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TITLE:

Facial Nerve Surgery in the Rat Model to Study Axonal Inhibition and Regeneration

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KEYWORDS:

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SUMMARY:

This protocol describes a reproducible approach to facial nerve surgery in the rat model, including descriptions of various inducible patterns of injury.

ABSTRACT:

This protocol describes consistent and reproducible methods to study axonal regeneration and inhibition in a rat facial nerve injury model. The facial nerve can be manipulated along its entire length, from its intracranial segment to its extratemporal course. There are three primary types of nerve injury used for the experimental study of regenerative properties: nerve crush, transection, and nerve gap. The range of possible interventions is vast, including surgical manipulation of the nerve, delivery of neuroactive reagents or cells, and either central or end-organ manipulations. Advantages of this model for studying nerve regeneration include simplicity, reproducibility, interspecies consistency, reliable survival rates of the rat, and an increased anatomic size relative to murine models. Its limitations involve a more limited genetic manipulation versus the mouse model and the superlative regenerative capability of the rat, such that the facial nerve scientist must carefully assess time points for recovery and whether to translate results to higher animals and human studies. The rat model for facial nerve injury allows for functional, electrophysiological, and histomorphometric parameters for the interpretation and comparison of nerve regeneration. It thereby boasts tremendous potential toward furthering the understanding and treatment of the devastating consequences of facial nerve injury in human patients.

INTRODUCTION:

Cranial nerve injury in the head and neck region can be secondary to congenital, infectious, idiopathic, iatrogenic, traumatic, neurologic, oncologic, or systemic etiologies¹. Cranial nerve VII, or the facial nerve, is commonly affected. The incidence of facial nerve dysfunction can be significant, as it affects 20 to 30 per 100,000 people each year². The main motor branches of the facial nerve are the temporal, zygomatic, buccal, marginal mandibular, and cervical branches; depending on the branch involved, the consequences can include oral incompetence or drooling, corneal dryness, visual field obstruction secondary to ptosis, dysarthria, or facial asymmetry^{2,3}. Long-term morbidity includes the phenomenon of synkinesis, or involuntary movement of one facial muscle group, with attempted voluntary contraction of a distinct facial muscle group. Ocular-oral synkinesis is the most common of the aberrant regeneration as a sequela of facial nerve injury and causes functional impairment, embarrassment, diminished self-esteem, and poor quality of life³. Injury to individual branches dictates the functions that are selectively compromised.

The clinical treatment of facial nerve injury is not well standardized and is in need of further research to improve outcomes. Steroids can alleviate acute facial nerve swelling, whereas Botox is useful for temporizing synkinetic movements; but, the primary reconstructive options in the practitioner's armamentarium involve surgical intervention through nerve repair, substitution, or reanimation³⁻⁶. Depending on the type of facial nerve injury sustained, the facial nerve surgeon may utilize a number of options. For simple transection, nerve reanastomosis is useful whereas cable-graft repair is better suited for a nerve defect; for a restoration of function, the surgeon may choose either static or dynamic facial reanimation procedures. In many cases of facial nerve injury and subsequent repair, even in the hands of experienced facial nerve surgeons, the best outcome still results in persistent facial asymmetry and functional compromise⁷.

These suboptimal outcomes have spurred extensive research on facial nerve regeneration. Broad topics of interest include perfecting and innovating nerve repair techniques, determining the effect of various nerve regeneration factors, and assessing the potential of specific neural inhibitors to help combat the long-term outcome of synkinesis⁸⁻¹¹. While in vitro models can be used to assess some characteristics of pro-growth or inhibitory factors, true translational research on this subject matter is best accomplished via translatable animal models.

The decision of which animal model to utilize can be challenging, as researchers have utilized both large animals, such as sheep and small animal models, such as mice^{12,13}. While large animal models offer ideal anatomic visualization, their use requires specialized equipment and personnel not readily or easily available. Furthermore, powering a study to demonstrate effect could be highly cost-prohibitive and potentially not within the feasible scope of many scientific centers. Thus, the small animal model is most frequently utilized. The mouse model can be utilized for assessing a number of outcomes related to facial nerve surgery; however, the limited length of the nerve can restrict the scientist's ability to model certain patterns, such as large-gap injury¹⁴.

