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MAPPING OF THE SUPERIOR LARYNGEAL TERMINAL NERVE ENDINGS USING LIPOPHILIC TRACERS

by

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ABSTRACT

Mapping of the Superior Laryngeal Terminal Nerve Endings Using Lipophilic Tracers

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Introduction: Damage to laryngeal nerves during thyroid surgery often results in sensory and motor nerve function deficits for patients. Better knowledge of the anatomical presentation of these nerves with a focus on terminal nerve endings is crucial to improve outcomes. **Objective:** The purpose of this *ex vivo* study was to map the terminal branches of the superior and recurrent laryngeal nerves (SLN and RLN) using DiO (3,3`-dioctadecyloxacarbocyanine perchlorate) and DiI (1,1`-dioctadecyl-3,3,3`3`-tetramethylindocarbocyanine perchlorate) lipophilic tracers to identify branching patterns. **Methods:** Nine fresh adult human cadaver specimens were obtained from the Uniformed Services University of the Health Sciences Anatomy Laboratory for laryngeal dissection. The SLN and RLN were identified bilaterally prior to laryngectomy. Additionally, the bilateral branching pattern of the external branch of the SLN (EbSLN) was documented using a previously identified classification scheme. DiO and DiI tracers were applied to the EbSLN and RLN, respectively. Whole larynges were incubated in paraformaldehyde in 4% phosphate buffer solution (PBS) for 4-9 weeks. The larynges were sliced into 5μm sections and embedded in paraffin for confocal microscopy

evaluation. **Results:** Successful anterograde transmission of lipophilic neural tracers was identified in the EbSLN and RLN. Interpretation of the findings is limited by auto-fluorescence of the tissue surrounding laryngeal tissue hindering immediate tracing of the EbSLN and RLN to their endpoint innervations. **Conclusions**: The use of lipophilic tracers in an *ex vivo* setting is useful in examining and identifying neural tissue in larynges. This novel application currently does not allow specific mapping due to aberrant autofluorescence of the non-neural tissue surrounding the EbSLN and RLN. Further study of alternate embedding techniques and fluorescence analysis are needed to optimize the methodology.

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LIST OF ABBREVIATONS

cm	Centimeter(s)
CT	Connective Tissue
DiI	1,1`-dioctadecyl-3,3,3`3`-tetramethylindocarbocyanine perchlorate
DiO	3,3`-dioctadecyloxacarbocyanine perchlorate
EbSLN	External branch of the Superior Laryngeal Nerve
EMG	Electromyography
Fiji	Fiji Is Just ImageJ
H&E	Hematoxylin and Eosin
IONM	Intraoperative neuromonitoring
mm	Millimeter(s)
nm	Nanometer(s)
NPDS	Naval Postgraduate Dental School
PBS	Phosphate Buffer Solution
RLN	Recurrent Laryngeal Nerve
SMA	Smooth Muscle Actin
SLN	Superior Laryngeal Nerve
WRNMMC	Walter Reed National Military Medical Center

CHAPTER 1: Introduction

Thyroidectomy is the surgical removal of the thyroid gland. It may be accomplished by partial removal (partial thyroidectomy) or complete removal (total thyroidectomy) of the gland. It is a common procedure in the United States. Hence, most lay people have some knowledge of this procedure through personal experience or by them knowing someone who has been affected by a disease or condition requiring thyroidectomy.

There are various indications for thyroidectomy, some benign, and others malignant. Examples of benign entities include goiter, thyroid nodules, and hyperthyroidism whereas the various thyroid carcinomas and metastatic disease represent malignant entities.[1]

SIGNIFICANCE

Complications related to thyroidectomy often arise and are in part due to damage to the recurrent laryngeal nerve (RLN). Additionally, damage to the external branch of the superior laryngeal nerve (EbSLN) may occur and presents with function deficits. This is because the external branch of the superior laryngeal nerve courses in the vicinity of the thyroid gland superior pole and the vessels that supply it.[2,3] Moreover, in some instances, branches of the superior thyroid artery provide blood supply to the superior laryngeal nerve.[4]

Clinically, the complications of thyroidectomy that relate to damage to the superior laryngeal and recurrent laryngeal nerves range in severity and may include but

are not limited to change in phonation, hoarseness, vocal cord paralysis, and aspiration risk.[5,6,7,8,9] These are significant morbidities for the patient.

