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Preparation of the Rat Vocal Fold for Neuromuscular Analyses

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TITLE:

Preparation of the Rat Vocal Fold for Neuromuscular Analyses

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KEYWORDS:

rat, vocal fold histology, vocal fold, larynx, thyroarytenoid muscle, voice

SUMMARY:

This protocol describes methods used to prepare rat vocal folds for histochemical neuromuscular study.

ABSTRACT:

The purpose of this tutorial is to describe the preparation of the rat vocal fold for histochemical neuromuscular study. This protocol outlines procedures for rat laryngeal dissection, flash-freezing, and cryosectioning of the vocal folds. This study describes how to cryosection vocal folds in both longitudinal and cross-sectional planes. A novelty of this protocol is the laryngeal tracking during cryosectioning that ensures accurate identification of the intrinsic laryngeal muscles and reduces the chance for tissue loss. Figures demonstrate the progressive cryosectioning in both planes. To illustrate tracking methods, 29 rat hemi-larynges were cryosectioned and tracked from the emergence of the thyroid cartilage to the appearance of the first section that included the full vocal fold. The full vocal fold was visualized for all animals in both planes. There was high variability in the distance from the appearance of the thyroid cartilage to the appearance of the full vocal fold in both planes. Weight was not correlated to depth of laryngeal landmarks, suggesting individual variability and other factors related to tissue preparation may be responsible for the high variability in the appearance of landmarks during sectioning. This study details a methodology and presents morphological data for preparing the rat vocal fold for histochemical neuromuscular investigation. Due to high individual variability, laryngeal landmarks should be closely tracked during cryosectioning to prevent oversectioning tissue and tissue loss. The use of a consistent methodology, including adequate tissue preparation and awareness of landmarks within the rat larynx, will assist with consistent results across studies and aid new researchers interested in using the rat vocal fold as a model to investigate laryngeal

neuromuscular mechanisms.

INTRODUCTION:

The rat larynx is a well-established model to investigate structural and functional neuromuscular laryngeal adaptations to development, aging, disease, and pharmacological agents[1-5]. Consistency of histological methods is critical to this line of work, as there are multiple intricacies involved in muscle preparation and analysis as well as challenges associated with laryngeal size, shape, and topography of the muscles encapsulated within the laryngeal cartilages[1,6-11]. Due to the small size of the rat intrinsic laryngeal muscles, systematic embedding, freezing, and cryosectioning are critical to achieve consistent and accurate results. For example, when sectioning the rat vocal fold in the coronal plane, the neuromuscular junctions (NMJs) of four of the intrinsic laryngeal muscles are located within less than 1.8 mm of tissue depth[11]. Therefore, precise monitoring of laryngeal muscle anatomy during cryosectioning is imperative to accurately identify the section(s) of interest and prevent oversectioning of tissue. Oversectioning of the target muscle can result in inaccurate identification of number and topography of NMJs[11] or can result in overall reductions in sample size if the target muscle is discarded due to landmark orientation confusion[12]. As novel models for the study of laryngeal muscle and their respective adaptations are developed, standard operating procedures are essential to ensure results are precise, reliable, and reproducible across studies.

The objective of this article is to detail preparation of the rat vocal fold for optimal longitudinal and cross-sectional analysis. Detailed methods used regularly in our laboratory are described to identify target muscle landmarks during cryosectioning. Although similar methods are used in several laboratories, greater detail is provided here than in the literature to ensure reliable and accurate replication when implemented by novice investigators. The goal of this tutorial is to provide a standard methodology for immunohistochemical (IHC) evaluation of the rat vocal fold to improve consistency across laboratories and investigations.

PROTOCOL:

This study was performed in compliance with the Institutional Animal Care and Use Committee of New York University School of Medicine.

1. Dissect rat larynx

1.1. Euthanize rat according to the institutionally approved protocol. Shave the ventral neck from the mandible to manubrium and swab with alcohol to prevent fur contamination in the tissue specimens.

1.2. Under a dissecting scope with 10x magnification excise the entire larynx by creating a midline neck incision with a scalpel until the trachea is exposed.

1.3. Separate the ventral extrinsic laryngeal muscles at the midline to expose the larynx using forceps and dissecting scissors or a scalpel.

1.4. Sever the trachea caudal to the third tracheal ring and make an incision rostral to the hyoid bone to excise the whole larynx using dissecting scissors.

1.5. Remove the extrinsic laryngeal tissues (esophagus, thyroid gland, and extrinsic laryngeal muscles) from the larynx using microdissection tools (tweezers, pins, and microscissors) under magnification.