Thus, the rat murine prototype has emerged as the workhorse model through which the scientist can perform innovative surgical procedures or utilize inhibitory or pro-growth factors and assess effect across a broad range of outcome parameters. The rat facial nerve anatomy is predictably and easily approached in a reproducible fashion. Its larger scale, in comparison to the mouse model, allows for modeling of a wide range of surgical defects, ranging from simple transection to 5 mm gaps^{15,16}. This further allows for the application of complex interventions at the defect site, including the topical placement of factor, intraneural injections of factor, and the placement of isografts or bridges¹⁷⁻²³.

The docile nature of the rat, its reliable anatomy, and its propensity for effective nerve regeneration allows for the collection of many outcome measures in response to the aforementioned surgical patterns of injury²⁴. Via the rat model, the facial nerve scientist is able to assess electrophysiologic responses to injury, nerve and muscle histologic outcomes via immunohistochemistry, functional outcomes via tracking movement of the vibrissal pad and assessing eye closure, and micro- and macroscopic changes via fluorescent or confocal microscopy, among others^{11,22,23,25-29}. Thus, the following protocol will outline a surgical approach to the rat facial nerve and the injury patterns that can be induced.

PROTOCOL:

All interventions were performed in strict accordance with the National Institutes of Health (NIH) guidelines. The experimental protocol was approved by the University of Michigan's Institutional Animal Care & Use Committee (IACUC) prior to implementation. Ten-week-old adult female Sprague-Dawley rats were utilized.

1. Prior to the operative day

1.1. Ensure an appropriate stock of sterilized surgical instruments, analgesic medications, anesthetic medication, and oxygen prior to the operating day. Please see **Table of Materials** for a complete list.

2. Preoperative setup

2.1. Ensure an adequate working space, including room for at least two individuals (the surgeon and an assistant).

NOTE: There is need for a dedicated operating table, room for the anesthesia machine setup, and adequate storage space for sterilized and backup supplies.

2.2. Calibrate an operating microscope for use during the procedures. Make sure the surgeon has the ability to adjust the handles of the microscope and the zoom/focus buttons by placing a sterilized cover over the handles/buttons

NOTE: We utilized sterilized aluminum foil over the handles/buttons.

3. Anesthesia and preparation

3.1. Place the animal in the anesthesia chamber and induce general anesthesia via 1.8% isoflurane and 0.9 L/min oxygen.

3.1.1. Confirm an adequate plane of anesthesia via an assessment of spontaneous breathing and an evaluation of consciousness by assessing the animal's grimace response to a toe pinch.

3.2. Apply eye lubricant bilaterally to guard against corneal irritation or dryness.

3.3. Shave the operative site(s) with a razor or automatic clipper.

3.3.1. Establish a method for rat identification at this time, either via an ear tag or tail label/markings.

3.4. Administer a subcutaneous injection of 0.05 mg/kg buprenorphine along the animal's back for prophylaxis against postoperative pain.

4. Surgical approach and injury patterns

4.1. Transfer the animal to the operating table and continue the gas flow via a nosecone. Ensure that a warming pad is positioned underneath the animal and the sterile field to maintain its body temperature.

4.2. Place sterilized gauze (rolled up and fastened with tape) to use as a neck roll for the rat; this will provide an enhanced exposure of the surgical field. Note that the appropriate positioning of the animal is paramount for efficient nerve identification and dissection.

4.3. Prepare the animal's facial skin for the procedure. Use chlorhexidine or an iodine-based solution to scrub the surgical site 3x, alternating with 70% ethanol, to ensure disinfection.

4.4. Plan and mark the surgical incision if desired. Manipulate the ipsilateral ear in an anterior-posterior direction to determine the natural folding of the postauricular skin.

4.5. Fashion a 4–5 mm incision in the postauricular crease using sharp iris scissors or a number 15 blade. This can be expanded later in the procedure as necessary.

4.6. Bluntly dissect through the immediate subcutaneous fascia and place a micro-Weitlaner retractor to enhance exposure. Note that there may be small caliber blood vessels in this area; these are best avoided by retracting superiorly or inferiorly via the Weitlaner retractor.

4.7. Identify the anterior digastric muscle as it travels in an inferior-to-superior direction toward its insertion along the skull base.

4.7.1. Spread gently through the muscle belly along its insertion point to reveal the tendon of the anterior digastric belly. Note that the tendon appears as a filmy white process emanating from the muscle with a solid insertion onto the skull base.