Accordingly, various techniques have been employed to improve identification and visualization of these nerves intraoperatively and improve knowledge of anatomical distribution in *in vivo* and *ex vivo* settings. For example, Sihler's technique, is an *ex vivo* whole mount staining technique which has been used to stain nerves distinctly from the surrounding tissue.[10] The advantage is that it has a proven track record of being successful and allows visualization of small nerves with the naked eye.[10] The disadvantage is that it requires a multi-step staining protocol and is overall time-consuming and technique sensitive.[11]

Intraoperative neuromonitoring (IONM) and electromyography (EMG) assessment of the nerves and muscles innervated by these nerves, respectively, are examples of procedures utilized in an *in vivo* setting.[12] Despite these advances for the thyroidectomy procedure, still more knowledge is needed as ongoing injury to these nerves persists. Therefore, advanced knowledge of the anatomical presentation of these nerves with a focus on terminal nerve endings is crucial to improved outcomes.

STUDY AIM

The aim of this study was to map the terminal branches of the superior laryngeal nerve, particularly the external branch of the superior laryngeal nerve, using 3,3`-dioctadecyloxacarbocyanine perchlorate (DiO) and additionally map the recurrent laryngeal nerve using 1,1`-dioctadecyl-3,3,3`3`-tetramethylindocarbocyanine perchlorate (DiI).[13] DiO and DiI are neural carbocyanine lipophilic tracers that can travel through nerve tissue and laterally diffuse across the cell membrane to highlight nerve cells.[13] DiO

has a fluorescence excitation of 484 nm and fluoresces green. While DiI has a fluorescence excitation of 549 nm and fluoresces red.

CHAPTER 2: Materials and Methods

This was a multidisciplinary research study, coordinated with the Department of Otolaryngology at Walter Reed National Military Medical Center (WRNMMC) and the Oral and Maxillofacial Pathology Department at the Naval Postgraduate Dental School (NPDS), located in Bethesda, MD.

The study received approval from the Uniformed Services University of the Health Sciences' Human Anatomical Materials Review Committee for use of cadavers received through the Anatomical Gift Program. Funding support was received from the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.

STUDY DESIGN

Nine random, fresh adult human cadaver specimens were obtained for laryngeal dissection. The exclusion criteria were cadavers age 17 and under, as well as cadavers with missing, diseased, or damaged larynges and/or adjacent tissues.

At the time of dissection, the external branch of the superior laryngeal nerve was identified bilaterally, and the branching configuration was documented per side, using the pattern described by Cernea (1992) as described below. The branching pattern was not recorded at the time of dissection for three cadavers and thus is not included.

- "Type 1 EbSLN: crosses the superior thyroid vessels more than 1 cm above the edge of the thyroid superior pole
- Type 2a EbSLN: crosses the vessels less than 1 cm above the upper edge of the thyroid superior pole

- Type 2b EbSLN: crosses the superior thyroid pedicle below the upper border of the superior thyroid pole"[14]

Additionally, the RLN was visually identified bilaterally and the distance of the EbSLN to the skull base was determined bilaterally. The whole larynges were then removed from the cadavers. The nerves were spliced using microsurgical techniques. DiO and DiI from the NeuroTrace® Multicolor Tissue-Labeling Kit (Molecular Probes, Inc.) were inserted into the EbSLN and RLN, respectively (**Figure 1**). The whole laryngeal samples were then incubated in paraformaldehyde in 4% phosphate buffer solution (PBS) in a container that impeded light, at room temperature, for four to nine weeks. Per ThermoFisher Scientific specifications, DiO and DiI travel approximately 0.5 mm per day in fixed tissue.[13]

After incubation, decalcification was performed using TBD-2™ Decalcifier (Thermo Scientific), and the larynges were sectioned into 0.2 cm sections from superior to inferior taking care to note at what point in the tissue the sections were made for easier mapping of terminal innervations.

The laryngeal sections were then put into cassettes to be embedded in paraffin and sliced into five micrometer sections for placement on microscopic slides. One hematoxylin and eosin (H&E) stained microscopic slide and one each of S100, cytokeratin marker AE1/AE3, and smooth muscle actin (SMA) immunohistochemical slides were made (**Figure 2**).[15] S100 is a marker of Schwann cells and melanocytes and stains positively in neural tissue (**Figure 3**).[16] SMA is a smooth muscle and myofibroblast marker (**Figure 4**).[17] The H&E slides were viewed using a Carl Zeiss LSM 700 confocal microscope with ZEN 2010 software to identify DiO and DiI

fluorescence in nerve tissue. The S100, EMA, and SMA immunohistochemical slides were viewed with a light microscope for comparison.

