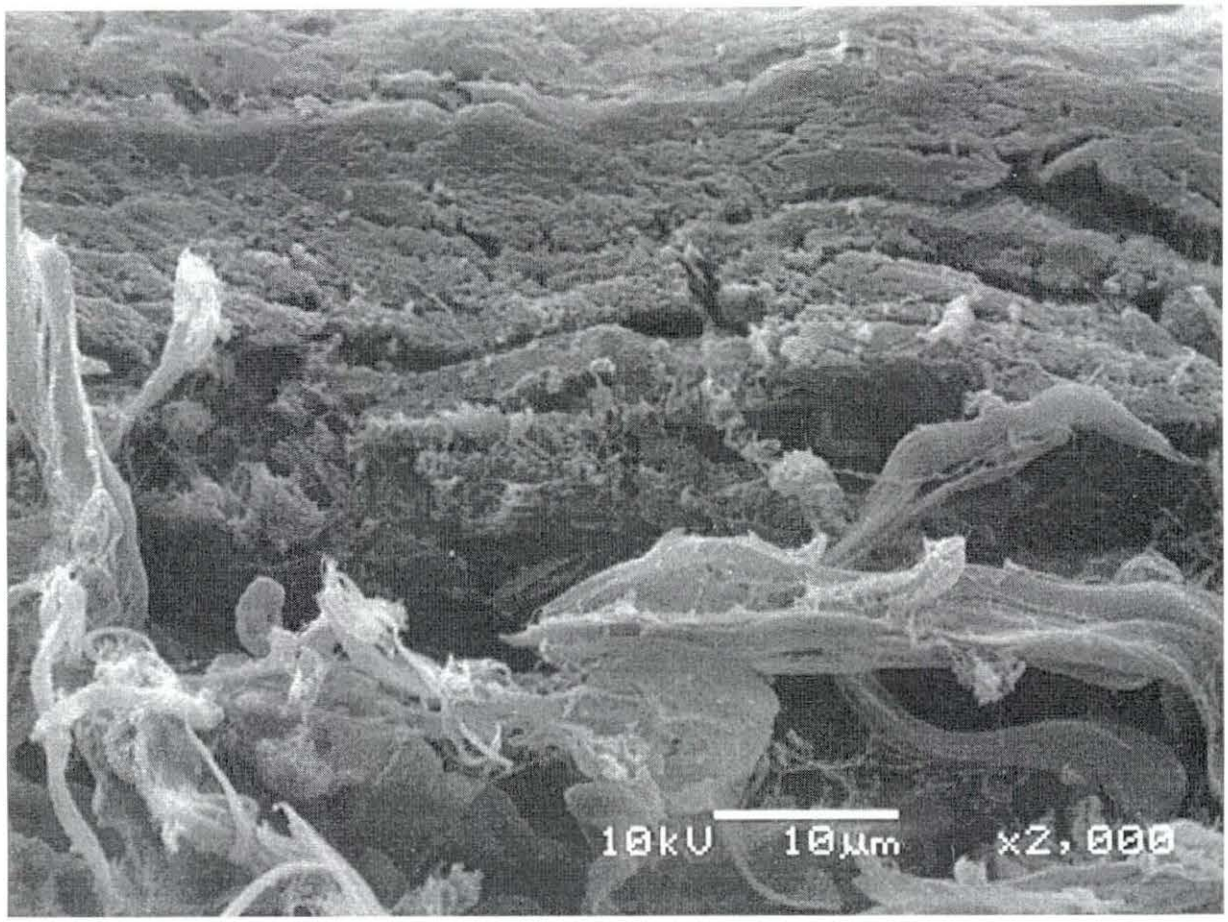

Figure 4.10 - Rat 5 Control epineurium - wet cut sample with collagen fibrils. See Figures 4.8 and 4.12 also. Compare Figures 4.10 and 4.11. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

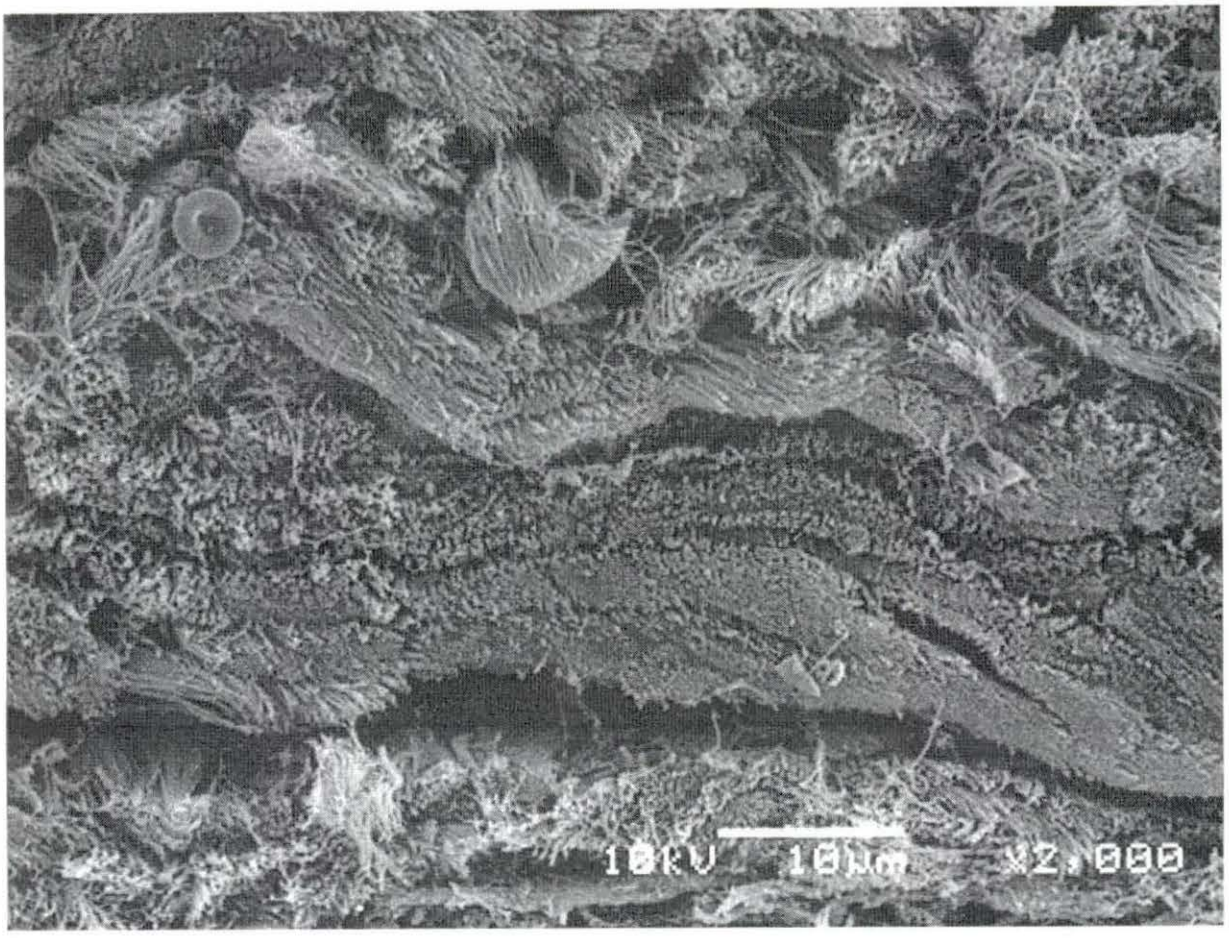

Figure 4.11 - Rat 5 Control epineurium - freeze fractured with individual collagen fibrils evident. See Figures 4.9 and 4.13 also. Compare Figures 4.10 and 4.11. 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold.

The endoneural region indicates the presence of significant microstructural differences related to the method of preparation. The wet cut section displays protruding fibers of the endoneural longitudinal collagen fibrils (Figure 4.12). The axonal information was not obliterated by these long endoneural fibrils. The freeze fractured specimen has a relatively undistorted cross section (Figure 4.13).

During the fixation and ethanol dehydration stages for the processing, the tissue remained in fluids for varying lengths of time.

4.1.2 Regenerated cables

4.1.2.1 Cable shape and size Most of the regenerated strands were circular in shape. Some of the tissue viewed in cross section was layered into concentric rings (Figure 4.14). The cable tissue that was not circular in shape was elliptical (Figure 4.15). This shape may have been influenced by the wet cutting procedure.

The cross sectional diameter of the regenerated strands varied from 22 μm by 23 μm to 460 μm by 560 μm (Table 4.1). The regenerated strands narrowed as they approached the middle region of the gaps; however, the changes were nonuniform. The proximal sample of Rat 9 (Figures 4.16-18) was a good example of this phenomenon where the diameter of the orange color coded strand was 200 μm at the stump-strand junction and 60 μm at 1.25 mm from the proximal stump into the mid gap region (Table 4.1- middle proximal region, 3.5mm into the gap region, 100 x 100 μm and Table 4.2).

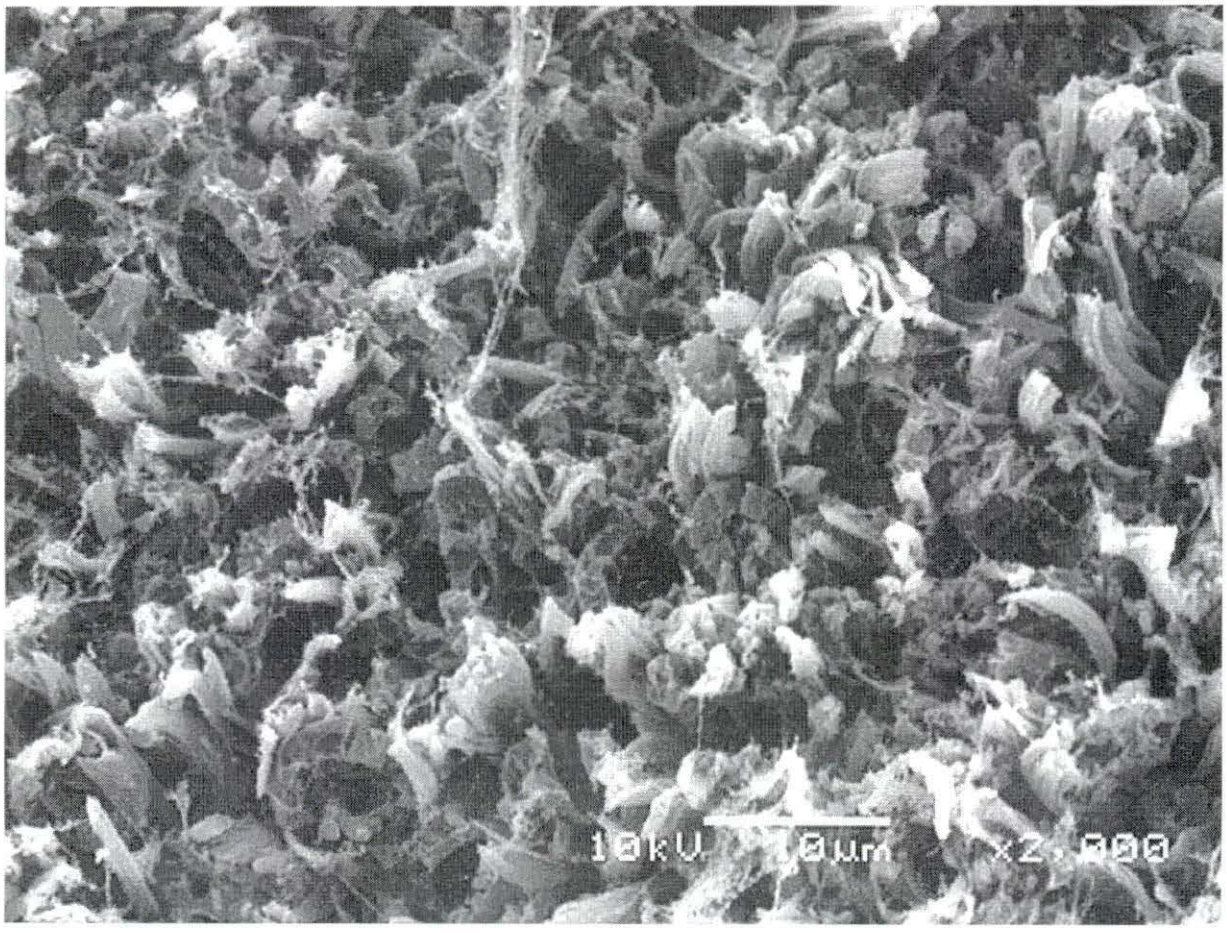

Figure 4.12 - Rat 5 Control endoneurium - wet cut sample with drawing effect seen primarily for the collagen fibrils. See Figures 4.8 and 4.10 also. Compare Figures 4.12 and 4.13. 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

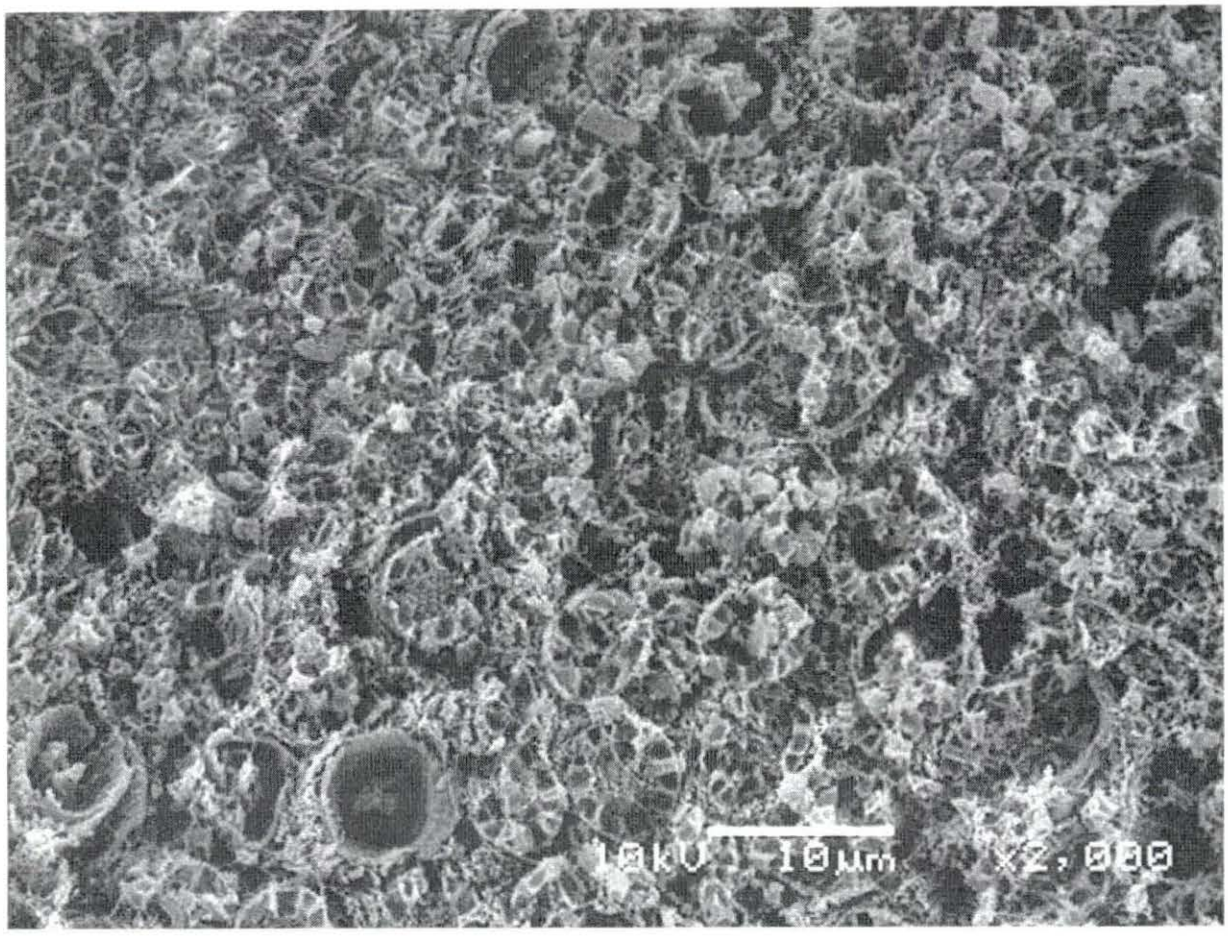

Figure 4.13 - Rat 5 Control endoneurium - freeze fractured with well-defined features. Also see Figures 4.9 and 4.11. Compare Figures 4.12 and 4.13. 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold.