4.8. After identification of the anterior digastric muscle and its tendon, adjust the Weitlaner retractor to further retract the muscle belly. Note that the subsequently exposed region is the three-dimensional space where the main trunk of the facial nerve lies.

NOTE: This region is bounded superiorly and medially by the skull base, laterally by the anterior digastric muscle, posteromedially by the ear canal, and inferiorly by the structures of the neck, including the superficial temporal artery.

4.9. After adequate exposure, identify the main trunk of the facial nerve as it travels inferiorly from underneath the tendon of the digastric muscle, where it exits the stylomastoid foramen from the skull base. Note that the nerve appears as a pearly white cord, encased in the animal's parotid-masseteric fascia. Practice caution when further exposing the nerve, for the following reasons.

4.9.1. Avoid aggressive dissection, or perpendicular spreads, to guard against stretch-mediated neuropraxia injury.

4.9.2. Avoid aggressive posteriorly and medially directed dissection to guard against violating the thin tissues overlying the ear canal as this could introduce middle ear flora into the surgical field.

4.9.3. Avoid damaging the superficial temporal artery through broad medially and inferiorly directed dissection. Note that an injury will be identified by brisk, pulsatile bleeding.

4.9.3.1. If the artery is injured, apply prompt pressure with a cotton-tipped applicator or sterile gauze via forceps. Hemostatic agents or liquid fibrin sealant can be placed in near proximity. Keep in mind that the animal may require a subcutaneous injection of 0.9% sterile saline for fluid stabilization.

4.10. Trace the main trunk distally by dissecting along the nerve in an inferior direction, distally from the exit of the stylomastoid foramen.

4.10.1. Extend the original incision to allow for a full exposure of the nerve and its branches. Take care to avoid a disruption of the parotid gland as this could result in postoperative sialoceles.

4.11. Induce the desired injury patterns as follows.

4.11.1. For a crush injury, use smooth-surfaced jeweler's forceps to firmly grasp the nerve and compress it⁹. Apply constant and reproducible pressure to the nerve for a period of 30 s to ensure an appropriate crush injury.

4.11.2. For a simple transection, grasp the fascia overlying the nerve, or the immediate epineurium, with fine-toothed forceps, and use sharp microscissors to cleanly transect the nerve at the desired point with a single cut. Take care to avoid excess traction on the nerve with the forceps.

4.11.3. For a nerve gap model, create the desired nerve gap using a similar method to the simple transection injury. Use the sterilized shaft of a cotton-tipped applicator—cut to the desired nerve gap length—intraoperatively to ensure similarity of injury pattern between animals.

5. Wound closure

5.1. Irrigate the wound with sterile saline and dry it with sterile gauze.

5.2. Approximate the skin edges in a simple, subcuticular fashion with absorbable sutures, or use skin glue or wound clips, which are also acceptable for wound closure. Place a buried stitch by taking a deep-to-superficial bite of one skin edge and then a subsequent superficial-to-deep bite of the opposite skin edge.

6. Postoperative recovery

6.1. Administer a subcutaneous injection of nonsteroidal anti-inflammatory analgesic (such as 0.5 mg/kg carprofen) for postoperative pain control. Place the injection along the animal's back.

6.2. Cease the administration of the anesthetic agent and allow the animal to inhale oxygen for an additional 1 min.

6.3. Place the animal in a warmed (via a heat lamp), aseptic cage devoid of bedding material to avoid accidental ingestion. Note that the animal will typically demonstrate signs of recovery within 1–2 min and can appear disoriented, with a delayed recovery of hind-leg function.

6.4. Return the animals to their cages in the appropriate housing unit and administer postoperative analgesics on postoperative day #1 to ensure continued prophylaxis against pain.

6.5. Monitor the animals 2x per day to evaluate for signs of malnourishment, corneal irritation, or surgical site infection, and maintain appropriate surgical logs.