CHAPTER 3: Results

The cadavers' age, weight, sex, and height were documented (**Table 1**). The age ranged from 65 to 102 years old. Five cadavers were female and four were male. The weight ranged from 96 to 230 pounds. The average weight was 147.4 pounds with a standard deviation of 41.1. The height ranged from 5'0" to 6'5".

The topographical (Cernea) anatomy pattern found in the majority of cadavers was Type 1 (**Table 2**). The branching pattern was not recorded at the time of dissection for three cadavers and thus is not included.

The distance of the EbSLN to the skull base ranged from 4.5 to 6 cm and presented similarly in length between the right and left sides of the body (**Table 3**). The length of the EbSLN to the skull base was not recorded at the time of dissection for three cadavers and thus is not included. The lipophilic tracers were identified in both the external branch of the superior laryngeal nerve and the recurrent laryngeal nerve (**Figures 5 and 6**). The nerves were not yet able to be mapped due to limitation by auto-fluorescence of surrounding tissue. Autofluorescence was evidenced as the surrounding connective tissue (CT) and adipose tissue fluoresced when viewed with the confocal microscope (**Figure 5**).

CHAPTER 4: Discussion

One cadaver was dissected initially to test and determine the need for possible adjustments to the protocol. For the initial cadaver, 10% formalin was used for incubation and fixation. Use of 10% formalin did not allow gross visualization of anterograde movement of dye within the nerve, evidenced by the colored dye paste beyond the insertion point.

Six subsequent cadavers were dissected in groups of two. Changing the incubation solution and fixative from 10% formalin to paraformaldehyde in 4% phosphate buffer solution (PBS) for the remaining eight cadavers allowed gross visualization of forward movement of the colored dye paste in the nerve. Paraformaldehyde in 4% phosphate buffer solution and 10% formalin have both been used in human studies using lipophilic tracers for neural assessment.[18]

Two cadavers were dissected and used as controls. DiO and DiI were not applied to the EbSLN and the RLN in the control cadavers. However, they were sectioned, sliced, and embedded in paraffin.

The majority of the cadavers exhibited Type 1 Cernea pattern (**Table 2**). This is consistent with the findings of Cernea and Kirnea.[3,14] Sectioning the larynges from superior to inferior taking care to note what area of the specimen was placed in each slide was performed to allow easier discovery of the potential end innervations of these nerves when viewed with the confocal microscope, thus providing a more accurate picture of where the nerves terminate.

The carbocyanine lipophilic neural tracers were identified in both nerves with DiO identified in the EbSLN and DiI identified in the RLN. However, the autofluorescence of the tissue surrounding the DiO and DiI injected nerves upon confocal microscopy examination, has currently hindered immediate tracing of the EbSLN and RLN to their endpoint innervations. A method to isolate the dye on microscopy is currently being considered for the current samples and future study.

On certain slides, the nerve does appear to have a different excitation or brightness of the green on DiO and the same for DiI in red. However, it is extremely challenging to accurately visualize this difference with the naked eye. Therefore, other methods to differentiate the difference in excitation are being explored for the current samples as well as future research.

What has frustrated the research thus far is the fact that dye is identified in the nerve and anterograde is confirmed grossly by movement of the colored dye paste in the nerve. Nevertheless, with current resources, it has not been determined how to differentiate the dye fluorescence within the nerves from the autofluorescence within the surrounding tissue. We know anterograde movement of the dye within the EbSLN and RLN has been achieved. Yet, further study to isolate the differences in autofluorescence between our injected nerves with DiO and DiI and the surrounding tissue is required.

Steps in tissue processing, including, but not limited to, tissue decalcification and paraffin embedding may have contributed to the observed autofluorescence, making dye analysis of the processed tissue challenging. Gelatin-albumen solution is an embedding medium which has been used previously for tissue labeled with DiI

and evaluated with confocal microscopy.[19] This solution should be considered as an alternative embedding medium for future applications of this technique.

Although there is some variation of the fluorescence seen with the naked eye, it is subtle and difficult to definitively use at this moment. Alternate techniques using a biological-image analysis software program Fiji is just Image J (Fiji) to assign numeric values to the different densities of neural tissue with dye uptake (EbSLN and RLN) versus neural tissue with no dye (negative control) is being considered.[20] This will hopefully overcome the limitation of autofluorescence.