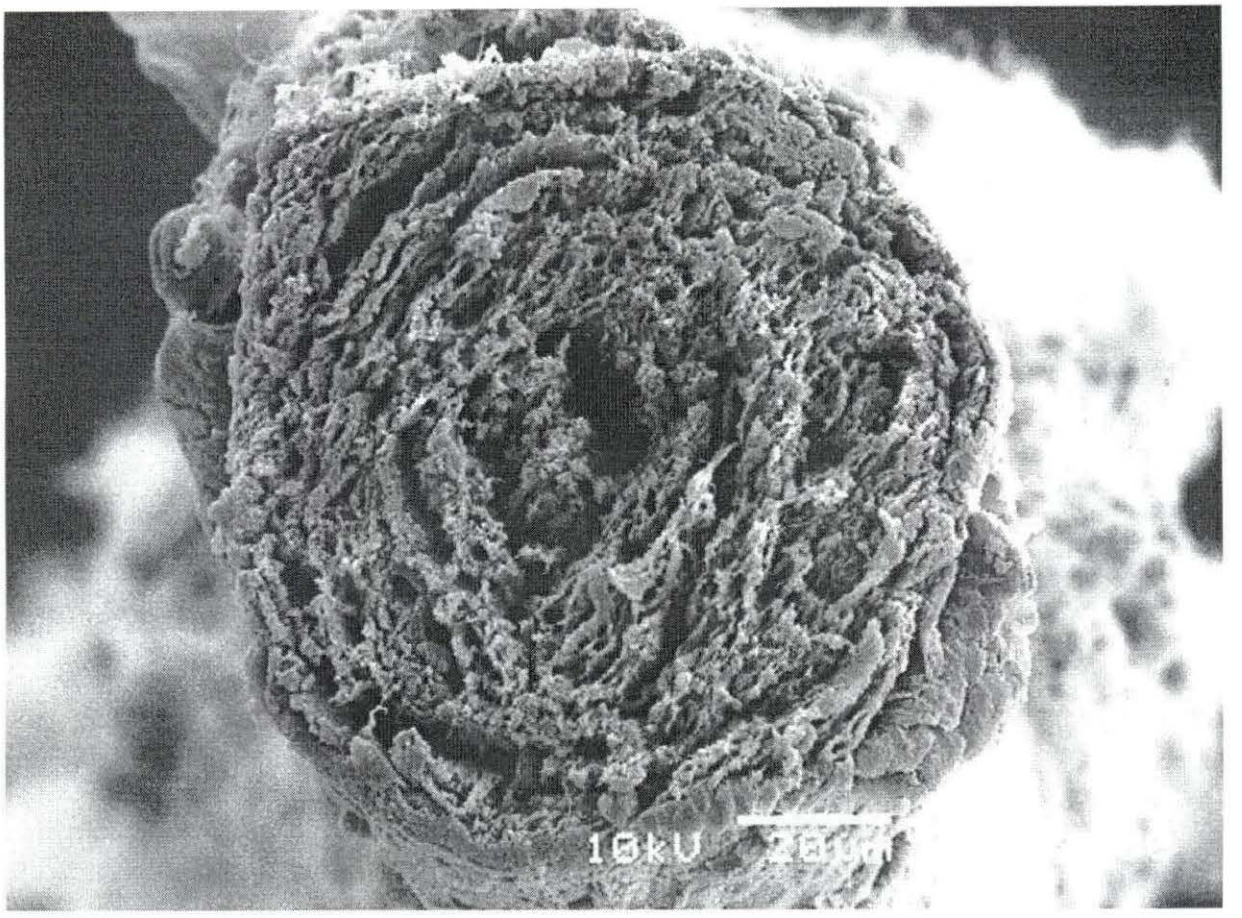

Figure 4.14 - Rat 9 Middle Proximal Orange color coded strand (collagen filled lumen) with a circular shape and concentric rings of tissue. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

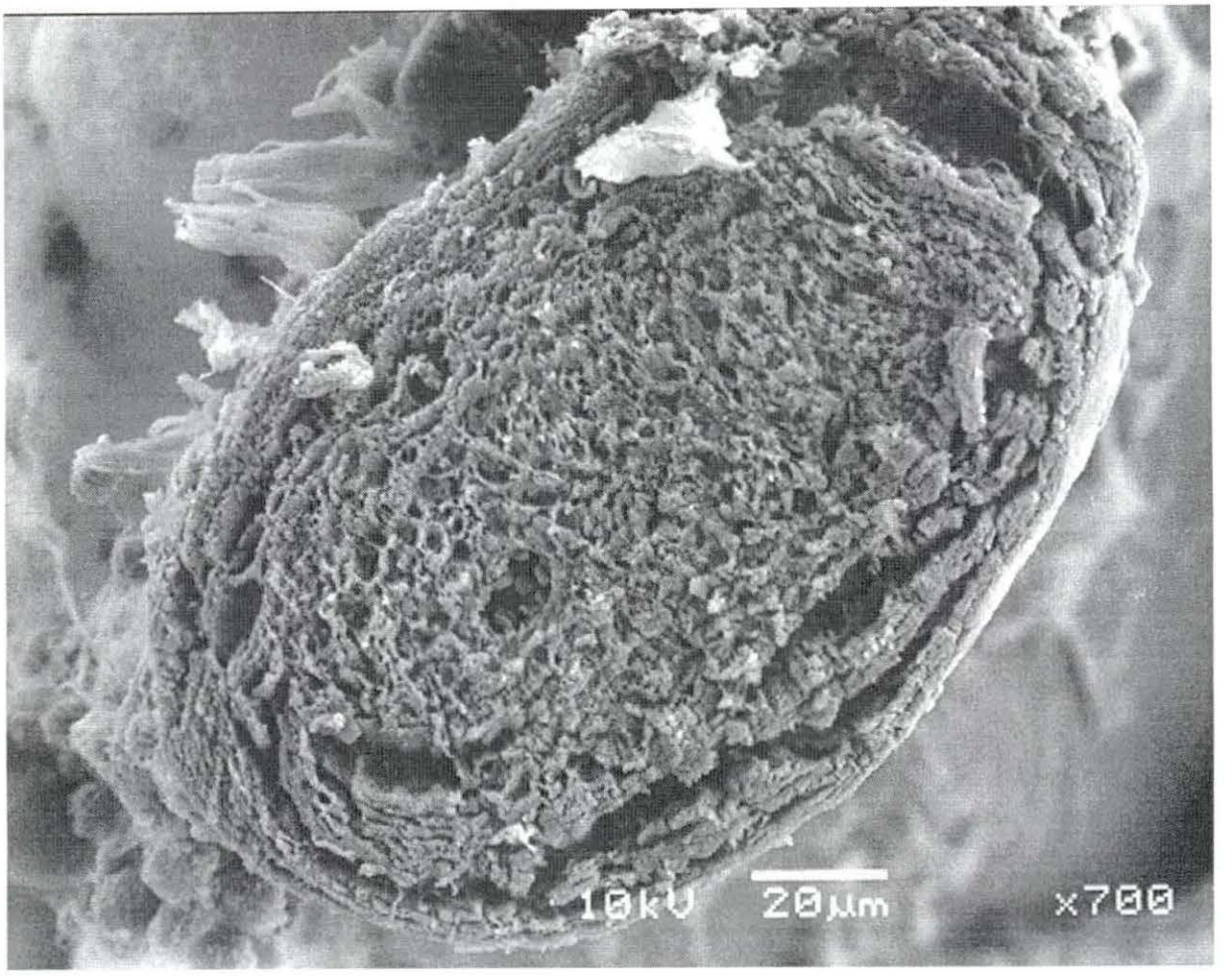

Figure 4.15 - Rat 11 Distal strand #2 (unknown lumen filling) in the gap region with an elliptical shape. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold.

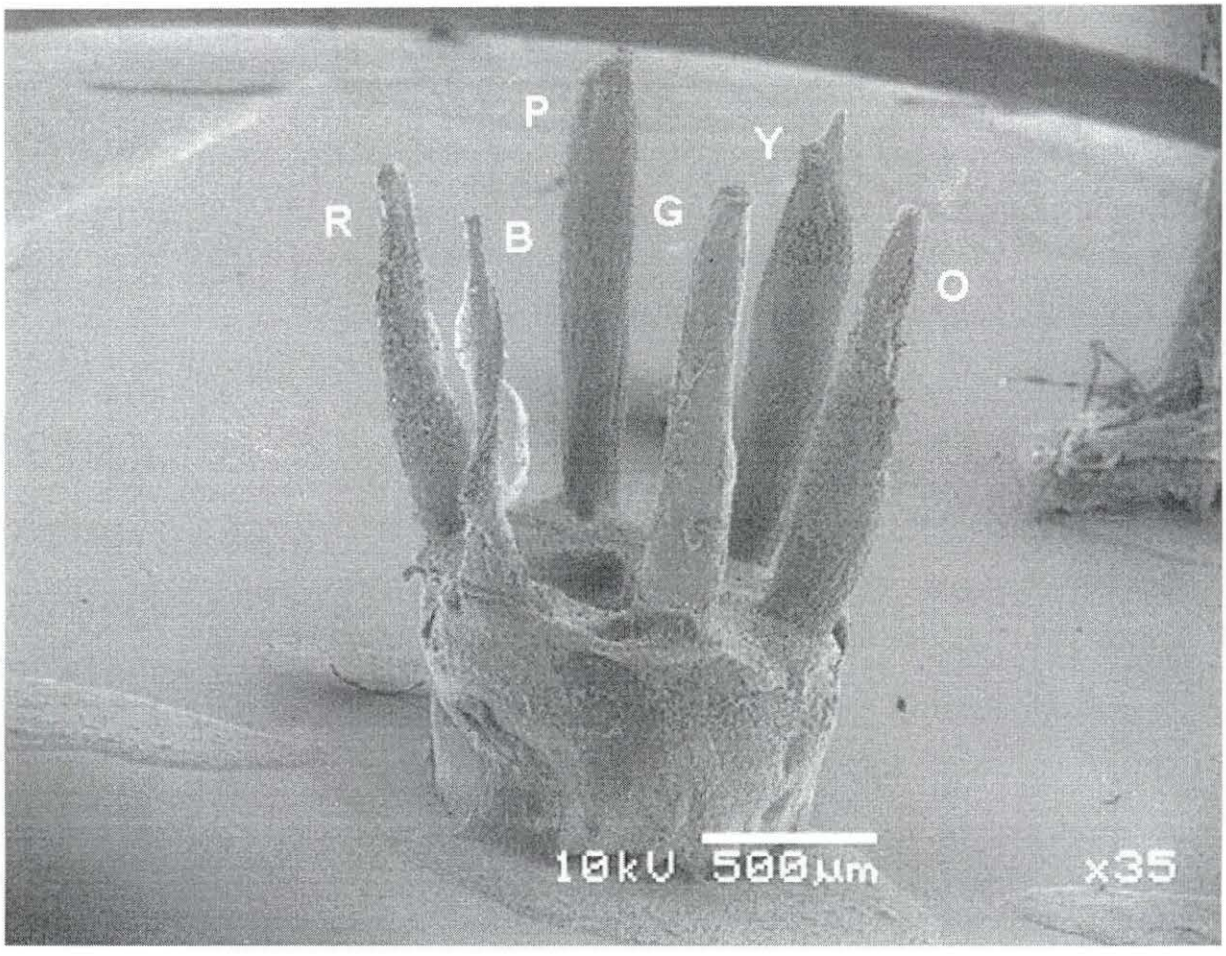

Figure 4.16 - Rat 9 Proximal section showing narrowing of stands as they approach the center of the gap region. Clockwise from the left side is red (R), purple (P), yellow (Y), orange (O), green (G) and blue (B) color coding. O, P and B correspond to cables found in collagen filled lumens. R, Y and G correspond to cables found in the mixture filled lumens.

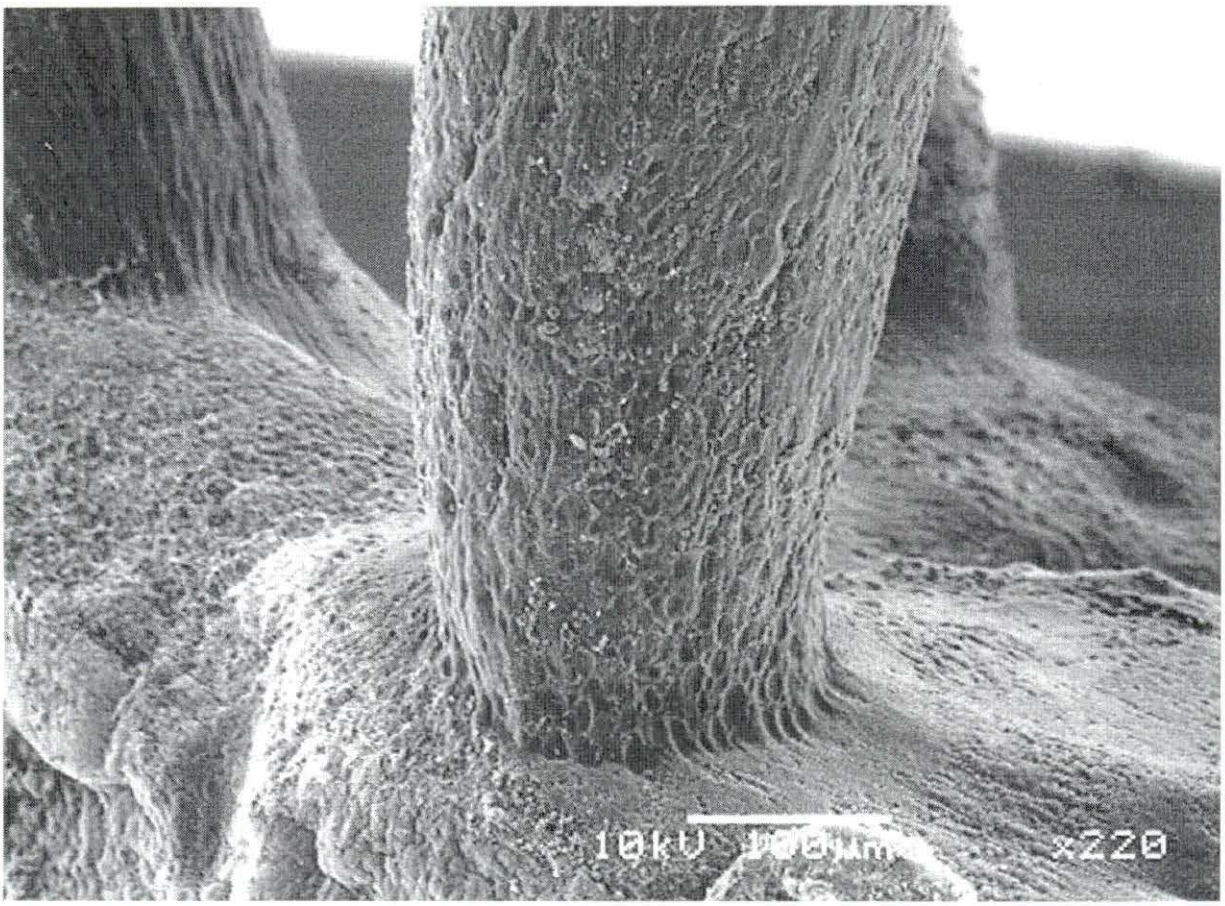

Figure 4.17 - Rat 9 side view of the red color coded strand - Junction between the proximal stump and the gap region (refer to the red color coded strand in Figure 4.16). Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Cable found in the mixture filled lumen (collagen, fibronectin and laminin).