6.5.1. Administer 0.9% sterile saline in a subcutaneous fashion if there is significant weight loss.

6.5.2. Apply lubricating eye ointment daily until the animal's blink reflex is re-established.

REPRESENTATIVE RESULTS:

Following the initial surgical procedure, there are two main types of outcome measures: serial measurements in the live animal and measurements that require sacrificing the animal. Examples of serial measurements include electrophysiological assays, such as a compound muscle action

potential measurement³⁰, assessments of facial muscle movement via laser-assisted or videography means⁹, or even repetitive live imaging of regrowth of the facial nerve in fluorescent transgenic animals^{31,32}. **Figure 1** illustrates live imaging of the main trunk of the facial nerve in an adult transgenic *Thy1-GFP* rat. A crush injury has been performed on the marginal mandibular branch, approximately 2–3 mm distal to the branch point of the first pes. Utilizing MetaMorph imaging software, we were able to quantify the fluorescent intensity at any point along the course of the facial nerve. In particular, it is possible to quantify fluorescence proximal and distal to an injury site, thereby serially assessing for the return of fluorescence as a marker for nerve regeneration. **Figure 2** illustrates the gradual return of fluorescence (measured as a ratio of distal fluorescence to proximal fluorescence across a simple transection site) at the 1, 2, 3, and 4 week time-points in the *Thy1-GFP* rat.

Histomorphometric analysis of the nerve proper or muscle requires sacrificing the animal after a predetermined length of time to demonstrate the desired effect across groups. **Figure 3** demonstrates cross-sectional images of the marginal mandibular division. This technique requires careful tissue handling, storage, preparation, sectioning, and staining to allow for a histomorphometric analysis across various groups. If appropriately performed, this technique allows for the quantification of the axonal diameter, amount of debris, nerve fiber, percentage of nerve, and density measurements.

FIGURE AND TABLE LEGENDS:

Figure 1: Facial nerve anatomy demonstrated in the *Thy1-GFP* rat 1 week after a crush injury at the marginal mandibular nerve. The site of the crush injury is demonstrated by the white arrow. Sections of the nerve are labeled as MT (main trunk), B (buccal), and MM (marginal mandibular) branches. The scale bar represents 1.5 mm.

Figure 2: Quantification of return of fluorescence as a ratio of the fluorescent intensity of the site immediately distal to the transection injury over the intensity of the site proximal to the transection injury. Four animals were studied, and a crush injury was modeled. The graph is plotted as mean proportion \pm standard error of the mean.

Figure 3: Axial cross-section of the marginal mandibular branch of the rat facial nerve following staining with toluidine blue. The scale bar represents 100 μ m.

DISCUSSION:

The rat facial nerve injury model has emerged as the most versatile system for the evaluation of neurotrophic factors due to its surgical accessibility, branching pattern, and physiological significance^{27,29,33–36}. The combination of video demonstration and application of transgenic animal data opens new possibilities for the scientific study of nerve regenerative phenomena. This model allows the systematic and detailed characterization of neural response to trauma, neurotrophic influences, immunomodulatory influences, and other aspects of the microenvironment. Although the primary objective of clinical facial nerve study relates to the recovery of motoneuron function, the model can also be used to better understand events at the

neuromuscular junction, nuances of axonal transport, and the interplay of axonal–glial influences^{27,36–44}. Mechanistic study using facial nerve has paradoxically led to important insights relating to central inflammation and neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease^{45–47}. There, the medical implications of work conducted in this model have notable implications for better understanding and eventually improving clinical care for patients afflicted with both peripheral and central nervous system disorders affecting both supporting cells and neuronal tissues.

There is an initial learning curve with performing facial nerve surgery in the rat model. While it is not necessary for the scientist to be surgically trained, they should be comfortable working underneath an operating microscope and utilizing binocular vision. The scientist should further be comfortable working with their nondominant hand, as adjusting the retraction with the Weitlaner retractor in the contralateral hand can significantly improve visualization. This is particularly true for identifying the main trunk of the facial nerve, as it is possible for the novice surgeon to lose landmarks and become disoriented in the three-dimensional space where the nerve exits the skull base. However, once experience is gained and the surgeon is consistently able to identify the tendon of the digastric muscle, then the procedures are quite straightforward. Where the procedure—a simple transection of the nerve—could take as long as 30 min when the scientist is initially learning, in the hands of an experienced surgeon, it can be as expedient as 5 min from incision to wound closure. With an experienced assistant managing the preparation and the anesthesia of the animals—as well as restocking supplies as needed—it is feasible to operate on several animals in a single sitting. If complex intraoperative maneuvers are performed, such as a stereotaxic intraneural injection, then the time required will increase.