CHAPTER 5: Conclusions

The use of lipophilic tracers in an *ex vivo* setting is useful in examining and identifying neural tissue in larynges. This novel application has not yet allowed specific mapping due to aberrant autofluorescence of the non-neural tissue surrounding the EbSLN and RLN. Overcoming this autofluorescence limitation is our next step because mapping the EbSLN and RLN will improve medical knowledge which has a direct impact on surgical management of service members/dependents with diseases or conditions that affect the thyroid gland and require thyroidectomy. Additionally, better anatomical anatomy knowledge improves care and treatment of warfighters with airway injuries.

Table 1. Demographic and Biographical Information

Cadaver #	Age (years)	Weight (lbs)	Sex	Height
1	68	150	M	5’9”
2	79	97	F	5’0”
3	102	96	F	5’5”
4	92	134	F	5’3”
5	85	230	M	6’5”
6	83	165	M	5’9”
7	65	175	F	5’8”
8	80	110	M	5’7”
9	76	170	F	5’5”

Table 2. Topographical Anatomy Pattern

Cadaver #	Pattern (Right)	Pattern (Left)
1	Type 1	Type 1
2	Type 1	Type 1
3	Type 1	Type 2a
4	Type 1	Type 1
5	Type 1	Type 1
6	Type 1	Type 1
7	Unknown	Unknown
8	Unknown	Unknown
9	Unknown	Unknown

Table 3. Length from Skull Base

Cadaver #	Length (Right) (mm)	Length (Left) (mm)
1	5	5
2	4.5	4.5
3	5	5.5
4	5	5.5
5	5	5
6	6	6
7	Unknown	Unknown
8	Unknown	Unknown
9	Unknown	Unknown

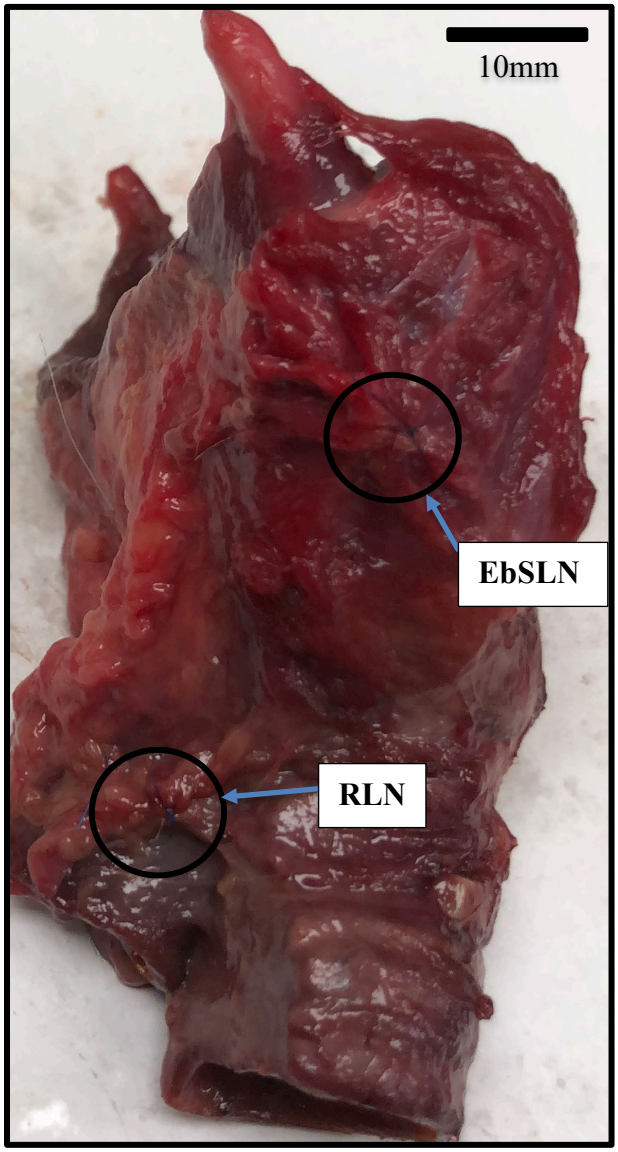

Figure 1. DiO and DiI Dye Insertion. DiO (EbSLN) and DiI (RLN) insertion in a whole larynx after removal from cadaver specimen.