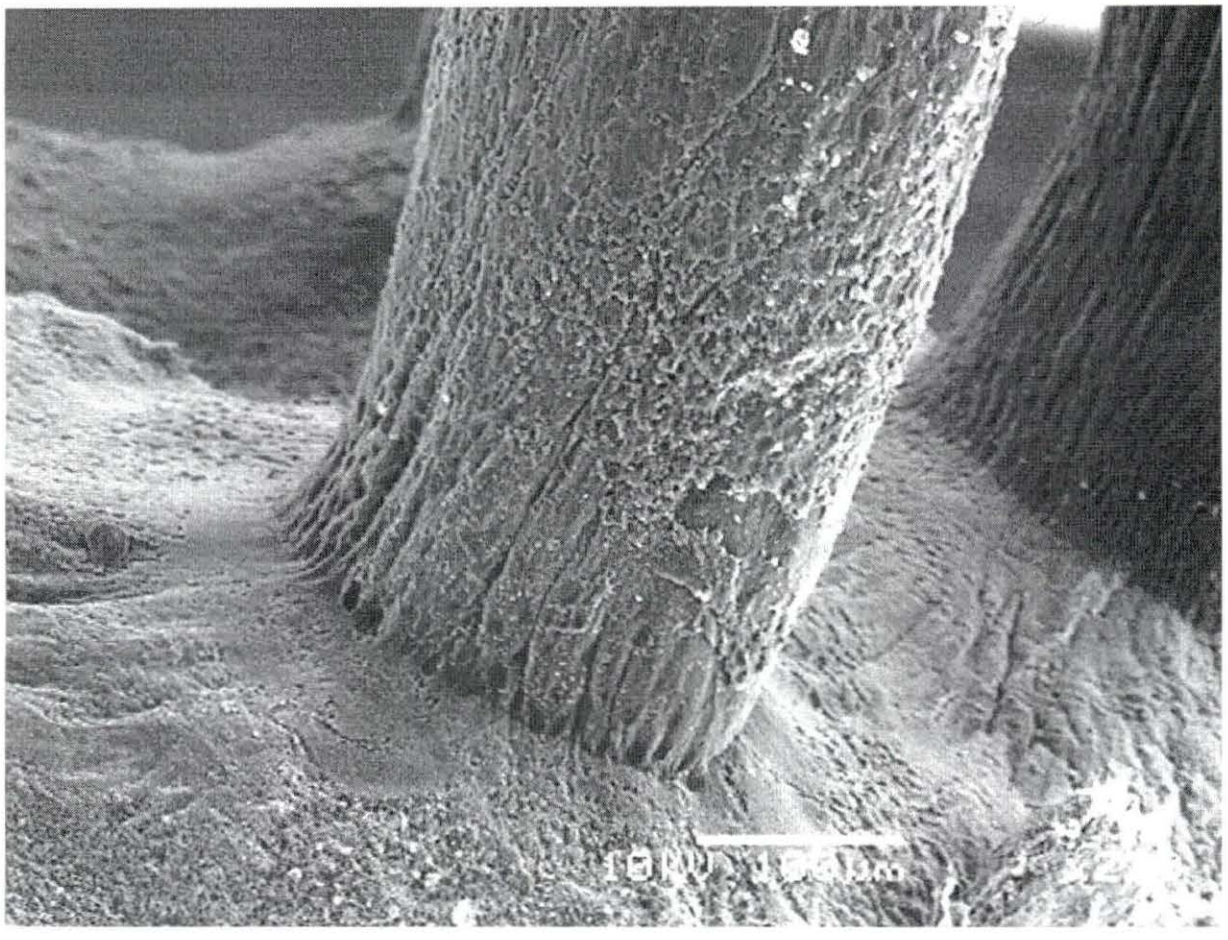

Figure 4.18 - Rat 9 side view of the blue color coded strand - Junction between the proximal stump and the gap region (refer to the blue color coded strand in Figure 4.16). Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Cable found in the collagen filled lumen.

Table 4.2 - Rat 9 proximal section strand diameters (from Figure 4.16)

Lumen Color	Size at Stump-strand Junction (μm)	Size at 750μm from Junction (μm)	Size at 1.25 mm from Junction (μm)
Orange	200	220	60
Purple	210	230	150
Blue	200	130	30
Red	250	100	100
Yellow	240	280	40
Green	230	170	120

Note: The orange, purple and blue lumens contained collagen. The red, yellow and green lumens contained a mixture of collagen, fibronectin and laminin.

The proximal sample contained a 0.5 mm long portion of the proximal stump from within the guide and about 1.25 mm long strands from the gap region. The orange, blue and purple color coded lumens contained collagen and the red, green and yellow color coded lumens contained collagen, fibronectin and laminin. The strand cables tend to narrow as they grow toward the center of the nerve guide (Figure 4.16). The distal sample contained 0.5 mm long strands from the gap region and a 0.5 mm long section of the distal stump from within the guide. The Distal section of Rat 9 (Figure 4.19) shows that cable tissue crossed the gap (this was the case for all of the lumens for this rat). There were no major differences between the appearance of the cables forming in the collagen filled lumens compared with those in the mixture filled lumens.

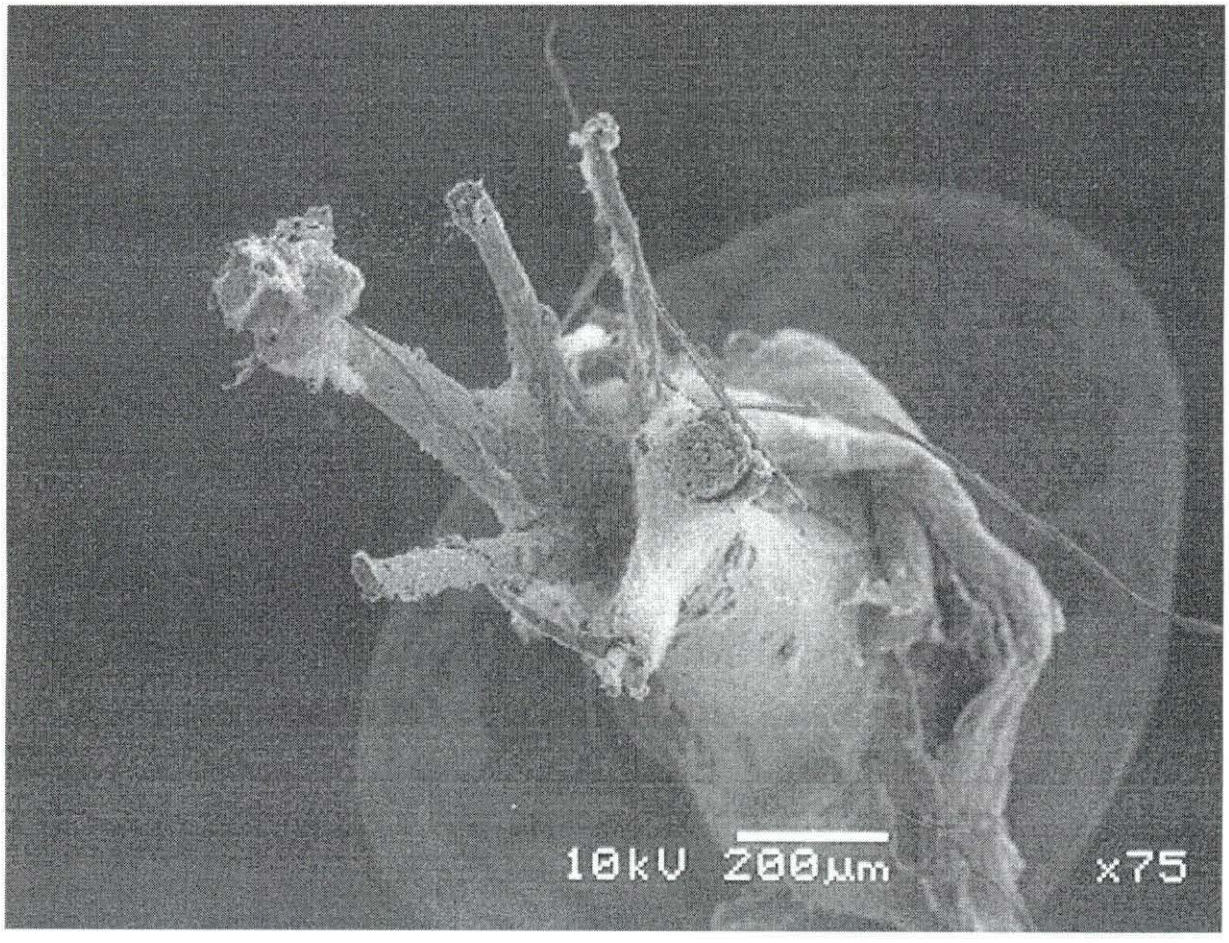

Figure 4.19 - Rat 9 Distal section.

4.1.2.2 Cable microstructure The fine structure of the regenerated tissue appeared in three categories: tissue, crystals and a mixture of tissue and crystals. There were no distinguishable trends in textual or size differences between the strands from the collagen filled lumens and the strands from the collagen, laminin and fibronectin filled lumens at the observed magnifications for the surface views.

There was evidence of blood vessels, porous structures and a definable rim (Figures 4.20 and 4.21). The central region was composed of less dense tissue with circular pores (Figure 4.22). The outer rim area consisted of a denser tissue that was layered (Figures 4.23 and 4.24). Some of the strands did not have a large rim (Figures 4.25 and 4.26). The central region of this strand (Figure 4.26) contained circular voids (possibly due to dissolution of the myelin around possible axons during the ethanol dehydration procedure).

Many of the regenerated strands contained plate-like crystal structures (Figures 4.28 and 4.29). This was most prevalent in Rat 7 (Table 4.1). The crystals varied in shape and texture. Most of the crystals were plate-like with different surface textures (Figures 4.30); however, changes in crystal shape were observed at different locations in the gap region even in a single lumen. In the Middle Distal Blue strand of Rat 8, needle-like structures were present (Figure 4.31) even though plate-like structures were evident in the Middle Proximal Blue section (Figure 4.30). The Middle Proximal Orange color coded section of Rat 8 contained a combination of plate-like crystals and tissue. The central region was a mixture of plates and tissue. The outer region of the strand consisted of layered connective tissue (Figure 4.32).

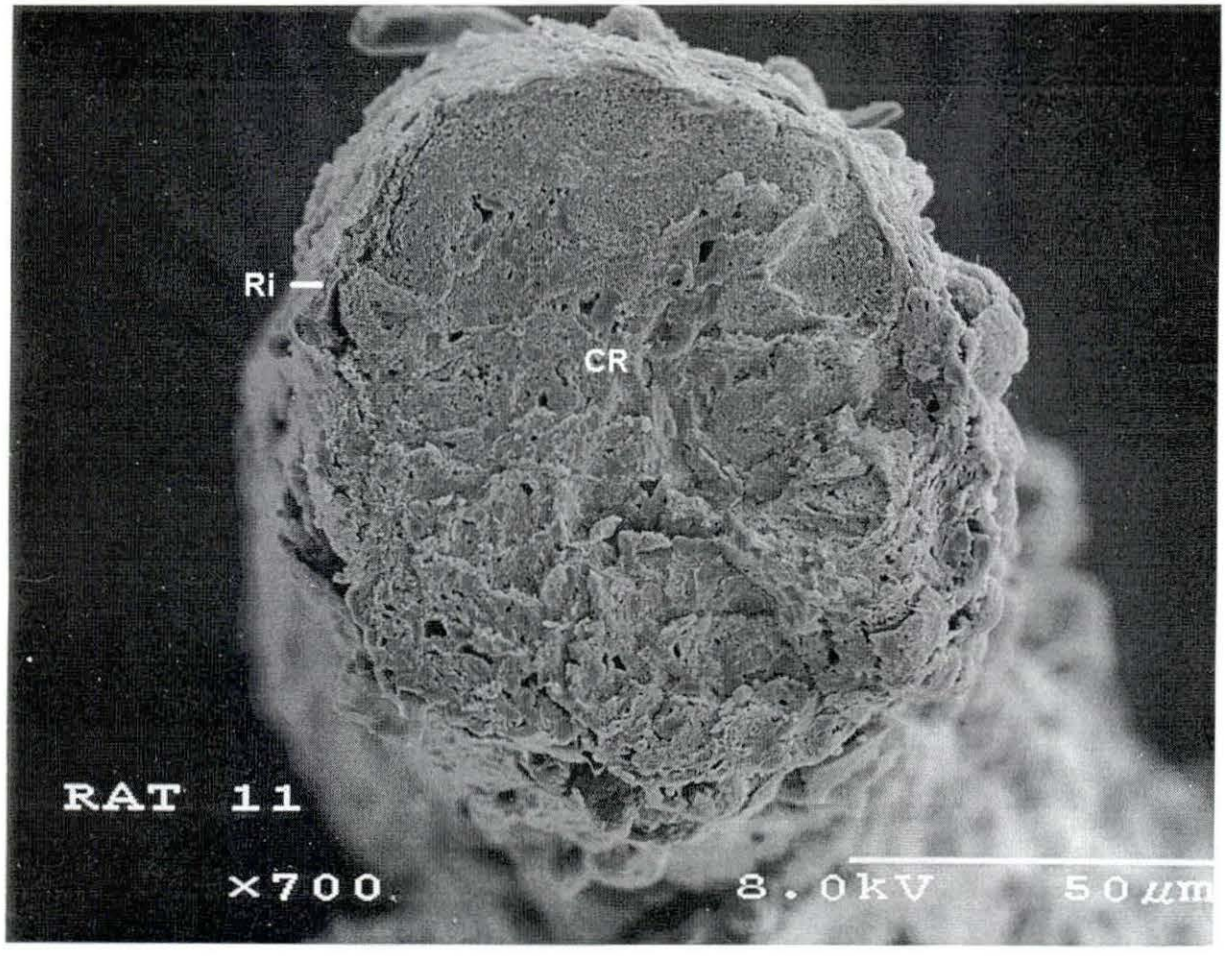

Figure 4.20 - Rat 11 Middle Distal Orange color coded strand cross section - Central region (CR) with rim (Ri) at outer edge of strand. 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold. Cable formed in collagen filled lumen.

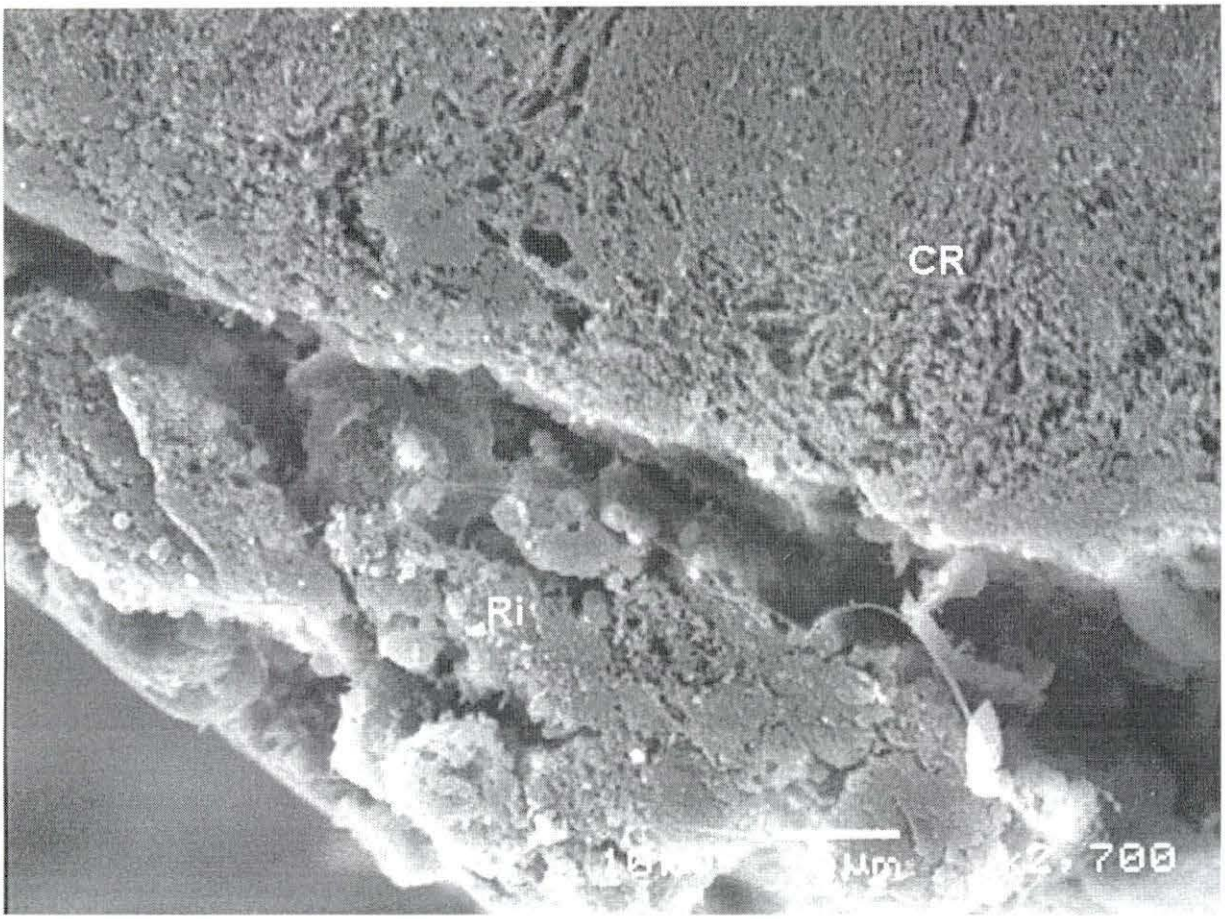

Figure 4.21 - Rat 11 Middle Distal Orange color coded strand - Junction of rim (Ri) and central region (CR). Refer to Figure 4.22 for a higher magnification view of the central region. 28 day fixation, 1 day dehydration, nitrogen freeze fractured, critical point dried, 200 Å palladium and gold. Cable formed in collagen filled lumen.