1.6. With microscissors, bisect the larynx dorsally between the arytenoids using the midline between the posterior cricoarytenoid muscles as a landmark. Pin lateral walls of the larynx to expose the vocal folds and then bisect ventrally through the midline of the thyroid cartilage between the anterior commissure of the vocal folds with microscissors (**Figure 1**).

NOTE: This step can be optional; it can be skipped to keep the larynx whole. Bisection of larynges allow multiple immunostaining techniques by separately using the right and left sides of the same larynx.

1.7. Rinse each hemi-larynx in phosphate buffered solution (PBS) for ~10 s and delicately dry with a task wiper to reduce ice crystal formation during freezing.

2. Fix and/or flash-freeze laryngeal tissue

NOTE: Fixation may not be ideal for all immunostaining protocols. Often laryngeal tissues are flash-frozen fresh immediately following dissection. Skip step 2.1 to flash-freeze laryngeal tissue without fixation.

2.1. To fix hemi-larynges place tissues in centrifuge tube filled with 4% formaldehyde in PBS for 1 h at room temperature on an orbital shaker at 70 rpm. Transfer tissues to a clean centrifuge tube and rinse 3x for 20 min in PBS. Then transfer to a clean centrifuge tube and submerge in a 20% sucrose/5% glycerol solution (~18 h or until tissue sinks) at 4 °C.

CAUTION: Formaldehyde is hazardous and should be used in a fume hood along with appropriate personal protective equipment.

2.2. Place all hemi-larynges in a uniform position into a cryo-mold filled with optimal cutting temperature (OCT) compound. For a hemilarynx, place the tissue with the medial surface of the vocal fold facing the bottom of the cryomold and the longitudinal aspect of the vocal fold parallel to the lower edge of the cryomold opening. For whole larynges, place the tissue with the posterior cricoarytenoids facing the bottom of the cryomold and the longitudinal aspect of the vocal fold parallel to the lower edge of the cryomold opening.

NOTE: Consistent laryngeal orientation within OCT compound is critical for cryosectioning of the rat vocal fold. Once the hemilarynx is embedded and frozen, it must be thawed to change its orientation, thereby introducing risks of tissue damage from multiple thaw-freeze cycles.

2.3. Flash-freeze tissues using isopentane (2-methylbutane) chilled in a steel beaker surrounded by liquid nitrogen.

NOTE: The isopentane reaches optimal temperature for tissue freezing when white precipitates start to form on the sides and bottom of the beaker[13]. Isopentane is used because it has a higher thermal conductivity than liquid nitrogen, which helps prevent cracking of the tissue block during rapid freezing. For a more detailed description of freezing tissue in OTC refer to Kumar et al.[13].

2.4. Wrap each mold in prelabeled foil and place in an individual freezer bag to prevent dehydration and immediately store on dry ice until transferred for storage in a -80 °C freezer.

3. Cryosection hemilarynx in cross-sectional plane

3.1. Set chamber temperature in the cryostat to -20 °C, which is in the middle of the temperature range (15−25 °C) recommended for muscle tissue sectioning by the manufacturer's manual.

3.2. Set cryostat section thickness to 10 µm thick sections.

NOTE: For muscle fiber cross-sectional analysis, 10 µm thick sections are optimal to allow for complete staining and robust imaging intensity of the labeled muscle fibers for fiber typing analysis[14-16]. Some protocols may require different section thickness depending on neuromuscular targets.

3.3. Transfer tissues to the cryostat chamber, add a uniform layer of OCT compound on the cryostat specimen disk (chuck), and place the embedded tissue block on top of the OCT compound on the specimen disk. To obtain cross-sections of the vocal fold for thyroarytenoid (TA) muscle fiber analysis, affix the specimen to the chuck so that the ventral thyroid cartilage faces the cryostat blade and the arytenoid cartilage faces the specimen disk.

NOTE: It is critical to note that these landmarks are not visible at this stage, due to the OCT compound becoming white and opaque when frozen. This lack of visibility is why it is critical to note the orientation of the hemilarynx during the flash freezing stage.

3.4. Trim OCT compound by advancing the specimen head by 100 µm until the ventral portion of the thyroid cartilage appears.

3.5. Then trim and track 30 µm sections from the onset of the thyroid cartilage until the lamina propria, medial TA muscles, and lateral TA muscle are exposed.

NOTE: Laryngeal landmarks should be tracked and noted from the onset of the thyroid cartilage every 100 µm to ensure the angle of sectioning is not oblique. **Figure 2** represents the two sets of laryngeal landmarks in the cross-sectional plane at 10x magnification.