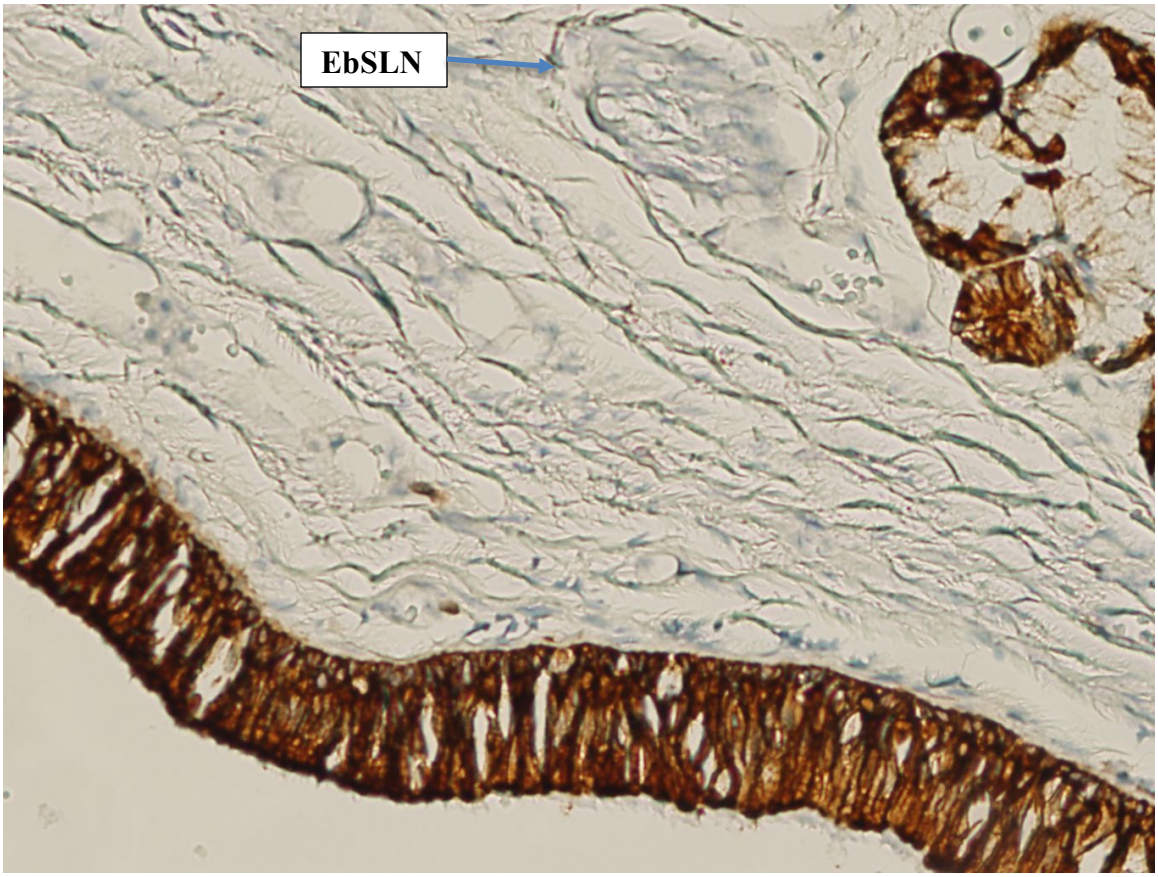

Figure 2. AE1/AE3 immunohistochemical staining. Epithelium of laryngeal tissue demonstrating positive reactivity to cytokeratin stain. Note that the EbSLN does not demonstrate reactivity. (AE1/AE3 10x)