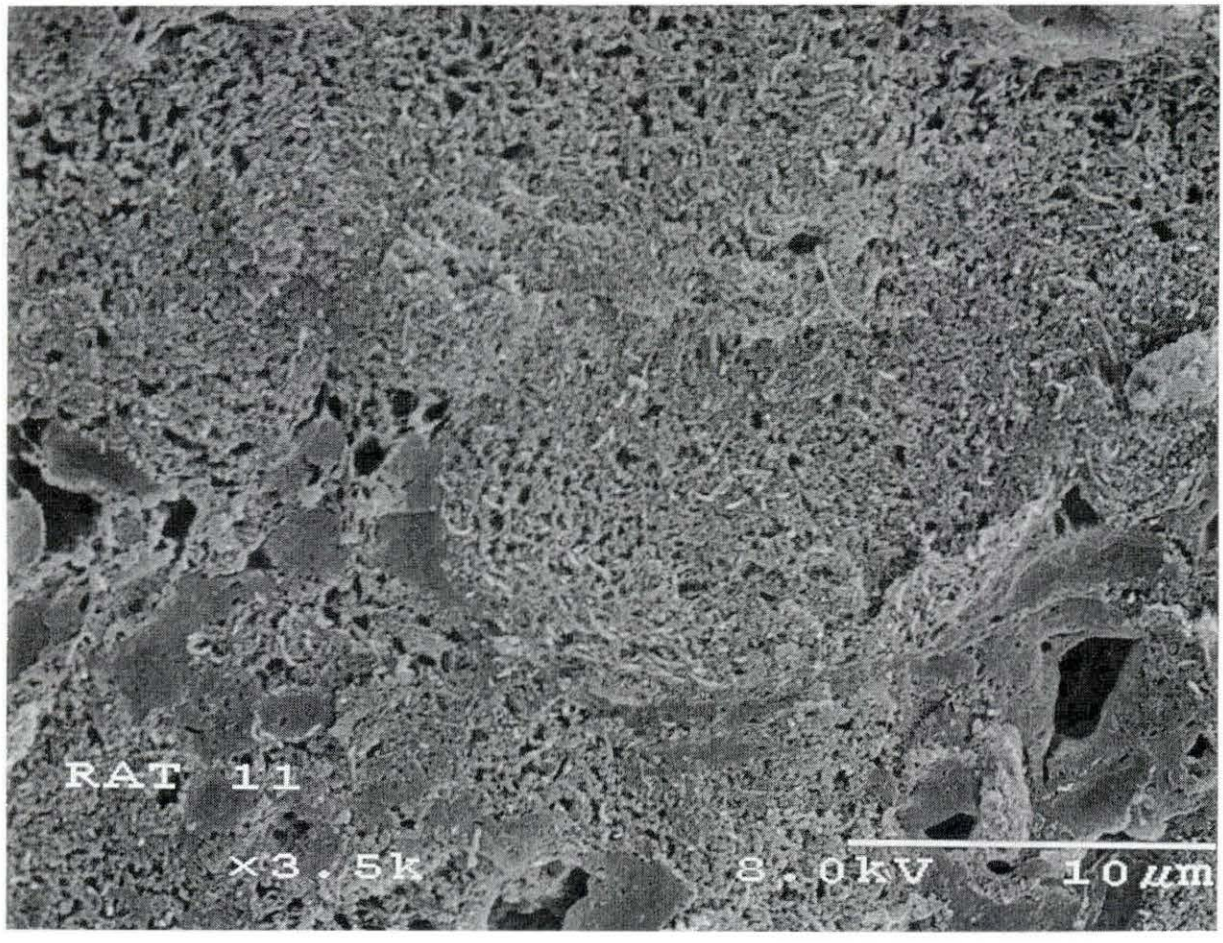

Figure 4.22 - Rat 11 Middle Distal Orange color coded strand central region (refer to Figures 4.20 and 4.21). The grey amorphous areas may be acellular material associated with the collagen gel matrix).

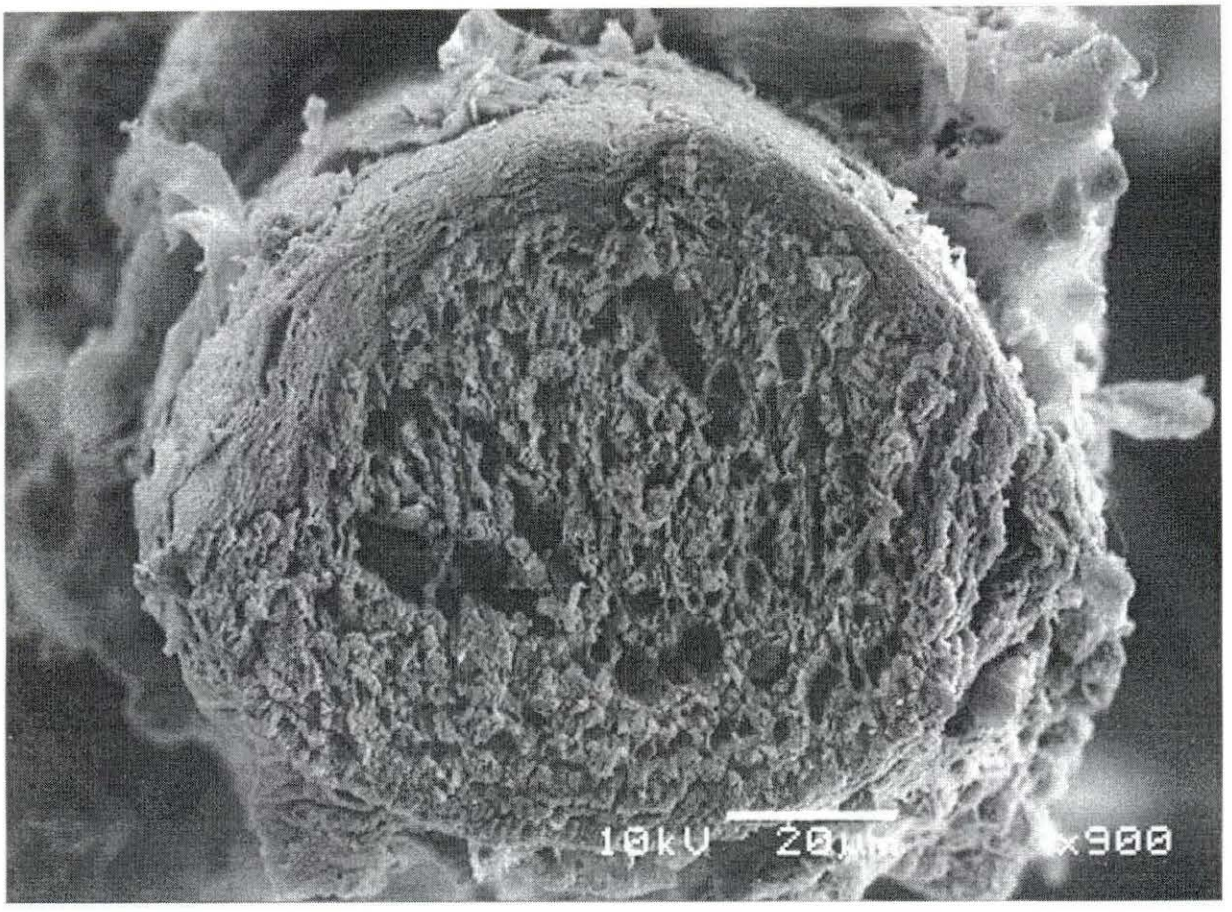

Figure 4.23 - Rat 10 Middle Proximal Red color coded strand cross section. Refer to Figure 4.24 for a higher magnification view. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Cable formed in the a lumen containing a mixture of collagen, fibronectin and laminin.

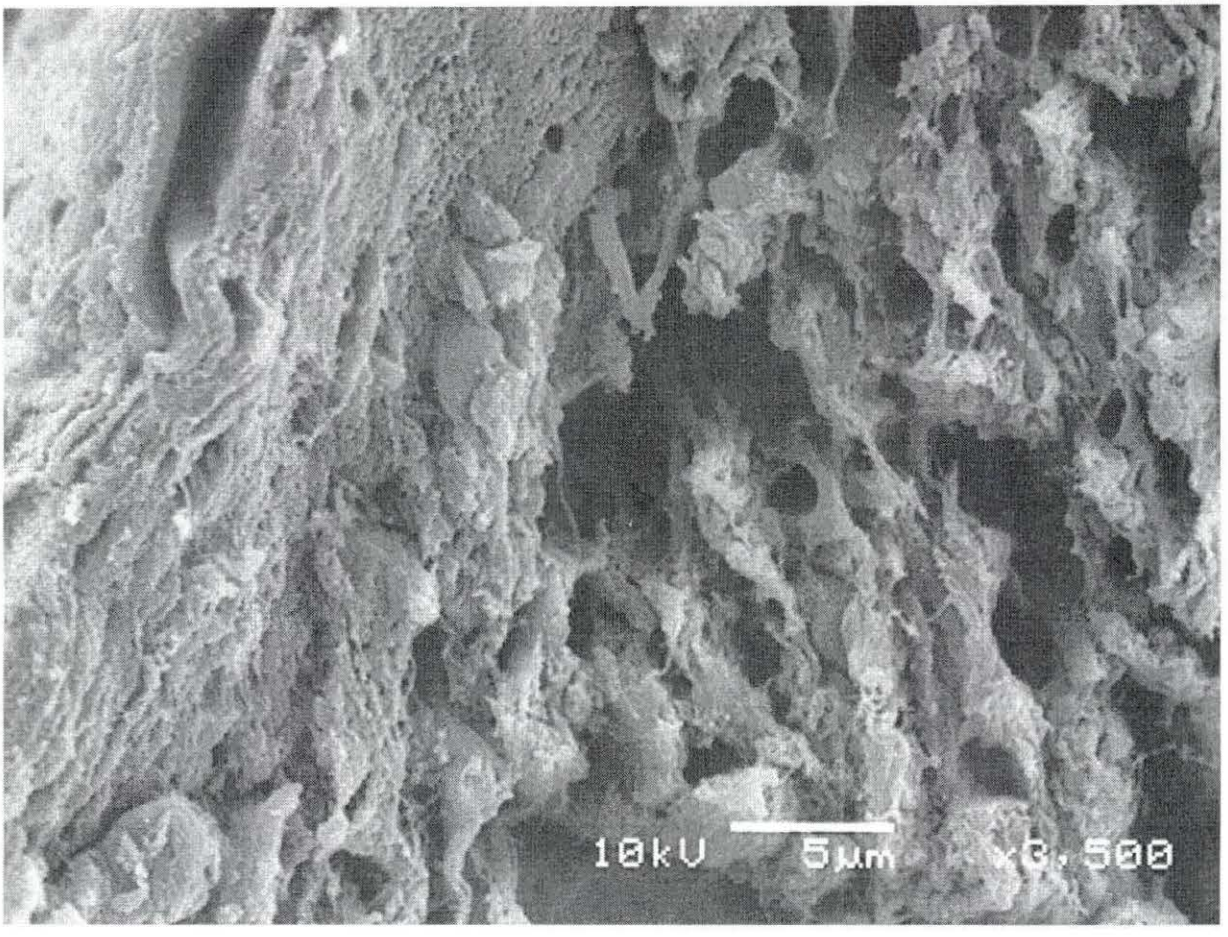

Figure 4.24 - Rat 10 Middle Proximal Red color coded strand - Junction of layered tissue rim and central region.

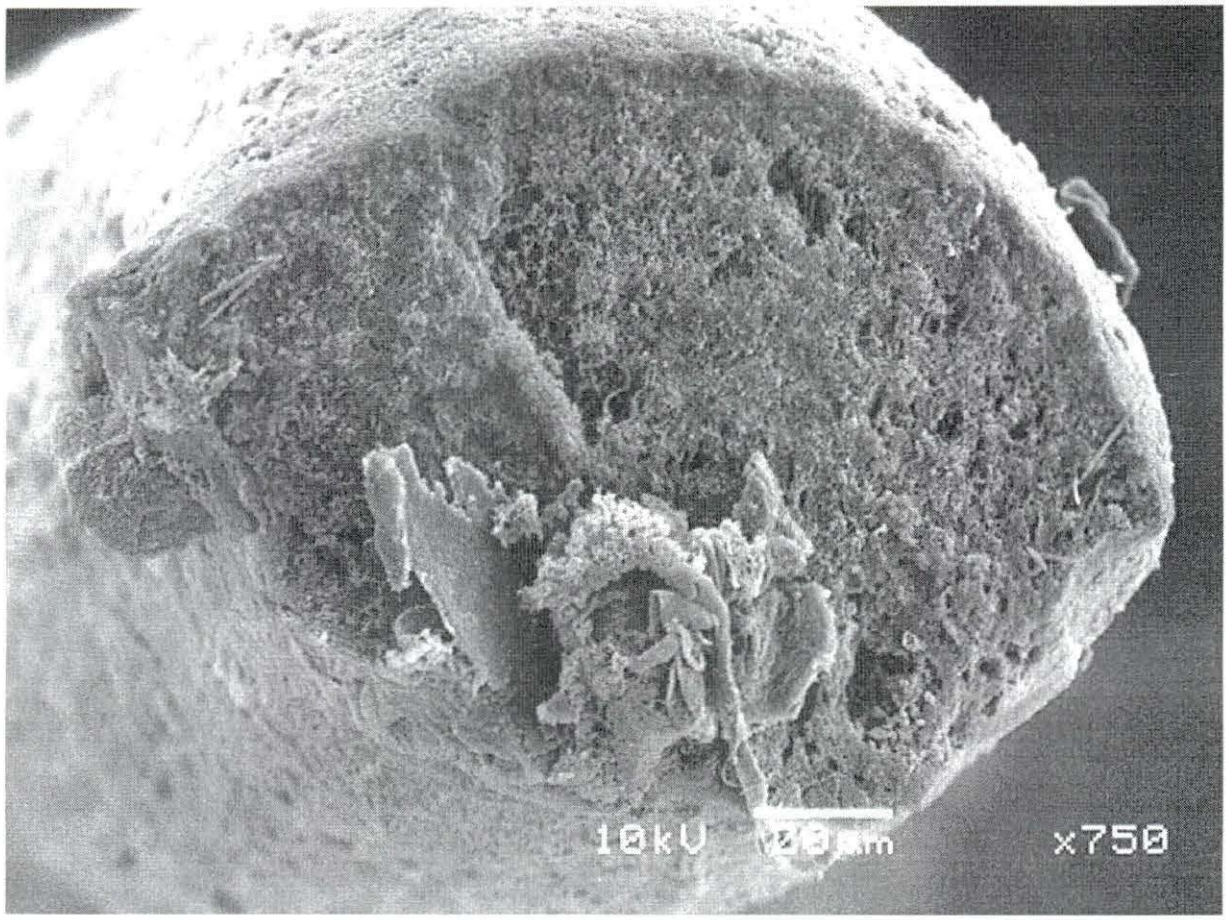

Figure 4.25 - Rat 10 Middle Proximal Orange color coded strand cross section. Also see Figures 4.26 and 4.27 for higher magnfication views. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Cable formed in the collagen filled lumen.