This group has experience working with the adult Lewis and Sprague-Dawley rats and the adult *Thy1-GFP* rat. These models have demonstrated impressive resilience, both intraoperatively and postoperatively. It would be prudent to allow time for the acclimation of the animal (typically 1 week) if ordered from a separate facility as mandated by the NIH Guide for Care and Use of Laboratory Animals, as operating too soon after a transfer could result in excessive stress and poor postoperative health. With unilateral facial nerve injury, the rat does not demonstrate untoward signs of malnourishment or corneal irritation postoperatively. Furthermore, they tolerate repetitive periods of anesthesia quite well⁴⁸, as previous protocols have demanded serial electrophysiological analyses of the return of whisker function until eventual sacrifice. Although the operators should attempt to maintain intraoperative sterility to the best of their abilities, we have not noted postoperative infections with any of the operated animals. On occasion, the animal will attempt to scratch at their incision site; however, it typically returns to baseline status with regrowth of hair within 1–2 weeks postoperatively. If the parotid gland is injured or inadvertently removed, then sialoceles will result, which may necessitate repeat anesthesia for drainage.

Modifications can be made to the location of injury, type of injury, or intervention performed. Injury can be induced at any point along the course of the facial nerve, from intracranial facial nerve transection to injury to the main trunk or any of its peripheral branches⁴⁹. The broad patterns of injury include crush injury, simple transection with or without repair, and gap-defect

with or without repair or bridge¹¹. The range of possible interventions is exhaustive. Briefly, intervention can be undertaken at the level of the nerve proper¹⁷, at the site of the injury²³, or at the muscular end-organ⁵⁰. The list of possible outcome parameters is equally lengthy. Workhorse histomorphometric parameters include axial-based quantification of nerve count, nerve density, and percentage of nerve, among others. Additional measures include histologic analyses of longitudinal sections to illustrate the extent of regeneration and neuromuscular junction quantification to demonstrate the reinnervation of target facial musculature⁵¹. Novel methods of assessing outcomes continue to be developed²². For example, Hadlock et al. have demonstrated a complex method for assessing the contracture of independent facial zones via scent- or puff-induced reactions; this has potential for the assessment and eventual treatment of troublesome synkinetic regeneration¹¹.

As with any animal model, there are limitations in translating the results to human patients. The mouse and rat models both exhibit the superior regenerative potential inherent in the rodent nervous system; this property allows the rodent to demonstrate regenerative results that could not possibly be achieved in humans and higher animals²⁴. Therefore, the facial nerve scientist must carefully select appropriate time-points for the evaluation of nerve regeneration and recovery; if a prolonged time-point is selected, the narrow window of opportunity where experimental groups may reliably demonstrate the effect from their unique intervention may be missed²⁴.

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DISCLOSURES:

The authors have nothing to disclose.