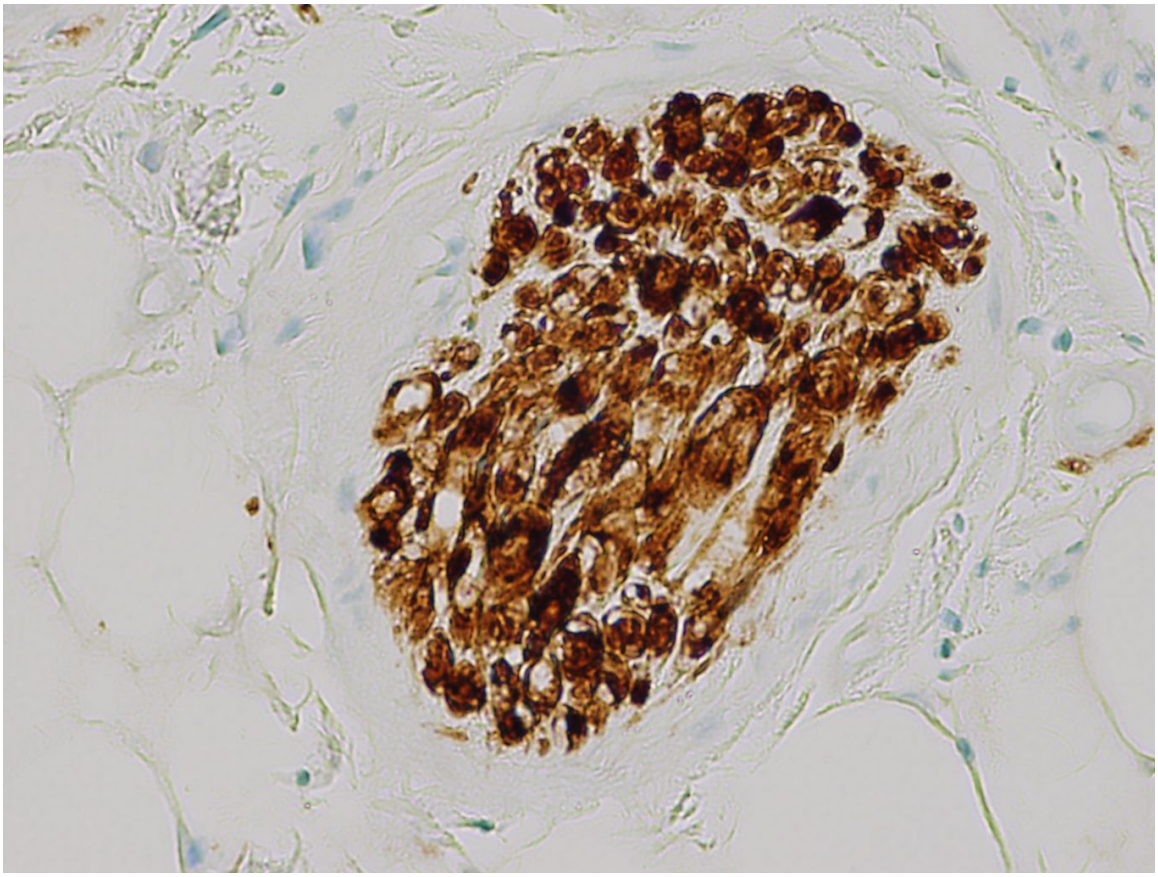

Figure 3. S100 immunohistochemical staining. External branch of the Superior Laryngeal Nerve with positive immunohistochemical reactivity to S100. (S100 40x)