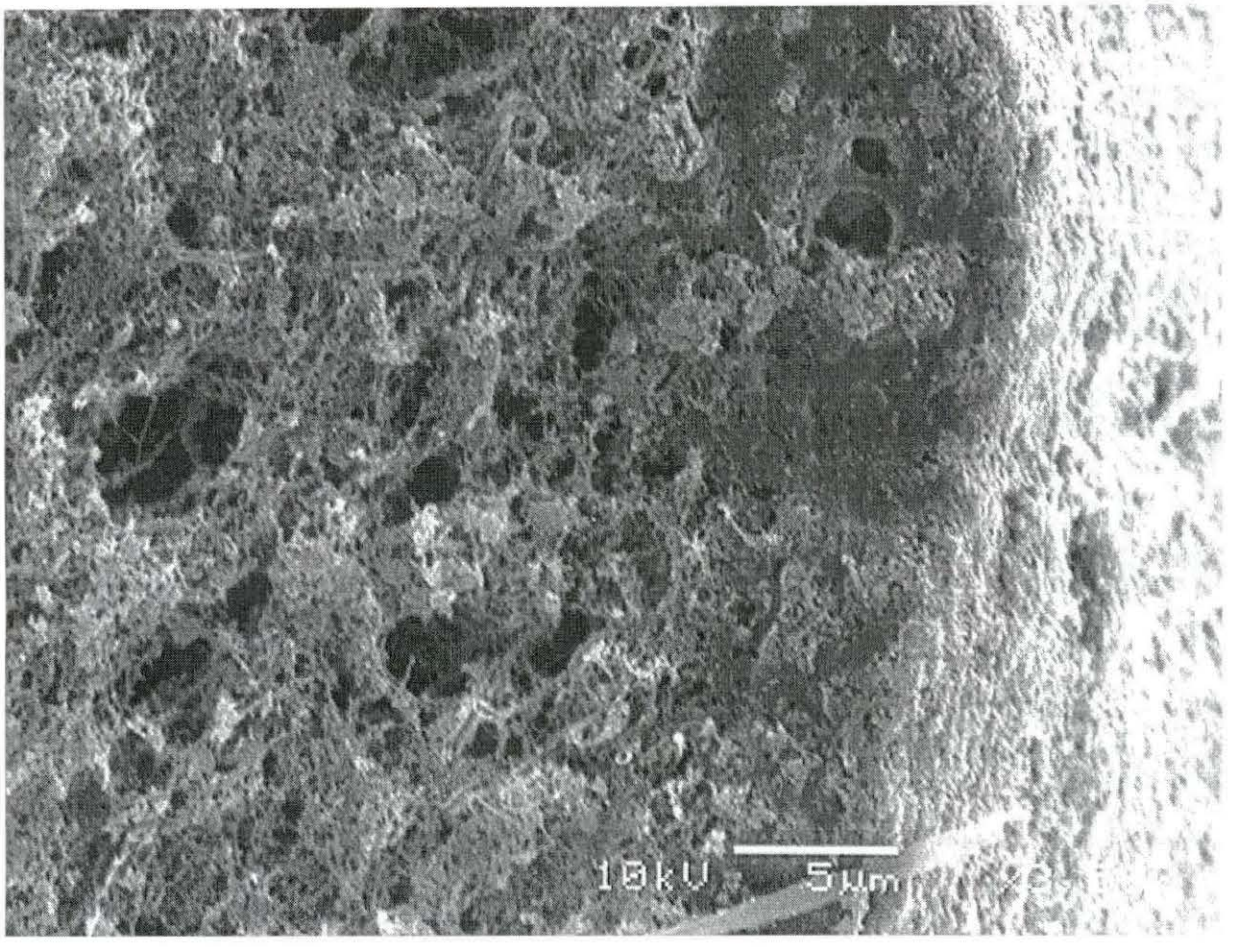

Figure 4.26 - Rat 10 Middle Proximal Orange color coded strand - Cable edge and central region. See Figures 4.25 and 4.27 also.

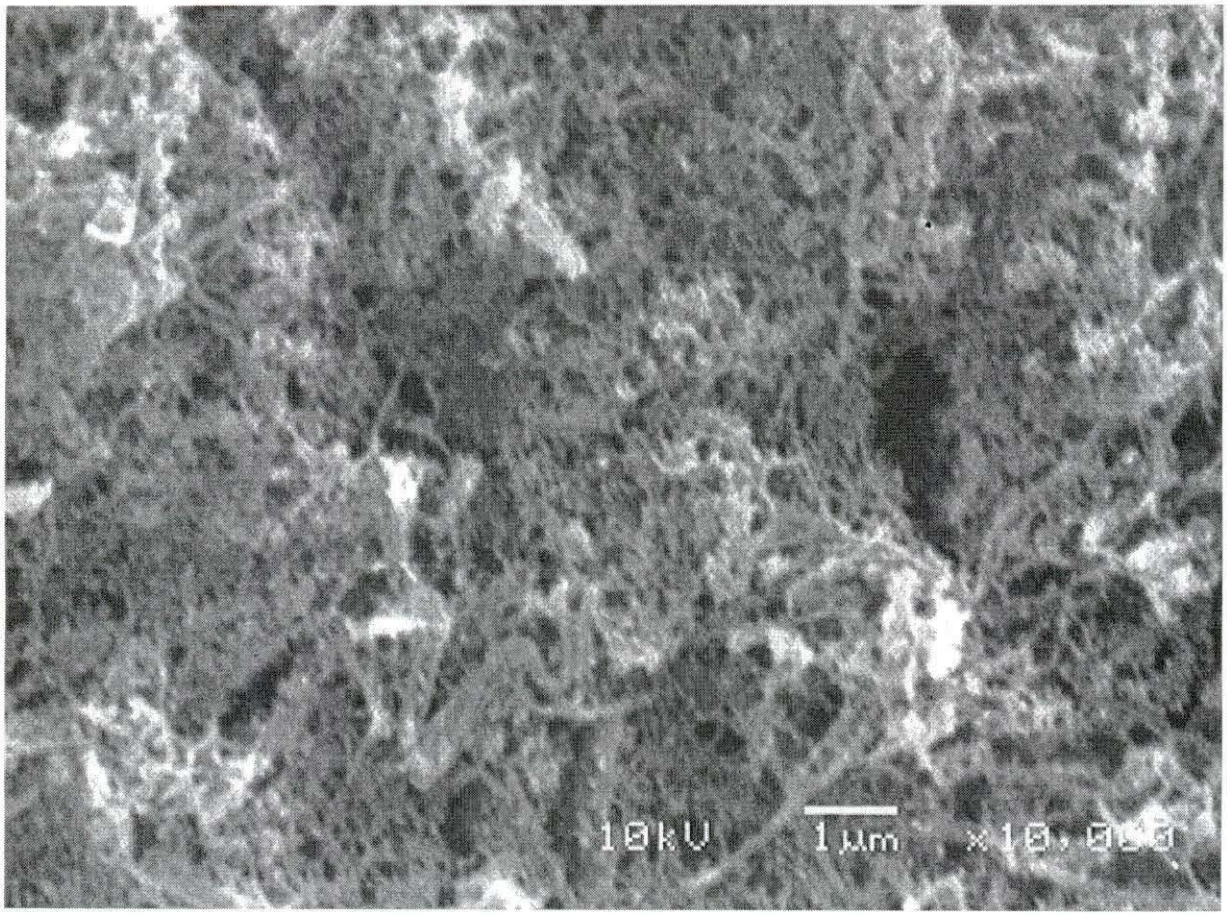

Figure 4.27 - Rat 10 Middle Proximal Orange color coded strand central region. Also see Figures 4.25 and 4.26.

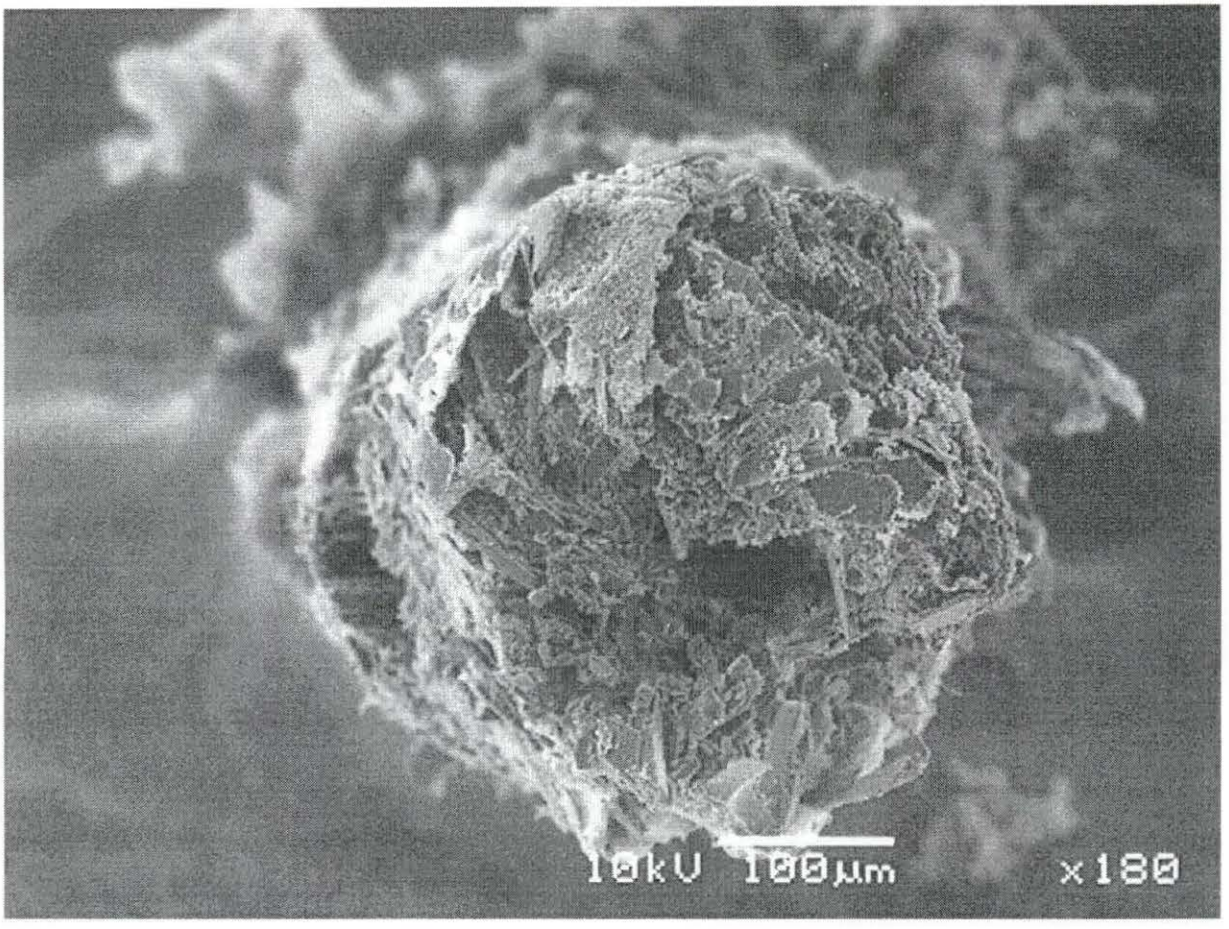

Figure 4.28 - Rat 11 Middle Proximal Red color coded strand cross section with plate-like crystals throughout. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Crystals are probably associated with a sodium bicarbonate solution cleaning procedure. Lumen contained a mixture of collagen, fibronectin and laminin.

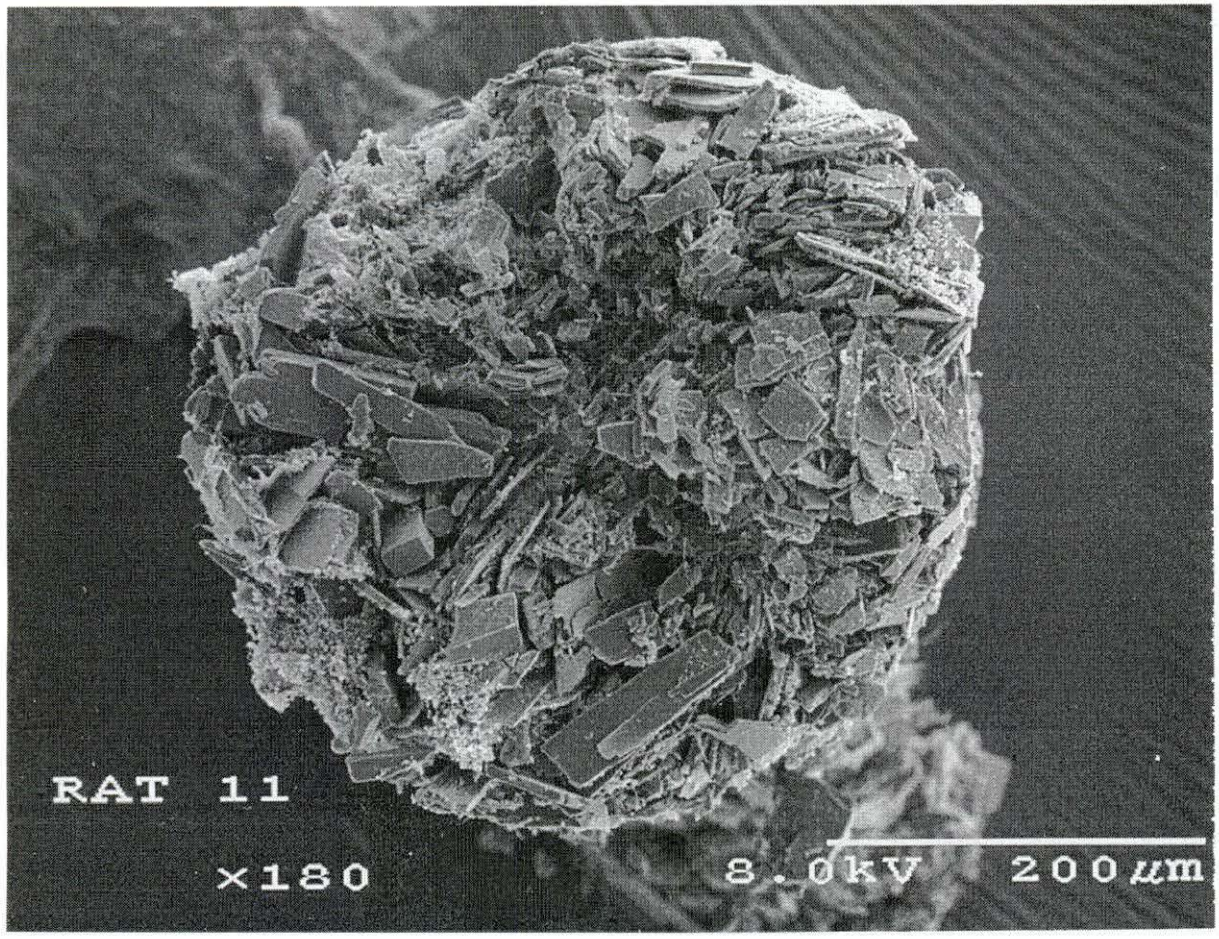

Figure 4.29 - Rat 11 Middle Distal Red color coded strand cross section with plate-like crystals throughout. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Crystals are probably associated with a sodium bicarbonate solution cleaning procedure. Lumen contained a mixture of collagen, fibronectin and laminin.