3.6. Once the target TA muscle is reached, collect sections on positively charged slides at 10 µm.

3.7. Store sections in PBS at 4 °C to retain moisture until they are ready to be stained.

NOTE: Fixed tissue can be stored in PBS up to one week depending on IHC target whereas unfixed tissue should be immediately processed.

4. Cryosection hemilarynx in longitudinal plane

4.1. With the cryostat chamber again set to -20 °C, change the section thickness to 30 µm.

NOTE: For NMJ analysis, a tissue thickness between 30−60 µm can be used to capture several complete NMJs within the laryngeal muscles without fragmentation of either the nerve terminal or motor endplate[11,12,17].

4.2. To obtain longitudinal vocal fold sections for NMJ analysis of the TA muscle, affix the specimens to the chuck so that the epiglottis is oriented towards the cryostat blade and the tracheal lumen faces down towards the specimen disk.

4.3. Trim the OCT compound by advancing the specimen head by 100 µm until the thyroid cartilage appears.

4.4. Trim and track sections of 30 µm from the onset of the thyroid until the lamina propria and medial and lateral divisions of the TA muscle are exposed.

NOTE: Five sets of laryngeal landmarks in the longitudinal plane are recommended to track tissue depth progression towards the target TA muscle. **Figure 3** represents the laryngeal landmarks in the longitudinal plane at 10x magnification.

4.5. Once the target TA muscle is reached, collect sections on positively charged slides at 30 µm.

4.6. Store sections in PBS at 4 °C to retain moisture until they are ready to be stained.

REPRESENTATIVE RESULTS:

The representative results were part of an ongoing investigation of the effects of vocal exercise on the laryngeal neuromuscular system. Twenty-nine male Fischer 344/brown Norway rats (12 9-month-old, 17 24-month-old) were weighed and euthanized with CO_2 inhalation followed by a bilateral thoracotomy.

The procedures followed the outlined protocol to label NMJs and fiber size of the lateral and medial TA muscles. The distance between laryngeal landmarks was tracked in both longitudinal and cross-sectional planes using laryngeal muscles and surrounding cartilages to determine progression during cryosectioning (**Table 1**). Tracking commenced at the first appearance of the thyroid cartilage in both directional planes. **Figure 2** illustrates the appearance of laryngeal

landmarks during cross-sectional cryosectioning in temporal order with the thyroid (**Figure 2a,b**) appearing prior to the medial TA muscle and to the lamina propria (**Figure 2c,d**). **Figure 3** illustrates the appearance of laryngeal landmarks during longitudinal cryosectioning in temporal order with the alar muscle (**Figure 3a,b**) appearing prior to the medial TA muscle (**Figure 3c,d**) and to the lamina propria (**Figure 3e,f**).

In both directional planes, distances among landmarks greatly varied for individual animals. Weight and laryngeal landmark appearances had weak to moderate correlations for young rats and weak correlations for aged rats (**Table 2** and **Table 3**). Distances among landmarks within each plane were moderately to strongly correlated for both age groups, but weakly correlated between the two dissection planes. Therefore, variability in landmark appearance could not be accounted for by weight or individual variations in laryngeal size.

FIGURE AND TABLE LEGENDS:

Figure 1: A rat larynx dorsally bisected between the arytenoid cartilages (ArC). The right side of the hemi-larynx is annotated with landmarks in the longitudinal plane (LZ1-LZ5) corresponding to the five longitudinal landmarks in **Table 1**. The left side of the hemi-larynx is annotated with landmarks in the cross-sectional plane (CZ1 and CZ2) that correspond to the beginning of the lateral TA muscle and full cross-section of the vocal fold respectively. VF = vocal fold, CrC = cricoid cartilage, AlC = alar cartilage, and T1 = first tracheal ring.

Figure 2: Two cross-sections imaged at 10x magnification in brightfield (right) and in the fluorescent 488 channel (left) following immunostaining for laminin to outline muscle fibers. The sections (from top to bottom) show the progression during cryosectioning in temporal order with the thyroid (**a,b**) appearing prior to the medial TA muscle and to the lamina propria of the vocal fold (**c,d**). ThC = thyroid cartilage, LTA = lateral thyroarytenoid, and MTA = medial thyroarytenoid.

Figure 3: Three longitudinal sections imaged at 10x magnification in brightfield (right) and in the fluorescent 488 channel (left) following immunostaining for neuromuscular junctions. The sections (from top to bottom) show the progression during cryosectioning in temporal order with the alar muscle (**a,b**) appearing prior to the medial TA muscle (**c,d**) and to the lamina propria (**e,f**) of the vocal fold. AlC = alar cartilage, ThC = thyroid cartilage, ArC = arytenoid cartilage, LTA = lateral thyroarytenoid, MTA = medial thyroarytenoid, and SCA = superior cricoarytenoid.