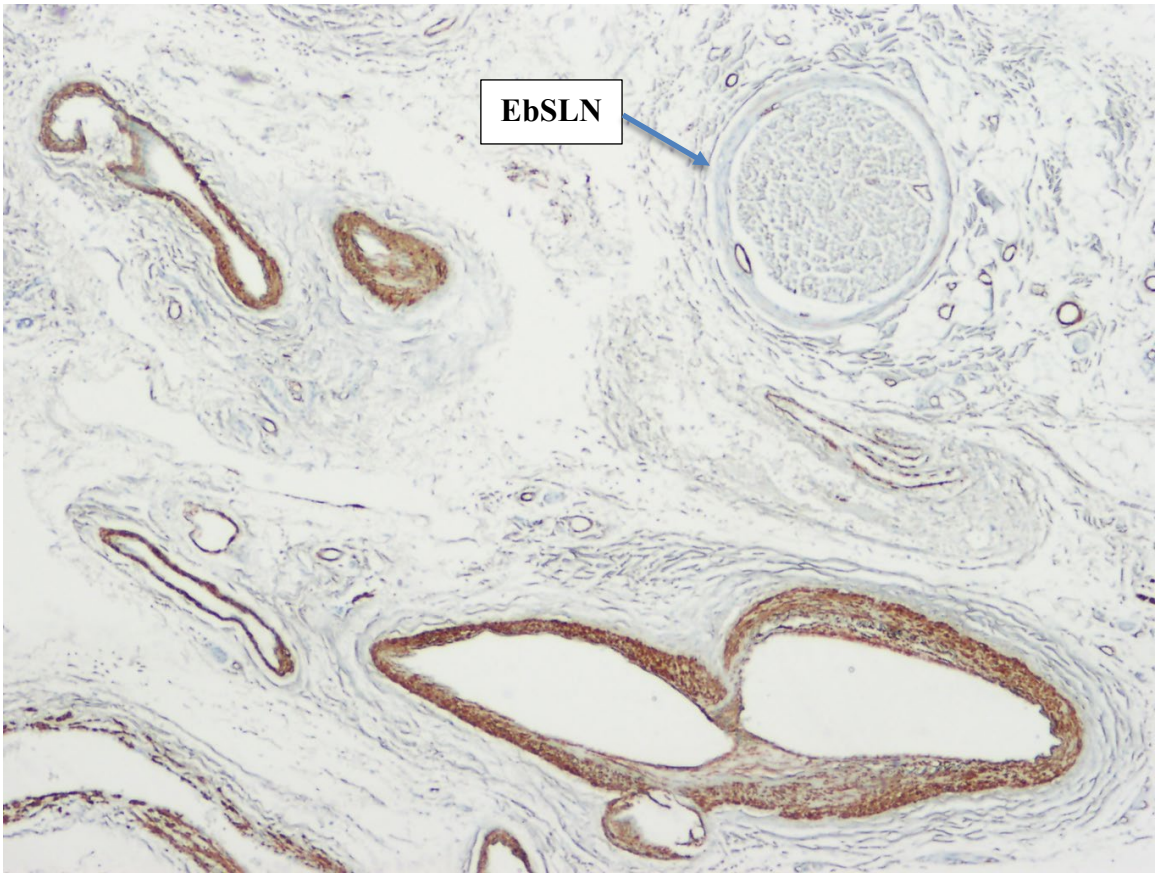

Figure 4. SMA immunohistochemical staining. Endothelial lining demonstrating positive reactivity to smooth muscle actin in multiple small vessels. Note that the EbSLN does not demonstrate reactivity. (SMA 10x)