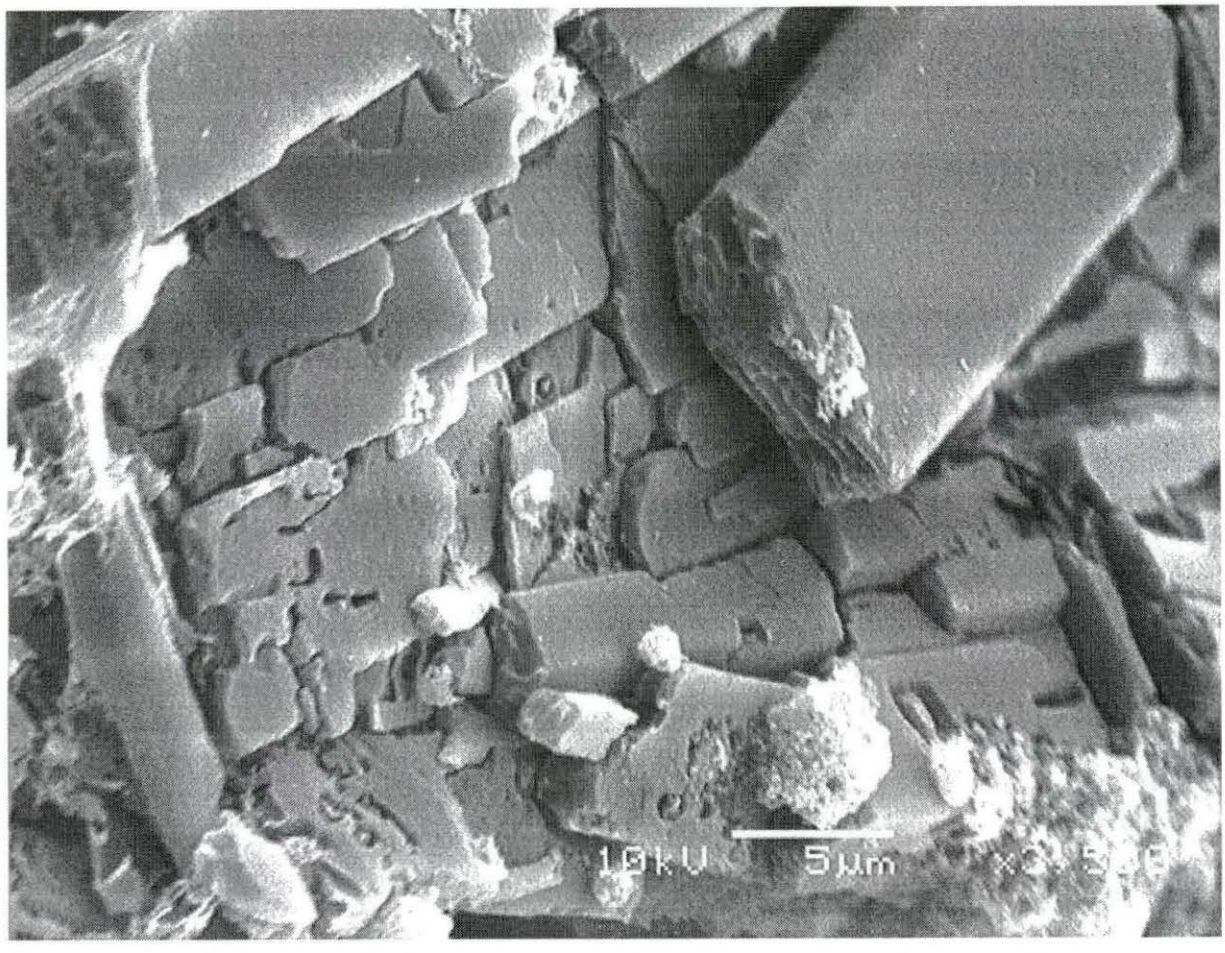

Figure 4.30 - Rat 8 Middle Proximal Blue color coded strand with plate-like crystals throughout the cross section. Wet cut, 28 day fixation, 107 days in ethanol, critical point dried, 200 Å palladium and gold. Lumen was collagen filled.

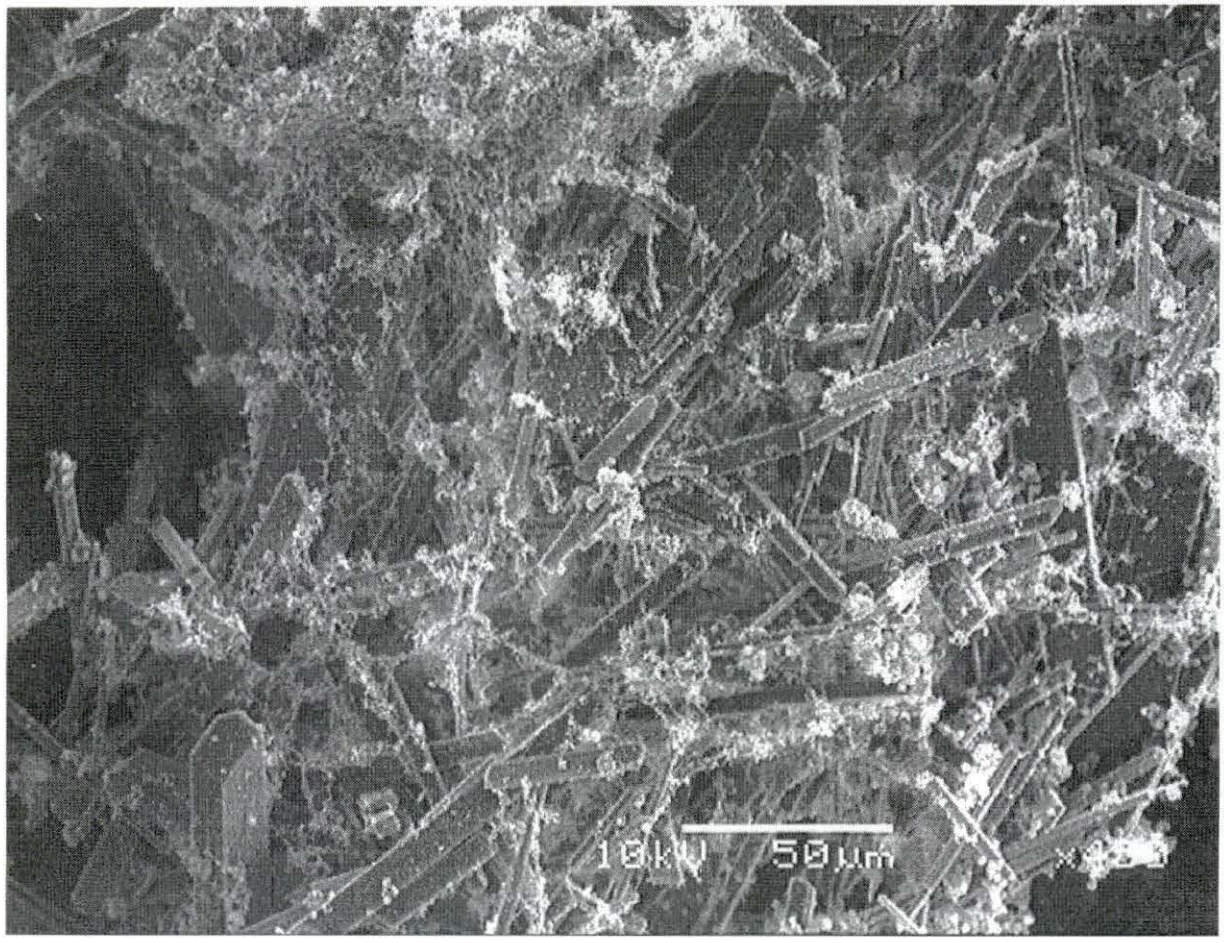

Figure 4.31 - Rat 8 Middle Distal Blue color coded strand with needle-like crystals throughout the cross section. Note that the crystals have a new shape for this location compared with that for the crystals that formed in the proximal location (Figure 4.30) for this cable.

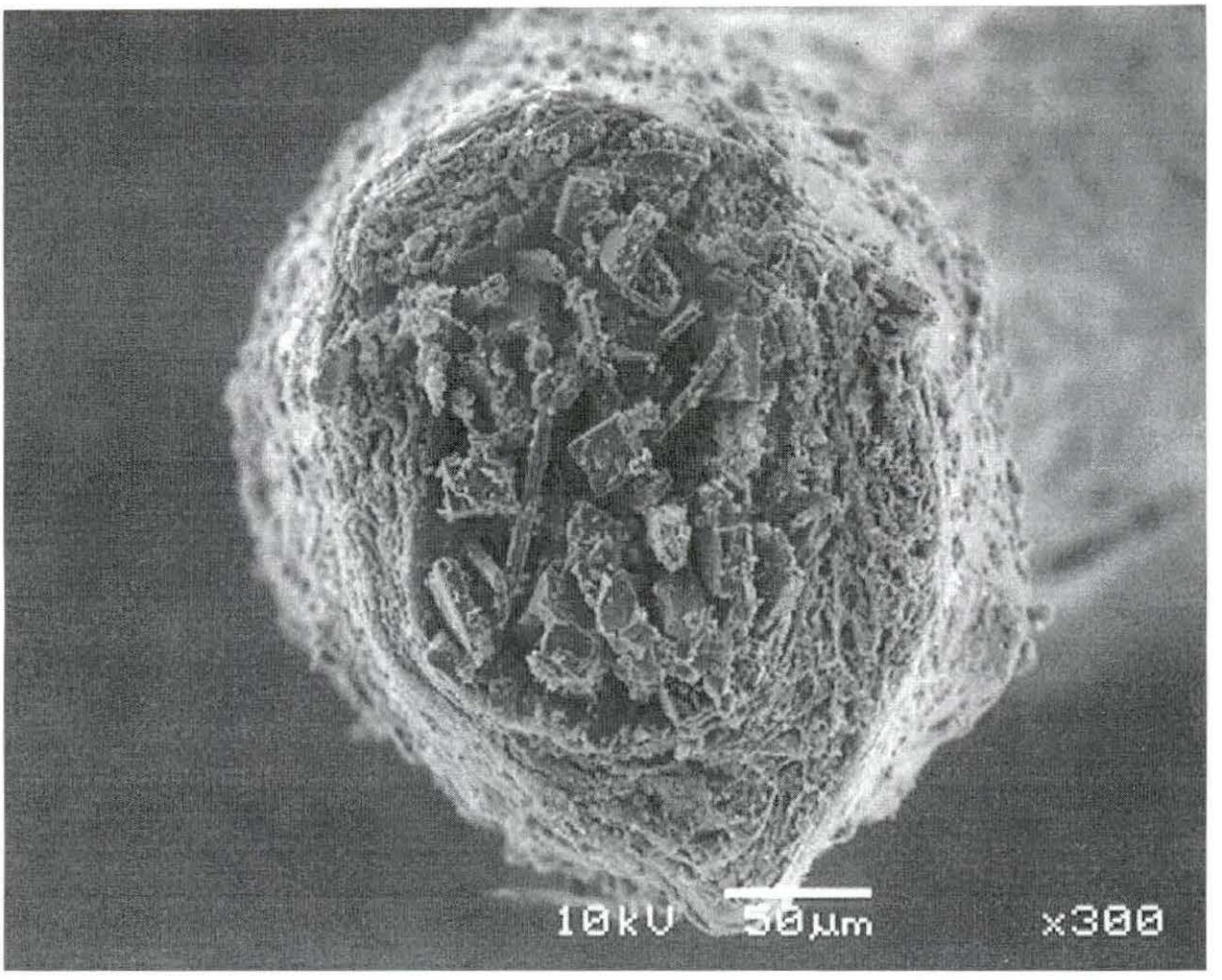

Figure 4.32 - Rat 8 Middle Proximal Orange color coded strand cross section composed of plate-like crystals in the central region and a rim of connective tissue. Wet cut, 28 day fixation, 1 day dehydration, critical point dried, 200 Å palladium and gold. Lumen was filled with collagen. Crystals are probably a contamination assocated with a sodium bicarbonate cleaning procedure.

Energy dispersive x-ray analysis of the crystals was accomplished with preliminary identification results of crystals based on the presence of ions in relatively high abundance (Figures 4.33-38).

4.2 Device

During the construction of the multiple lumen assembly, care must be taken to avoid introducing undesirable microstructural features. Incomplete filling of adhesive around the seven tubes of the nerve guide gap region can occur (Figure 4.39). Also, distortion of individual lumen cross sections from a normal circular cross section can occur (Figure 4.39). The assembly should be fabricated without air pocket distortion (Figure 4.40). When plugging the middle lumen, a protrusion of excess adhesive can influence the surface structure of the nerve stump because the nerve conforms to a rough surface on this scale (Figures 4.41 and 4.42; distal example and proximal example, respectively; center of field of view). In Figure 4.41, excess silicone rubber has filled the central seventh lumen of the guide. This represents a high feature at the nerve stump-gap region interface of the guide. Figure 4.42 shows the negative indentation in nerve tissue that has conformed to a similar positive surface feature (representative of a possible condition at either the proximal or the distal end) and represents the indentation in the center of this field of view.

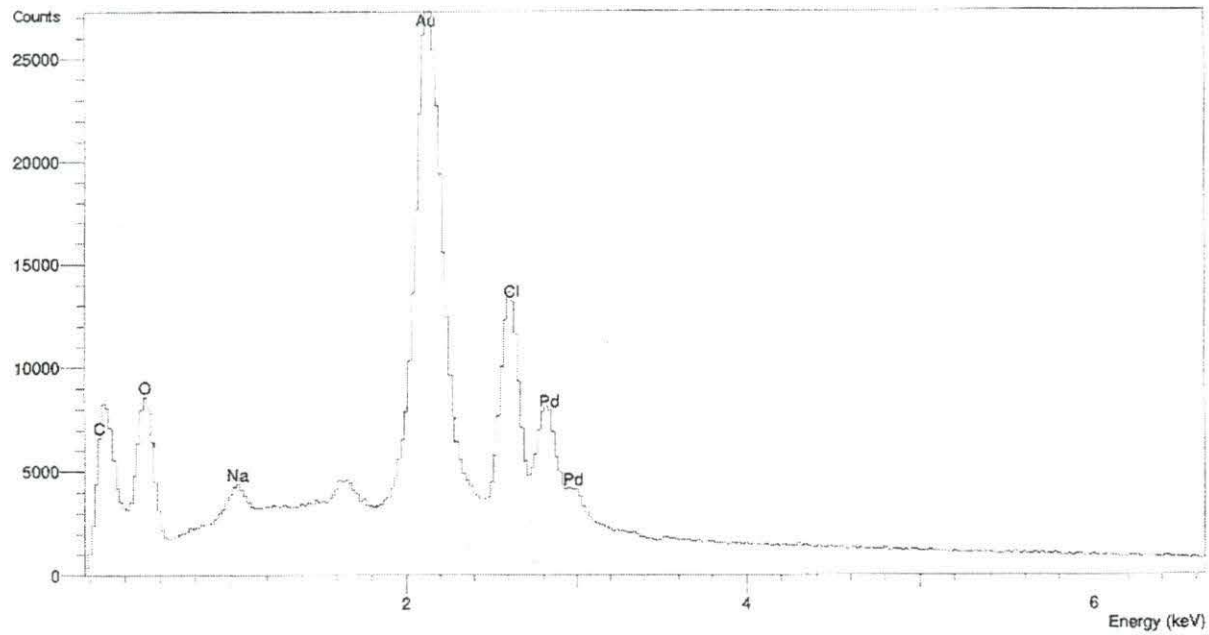

Figure 4.33 - Rat 7 Control energy dispersive x-ray analysis - nerve tissue - Au and Pd sputter coating - C, O, Na, Cl, Au and Pd are present.