Table 1: Distances in μm from the first appearance of the thyroid cartilage to each laryngeal landmark during cryosectioning (n = 29).

Table 2: Results of Pearson correlation between weight and the depth of laryngeal landmarks in the cross-sectional (CSA) and longitudinal planes for young male rats. LTA = lateral thyroarytenoid, MTA = medial thyroarytenoid, SCA = superior cricoarytenoid, and LP = lamina propria.

Table 3: Results of Pearson correlation between weight and the depth of laryngeal landmarks

in the cross-sectional (CSA) and longitudinal planes for old male rats. LTA = lateral thyroarytenoid, MTA = medial thyroarytenoid, SCA = superior cricoarytenoid, and LP = lamina propria.

DISCUSSION:

Preparing rat vocal folds for neuromuscular analysis can present with various challenges. Not only are laryngeal muscles small and surrounded by cartilage, thereby making it difficult to directly extract target muscle, high variability was also found between animals in the depth of laryngeal anatomical landmarks. For muscle the cross-section plane protocol, complete vocal fold sections appeared between 21−85 sections (10 µm per section) after the initial appearance of the ventral thyroid cartilage, which is quite a bit fewer than the 63−126 sections (35 µm per section) in the longitudinal plane for NMJ analysis protocols (**Table 1**).

Variability was noted in distances between laryngeal landmarks despite the uniform embedding, orienting, and sectioning of tissues for each type of protocol. Furthermore, differences in body weight did not account for variability in the wide ranges of tissue depth from one set of laryngeal landmarks to the next. This variability in distance between laryngeal landmarks may be due to individual differences in laryngeal anatomy across animals, small differences in orientation of larynges in the cryomold within the OCT compound at the time of dissection, or how the specimens were placed on the specimen disk within the cryostat when sectioning (i.e., the amount of OCT compound placed on the specimen disk prior to mounting or slight differences in angle of placement).

With an understanding that these slight differences in specimen preparation can lead to substantial variability in depth of laryngeal tissue landmarks, it is critical that novice investigators have a reference map from which to work. Outlined study protocols defining methods to identify the muscle(s) of interest and prevent protocol pitfalls—such as the ones outlined in this document—can improve reproducibility and prevent unwanted tissue loss.

Although this study focused on the TA muscle, this methodology is applicable for other intrinsic laryngeal muscles as well. For example, sectioning in the longitudinal vocal fold plane yields longitudinal muscle fiber sections of the alar, lateral TA, medial TA, lateral cricoarytenoid, and superior cricoarytenoid muscles, and cross-sections of the posterior cricoarytenoid muscles. Sectioning in the cross-sectional vocal fold plane yields cross-sections of the alar, lateral TA, medial TA, lateral cricoarytenoid, superior cricoarytenoid, and cricothyroid muscles, as well as longitudinal sections of the posterior cricoarytenoid muscles. Additionally, although this study did not include female rats, differences between male and female rats in laryngeal landmark appearance are not expected because sexual dimorphism within the rat larynx is muscle specific and not related to laryngeal framework anatomy[16,18].

The variability in distance between laryngeal landmarks can make cryosectioning the rat vocal fold difficult for novice investigators. This study demonstrated that despite consistency in how the rat larynges were frozen, embedded, and cryosectioned, the distance between laryngeal landmarks varied greatly; animal weight did not account for this variability. This study provides

detailed procedures with associated images on how to appropriately prepare laryngeal muscle tissue and identify laryngeal landmarks for neuromuscular histological investigation of the rat vocal fold.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1 - Hemilarynx.psd