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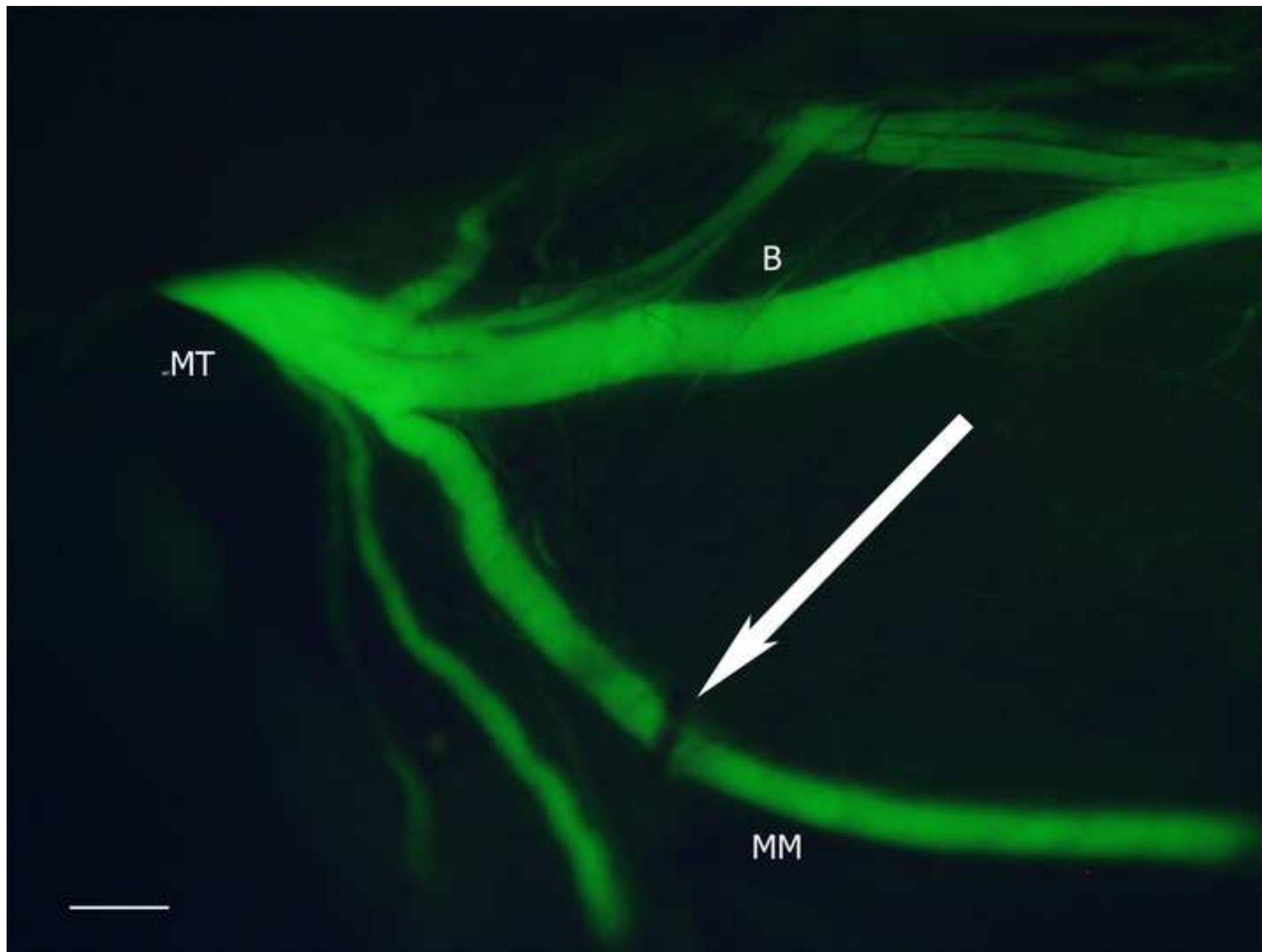
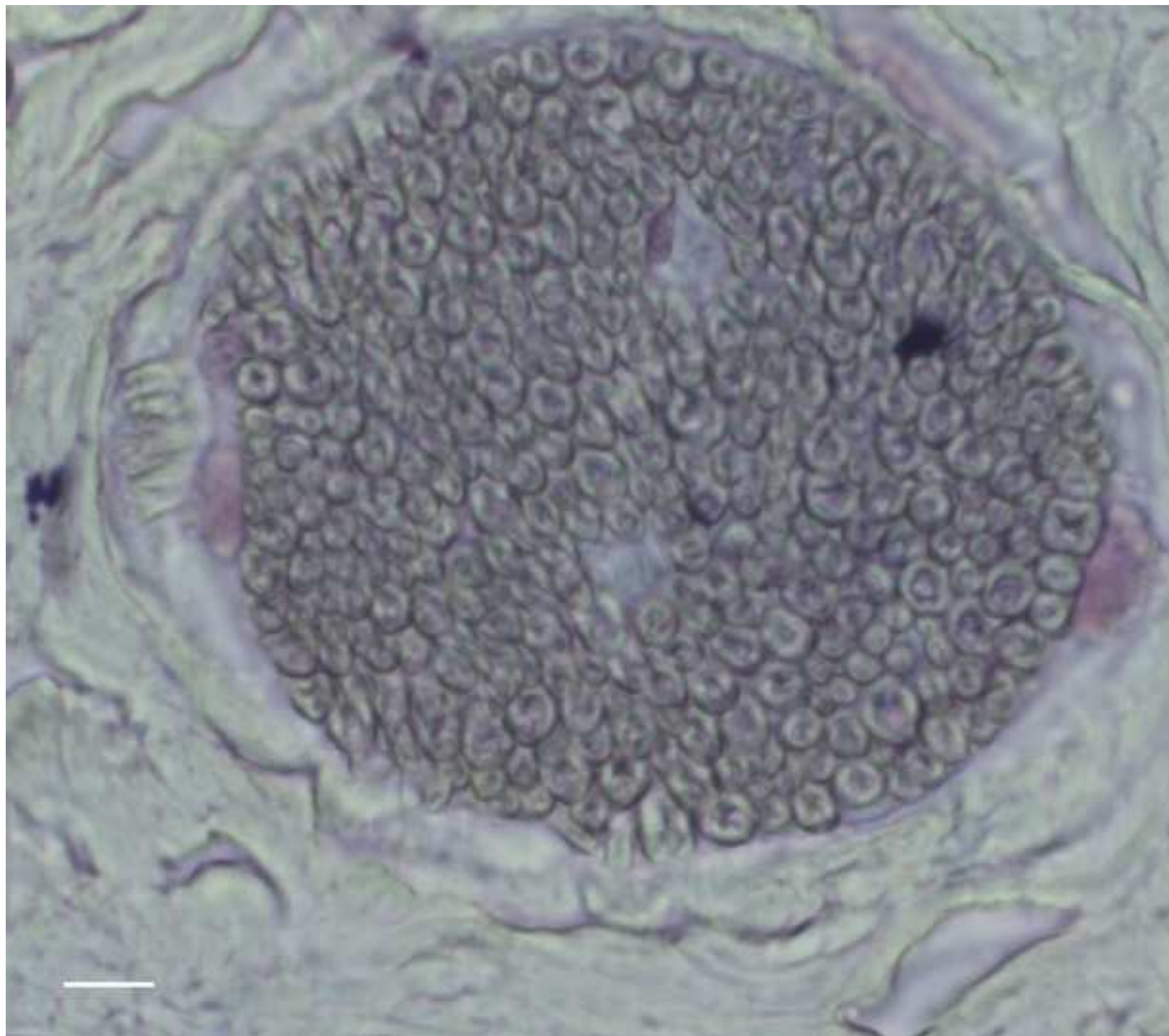
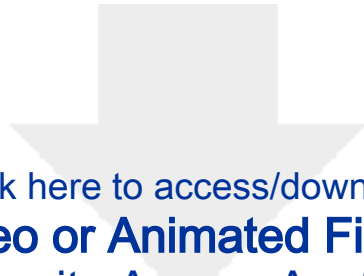