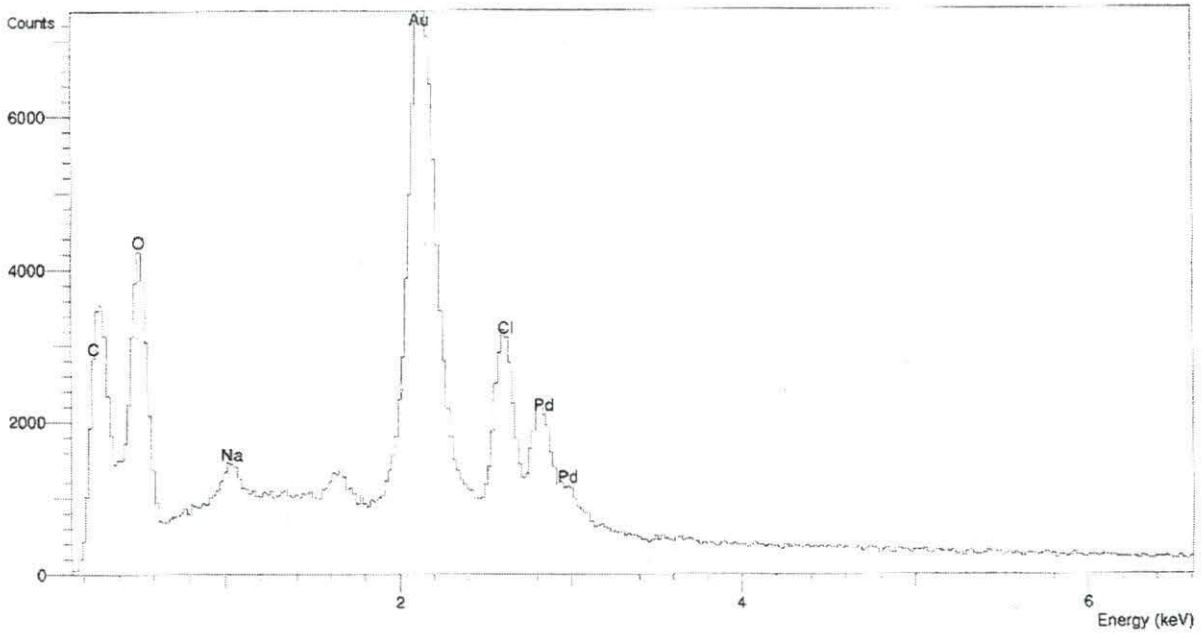

Figure 4.34 - Rat 11 Control energy dispersive x-ray analysis - nerve tissue - Au and Pd sputter coating - C, O, Na, Cl, Au and Pd are present.

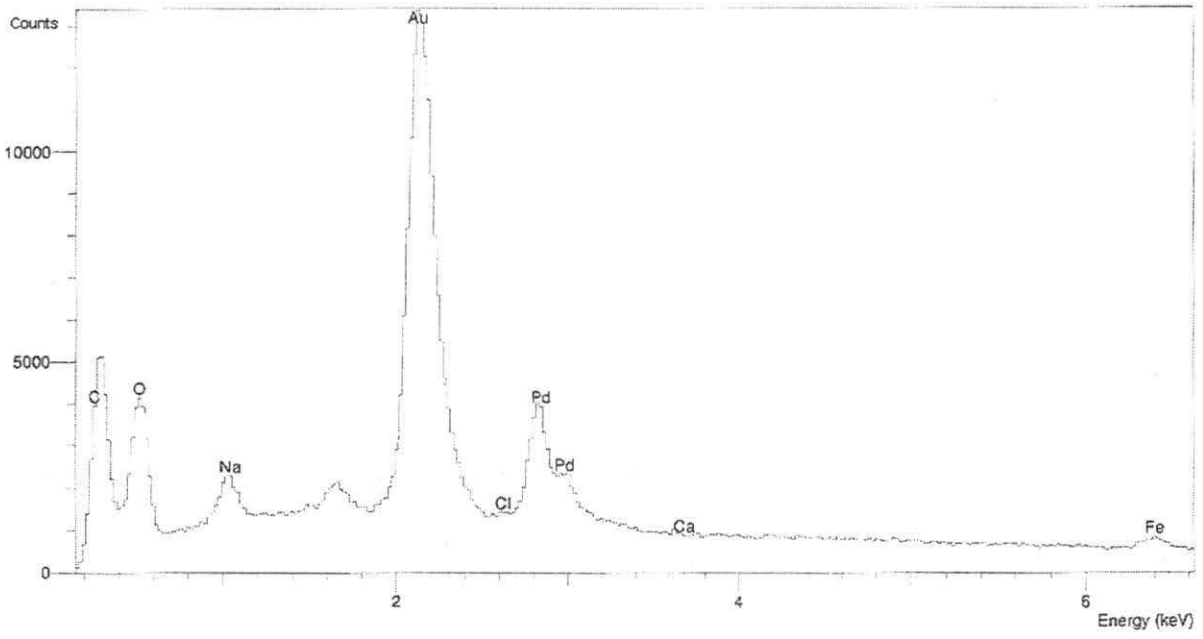

Figure 4.35 - Rat 7 Middle Distal Blue energy dispersive x-ray analysis - crystal - Au and Pd sputter coating - C, O, Na, Cl, Ca, Fe, Au and Pd are present.

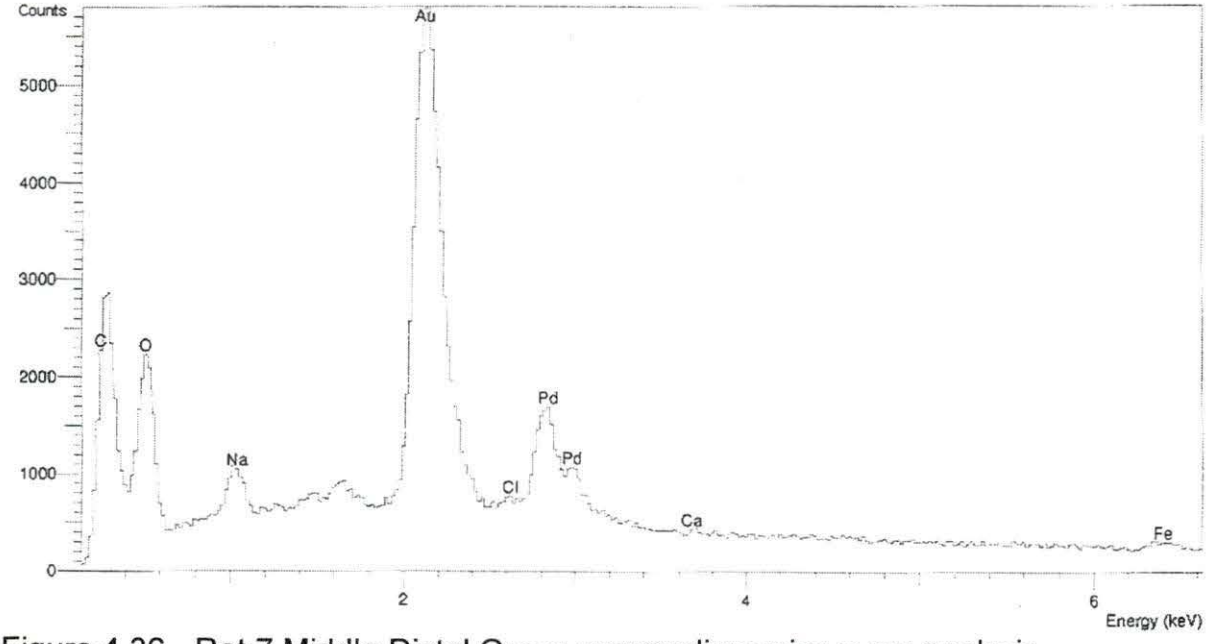

Figure 4.36 - Rat 7 Middle Distal Green energy dispersive x-ray analysis - crystal - Au and Pd sputter coating - C, O, Na, Cl, Ca, Fe, Au and Pd are present.

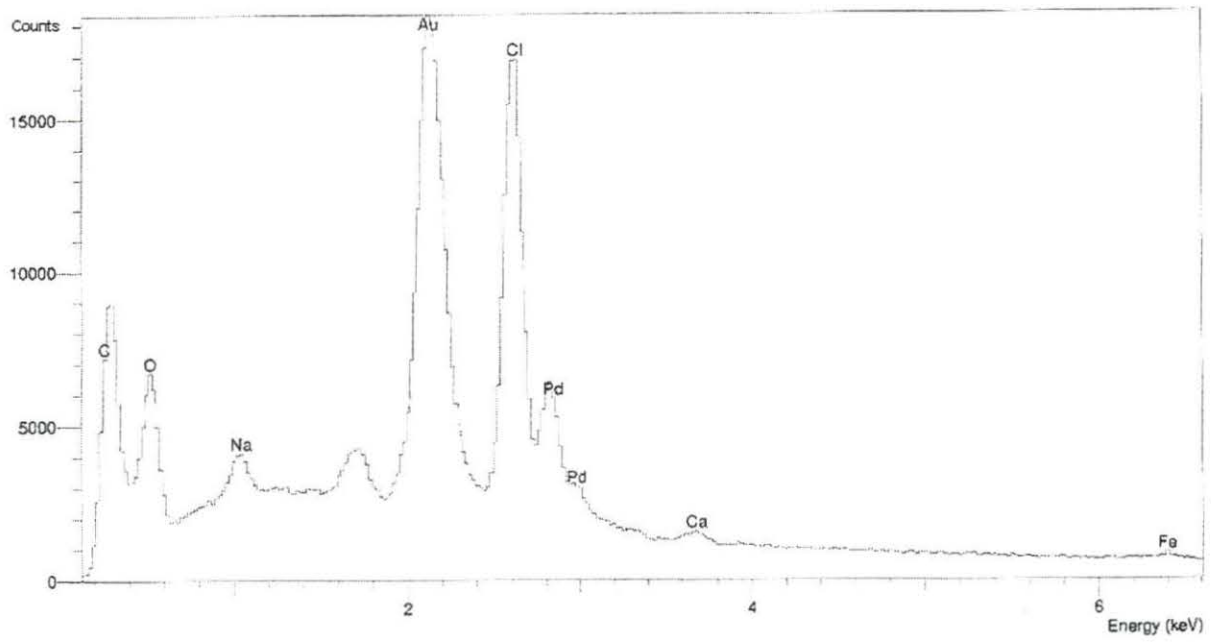

Figure 4.37 - Rat 11 Middle Distal Orange energy dispersive x-ray analysis - tissue - Au and Pd sputter coating - C, O, Na, Cl, Ca, Fe, Au and Pd are present.

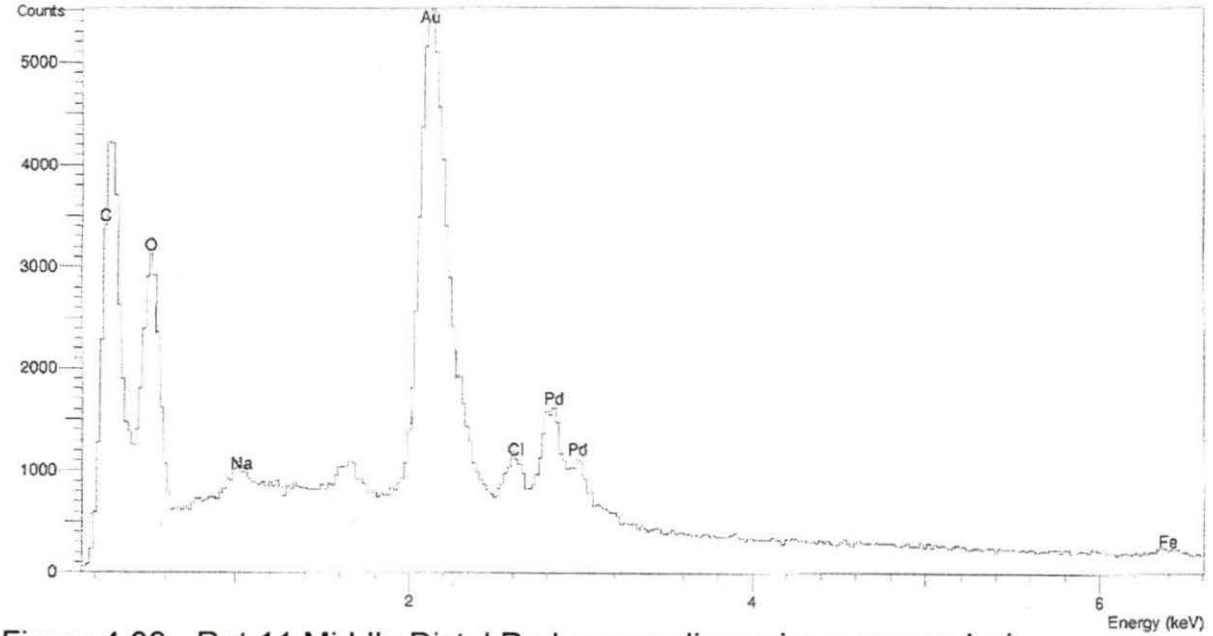

Figure 4.38 - Rat 11 Middle Distal Red energy dispersive x-ray analysis - crystal - Au and Pd sputter coating - C, O, Na, Cl, Fe, Au and Pd are present.

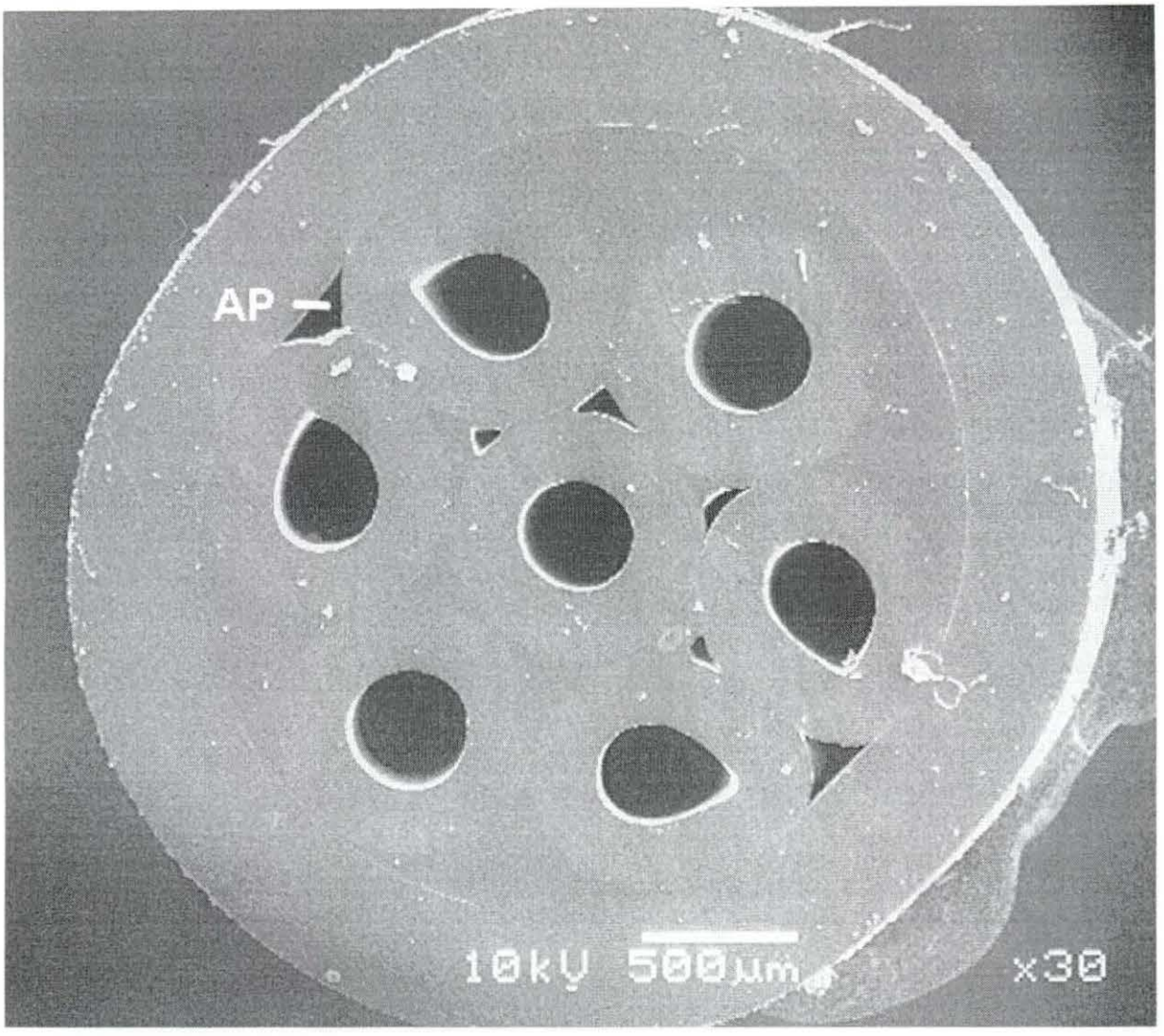

Figure 4.39 - Silicone rubber multiple lumen nerve guide - Segment from middle of the gap region with air pockets (AP) where an insufficient amount of adhesive was used during fabrication