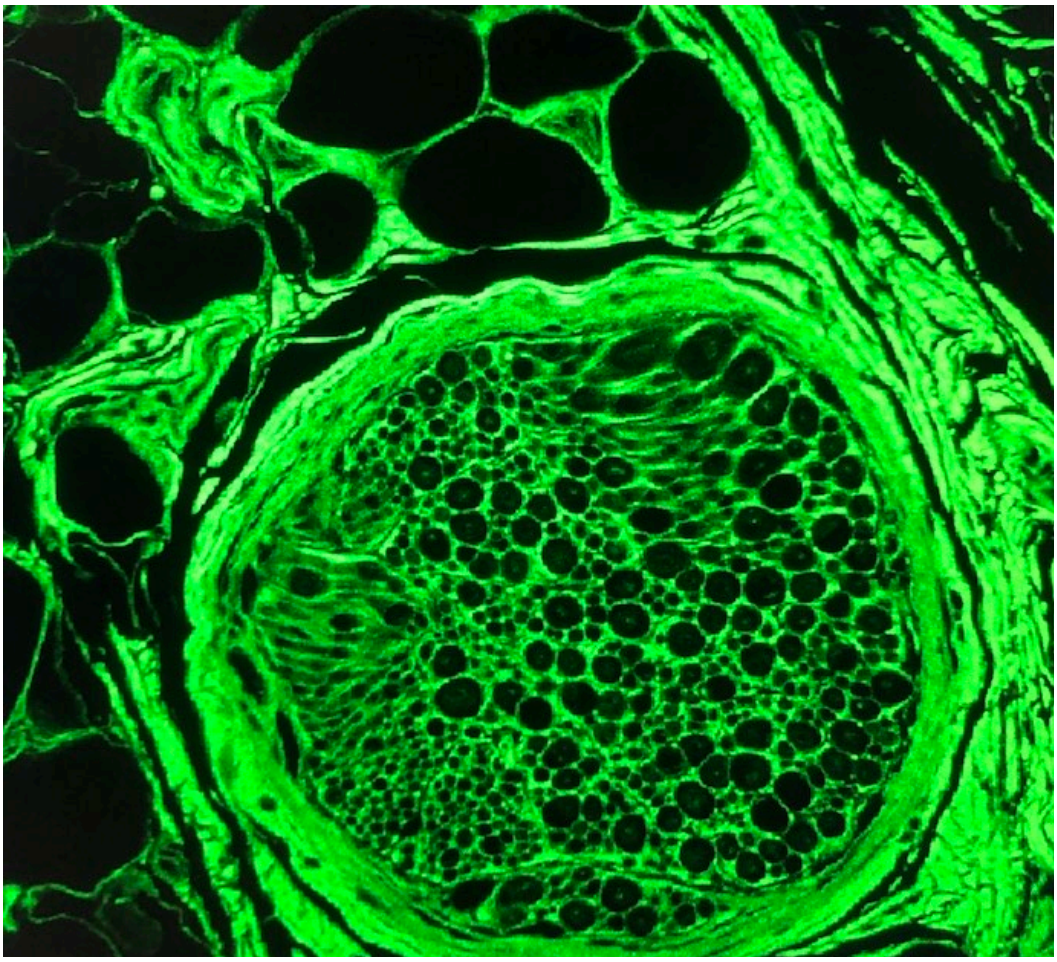

Figure5. EbSLN with DiO. External branch of the superior laryngeal nerve fluorescing with DiO. Autofluorescence of surrounding adipose and connective tissues. (Confocal microscope magnification: 100x)

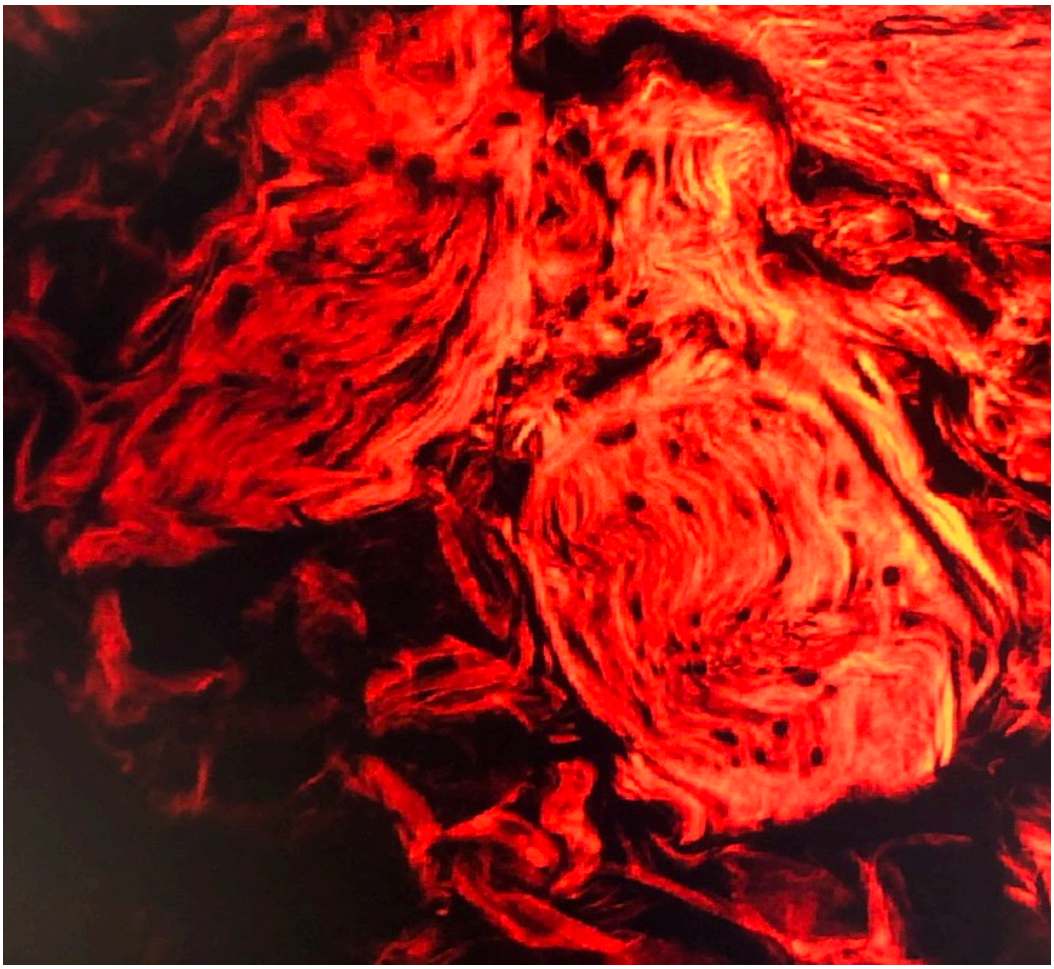

Figure 6. RLN with DiI. Recurrent Laryngeal Nerve fluoresced with DiI. Autofluorescence of surrounding adipose and connective tissue. (Confocal microscope magnification: 100x)

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