Figure 3

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Video or Animated Figure

Figure 2. Intensity Across Axotomy Site.emf



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.8% isoflurane	VetOne	13985-030-40	
11-0 nylon microsutures	AROSuture	TK-117038	
4-0 monocryl suture	VWR	75982-084	
Buprenorphine SR	ZooPharm	MIF 900-006	
Carprofen	Sigma-Aldrich	MFCD00079028	
Chlorhexidine	VWR	IC19135805	
Jeweler forceps	VWR	21909-458	
Micro Weitlaner retractor	VWR	82030-146	
Micro-scissors	VWR	100492-348	
Mini tenotomy scissors	VWR	89023-522	
Number 15 scalpel blade	VWR	102097-834	
Operating microscope	Leica		
Petrolatum eye gel	Pharmaderm	B002LUWBEK	
Sterile water	VWR	89125-834	
Tissue adhesive	Vetbond, 3M	NC9259532	
Water conductor pad	Aqua Relief System	ARS2000B	



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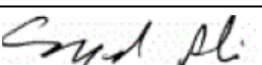
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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread for spelling and grammar issues.

2. Step 4.11.1: Please ensure that all text is written in the imperative tense.

The step has been updated to be in the imperative tense, as such on lines 222-225:

4.11.1. Crush injury: use smooth-surfaced jeweler’s forceps to firmly grasp the nerve and compress it. Apply constant and reproducible pressure to the nerve for a period of 30-seconds to ensure appropriate crush injury.

3. 4.8.1-4.8.4: Please write these steps in complete sentences and in the imperative tense.

The steps have been consolidated and now read in the imperative sense:

4.8.1. Note that this region is bounded superiorly and medially by the skull base, laterally by the anterior digastric muscle, postero-medially by the ear canal, and inferiorly by the structures of the neck, including the superficial temporal artery.

4. Step 6.1.1 is missing.

Step 6.1.2 has been amended to be 6.1.1.

5. Figure 2: Please define the error bars in Figure Legend.]

The error bars have been defined as such on line 303:

“... as mean proportion \pm standard error of the mean.”

We attest that this manuscript has not been published elsewhere, accepted for publication elsewhere, or under review for publication elsewhere. All authors listed have contributed sufficiently to the project to be included as authors. To the best of our knowledge, no conflict of interest, financial or other, exists.

Sincerely,

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