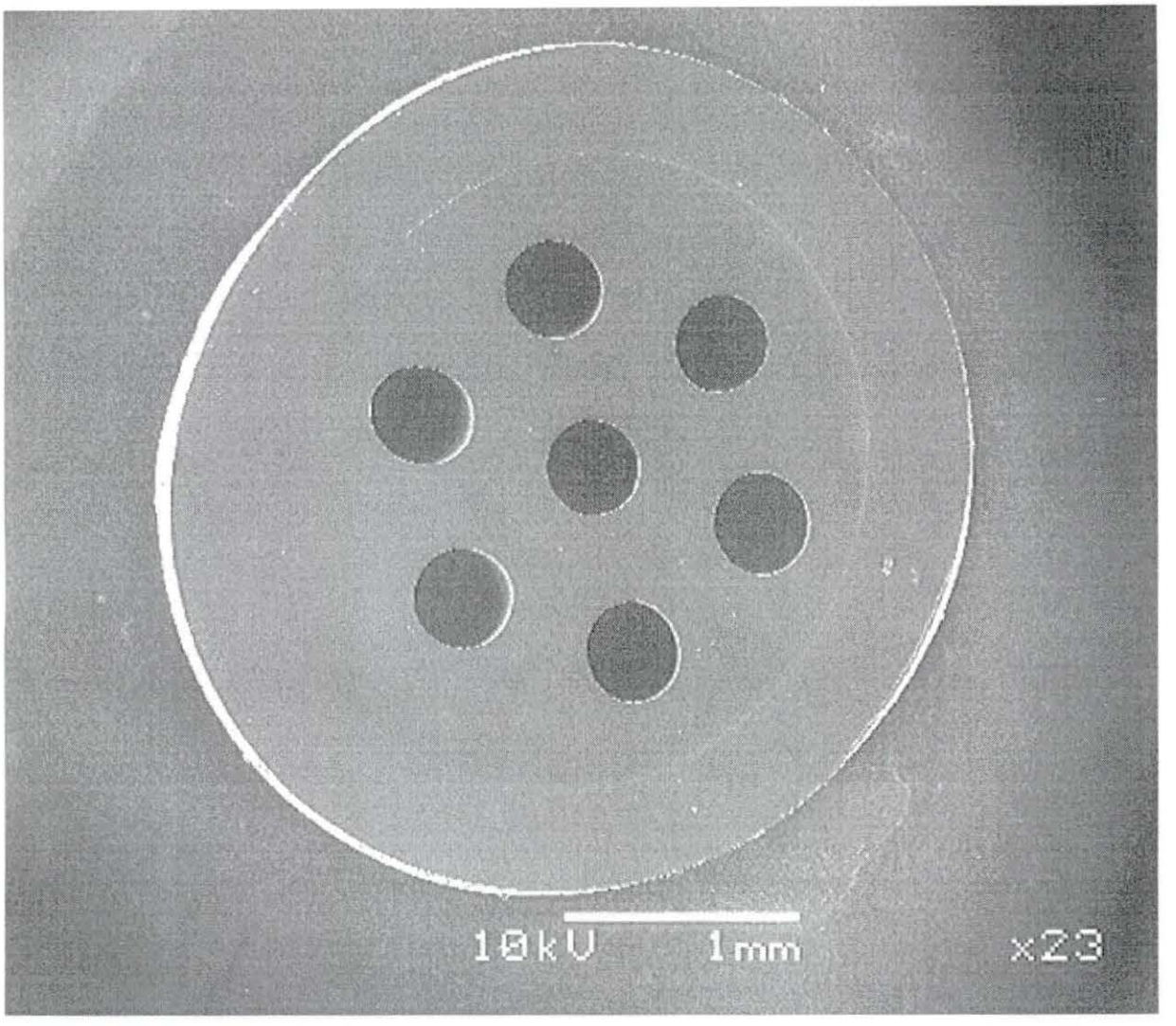

Figure 4.40 - Silicone rubber multiple lumen nerve guide - Segment from Middle Proximal gap region.

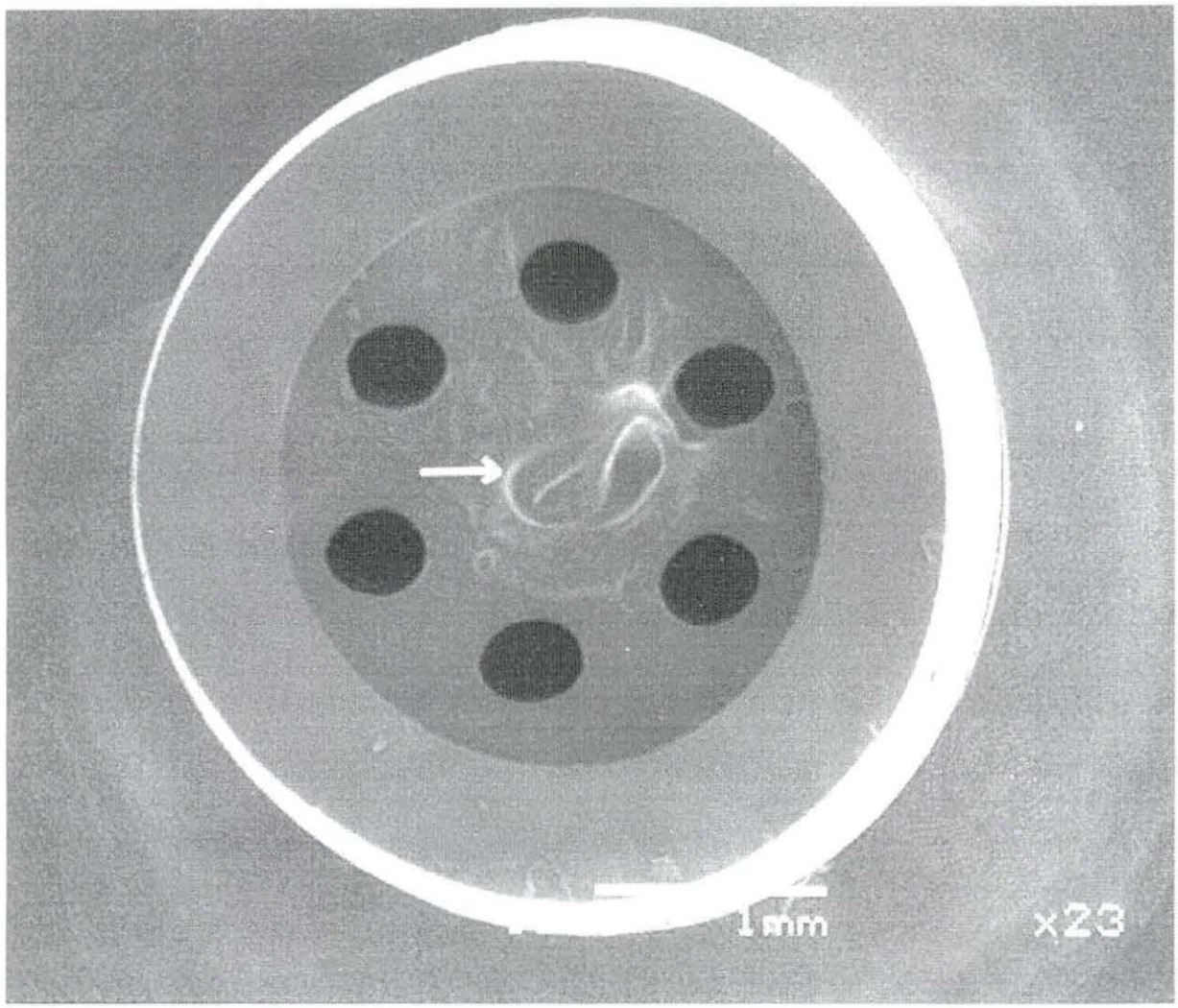

Figure 4.41 - Silicone rubber multiple lumen nerve guide with an excess of adhesive (arrow) in the distal gap-stump junction region.

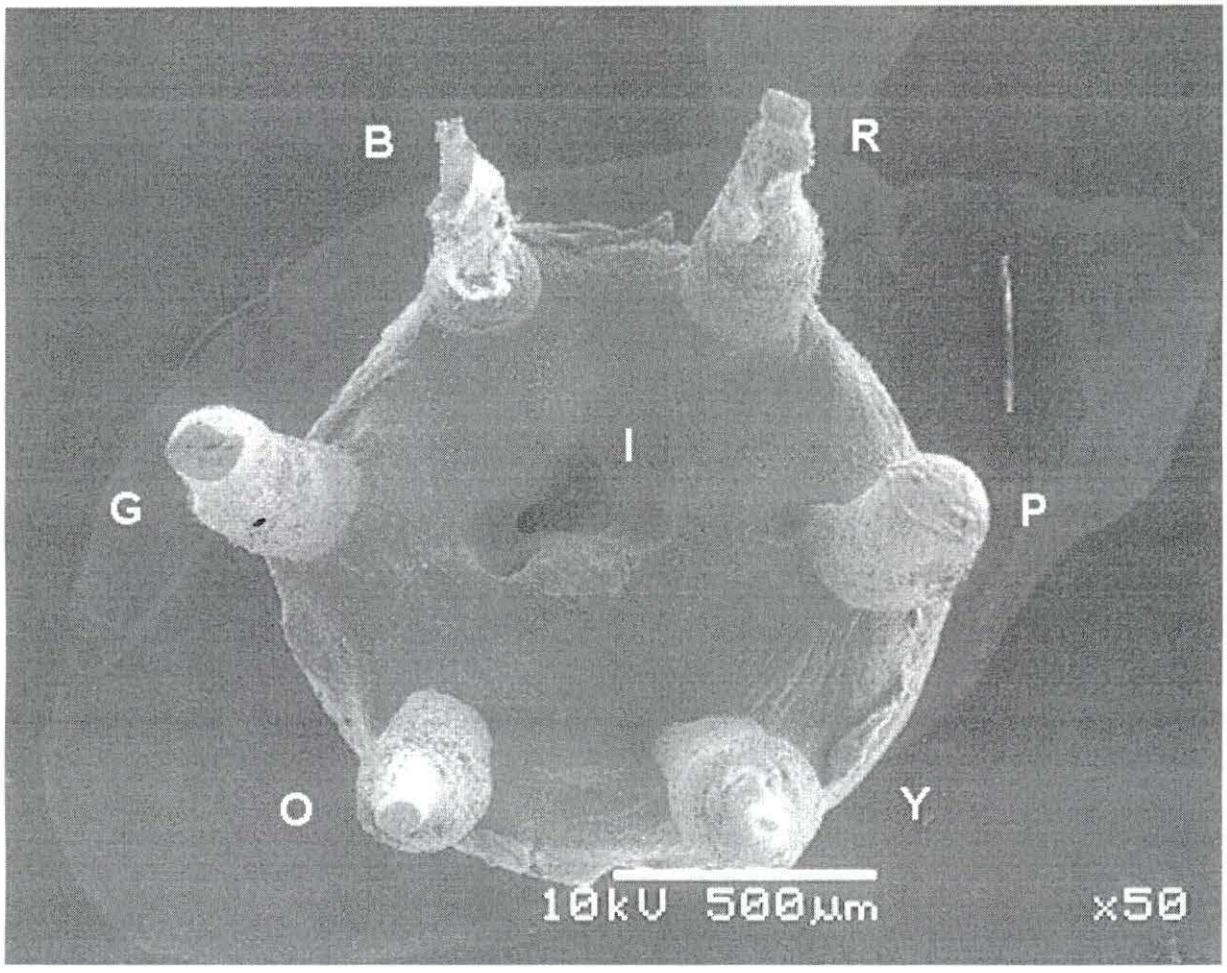

Figure 4.42 - Rat 9 Proximal section with indentation (I) caused by excess adhesive. Clockwise from the left: green (G), blue (B), red (R), purple (P), yellow (Y) and orange (O) color coded cables viewed on end.

5. DISCUSSION

The sciatic nerve did regenerate tissue across the large gap of 15.0 mm in the short time period of eight weeks for the 0.5 mm diameter conduits in the presence of both collagen gel and the gel mixture of collagen, fibronectin and laminin. Regenerated axons were not definitively evident in this scanning electron microscopy survey. This is the first SEM study of multiple lumen nerve guide regenerated cables.

The shapes and sizes of the regenerated strands were characterized in a point to point variation and with respect to the collagen gel and the collagen, fibronectin and laminin fillings. The cross sectional diameter of the regenerated strands changed for point to point in the gap region. Williams et al. (1983) observed that the regenerated tissue narrows as it approaches the midpoint of the nerve guide. This phenomenon was evident. The regenerated cables did tend to narrow toward the middle of the gap and were smaller on the distal side of the gap region than on the proximal side of the gap region.

At the magnifications (x35 to x10,000) used in this study, there were no discernible differences between the collagen filled lumens (orange, blue, purple color coding) and the collagen, laminin and fibronectin filled lumens (red, green, yellow color coding). There was no notable difference in the diameter of the strands or of rim formation, but some cables contained crystals whereas most of the cables contained only tissue.

Valentini et al. (1987) suggests that gel substrate, even if it contains neurite-promoting factors, impairs the regeneration process by physically impeding the diffusion of critical molecules, the migration of cells, or the elongation of axons. In the current study, tissue did bridge the 15.0 mm gap in eight weeks. Axons were not apparent.

Samples of a control, of a connective tissue strand and of a crystal strand were studied using energy dispersive x-ray analysis. By identifying the peaks on the energy dispersive x-ray graphs and their relative intensities, the identity of ions present in the tissue samples was found. The Au and Pd were evident because they were sputter coated onto the tissue samples to provide a thin film conductive coating on samples for SEM viewing. C, Ca, Cl, Fe, Na and O were also present. Unfortunately, no specific trend among the ions and the tissue samples was obvious. The most likely reason for the presence of the crystal structures in the gap region was associated with the lumen cleaning procedure prior to assembling the nerve guide. This process may have left a sodium bicarbonate residue inside the lumen.

The overall structure of the regenerated nerve strands compared to the controls was similar. All of the control nerves had distinct epineurium, perineurium and endoneurium connective tissue. Some regenerated strands consisted of a rim of layered tissue around the edge of the strand and a central region of fibrous tissue. The rim of the strand represents the formation of an epineurium and the fibrous central region may be a smaller scale of the perineurium or endoneurium.

Control axons were visible at magnifications of x3500 but regenerated axons were not conclusively apparent at magnifications of x3500 to x10000 and greater.

The size and fascicle patterns of the control nerves varied (Table 4.1). The number of fascicles even varied within the same rat peripheral nerve segment.

The axons in the control exhibited irregular shaped pits in the myelin sheath. This occurred because myelin has a high lipid content which is soluble in fat solvents. A portion of the myelin was dissolved away by the ethanol dehydration (Humason, 1972). This problem can be avoided by using osmic acid fixatives to preserve the myelin if a suitable procedure can be found for removing the small, flexible lumen samples prior to osmium fixation. Care should be taken to limit the amount of time the tissue is in the dehydration solutions.

During tissue preparation, the tissue was either left in the wet cut stage or freeze fractured stage. The features of the freeze fractured specimens were well-defined. The epineurium, perineurium, endoneurium and axons could be examined in detail. The wet cut specimens tended to have features obscured by compressed or drawn tissue artifacts. The epineurium was compressed and the axons were completely covered by endoneural fibers. These distinctions in the regenerated strands were not as apparent. In some cases, freeze fracturing the strand made the rim and central regions more discernible, but in others, it may have hindered the detection of axons. Some of the wet cut strands were deformed during the wet cutting. Some became elongated and appeared crimped.

6. CONCLUSIONS

This scanning electron microscopy survey of regenerated rat sciatic nerve tissue bridging a 15.0 mm gap in a multiple lumen nerve guide (8 week implantation period) characterized microstructural cable features for the epineural, perineural and endoneural regions of sciatic nerve tissue (control) and regenerated nerve tissue. Although there was no discernible difference between the regenerated cables for the collagen and the collagen, fibronectin and laminin filled lumens at the magnifications from this study, further studies to compare other growth stimulating chemicals and liquid and gel type matrixes are warranted. It is suggested that a longer implantation time (16 weeks), different dehydration procedures and possibly a different fixative (osmium) be used.

Further studies of the collagen gel and the chemical compositions of the chemicals used on the nerve tissue are necessary to fully establish the origin of the crystal structures that were found in most of the strands from Rat 11 and all of the strands from Rat 7.

Modifications can be made to the multiple lumen system to tailor the nerve guide to match the number of fascicles present in nerve stumps. The system can utilize lumens containing different chemicals for nerve guidance and nerve regenerating capabilities at the same time.

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