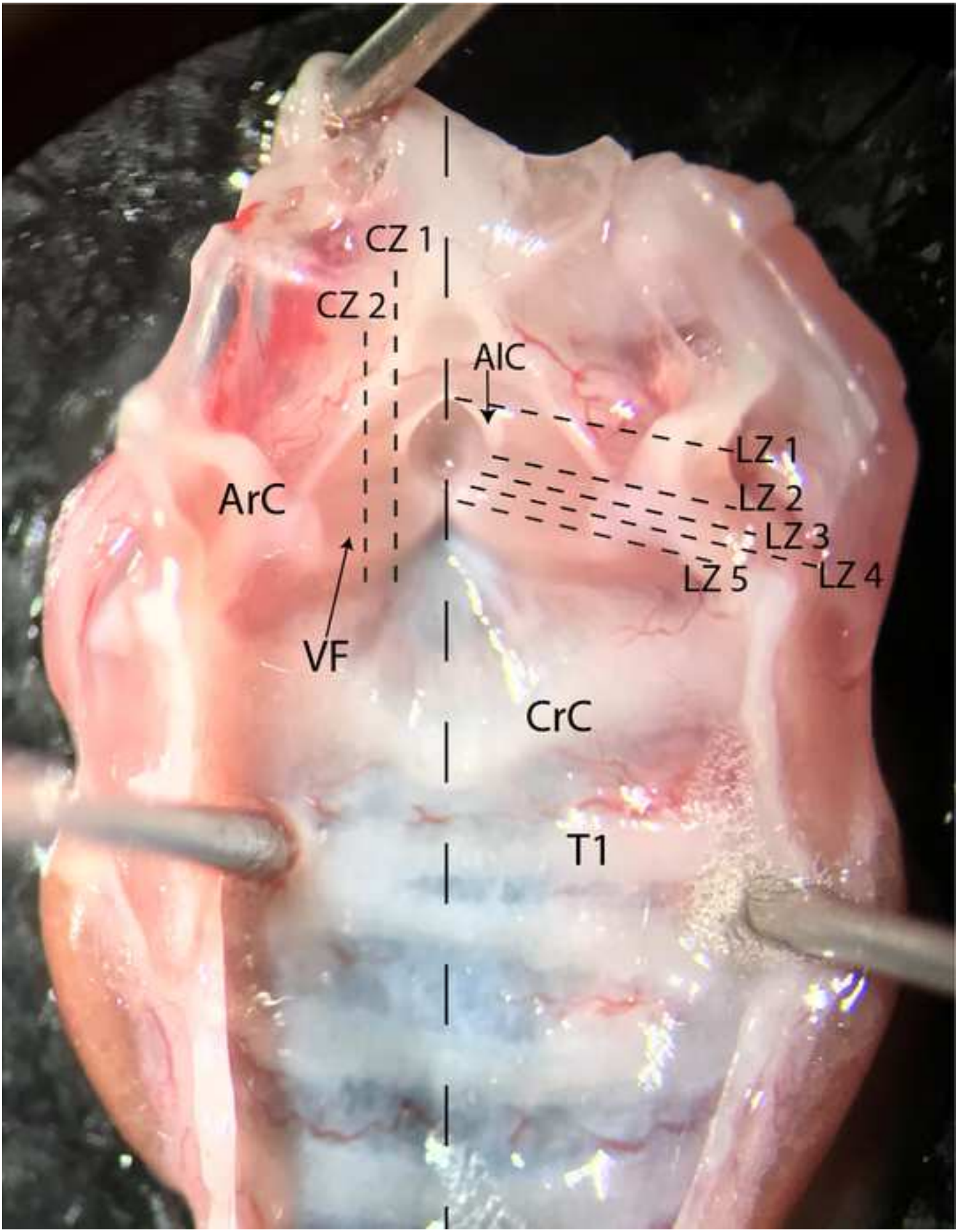

Figure 2

Your image file "Figure 2 - CSA.psd" cannot be opened and processed.
Please see the common list of problems, and suggested resolutions below.

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Longitudinal landmarks	Mean (standard deviation) in µm	Range in µm
1. All three major cartilages (thyroid, alar, arytenoid) appeared with emergence of muscle fibers	1,591 (665)	350–2,800
2. Superior cricoarytenoid (SCA), alar cricoarytenoid (ACA), and lateral thyroarytenoid (LTA) muscles appeared	2,344 (591)	91–3,500
3. ACA and LTA muscles extended completely without fragmentation	2,631 (532)	1505–3,640
4. Arytenoid cartilage enlarged, ACA disappeared, medial thyroarytenoid (MTA) muscle emerged	2,948 (606)	1765–4,305
5. Target full vocal fold section: LTA and MTA muscles extended completely without fragmentation and lamina propria emerged	3,131 (542)	2205–4410
Cross-sectional landmarks		
1. LTA muscle appeared	303 (138)	110–690
2. MTA muscle appeared and was ~50% of the LTA size with clear lamina propria and epithelium noted.	482 (167)	210–850

Table 2

		CSA		Longitudinal				
		LTA	MTA	Cartilages	Alar/SCA	LTA	MTA	LP
CSA	LTA	1						
	MTA	0.88	1					
Longitudinal	Cartilages	0.42	0.42	1				
	Alar/SCA	0.57	0.47	0.77	1			
	LTA	0.59	0.47	0.71	0.98	1		
	MTA	0.53	0.39	0.72	0.97	0.98	1	
	LP	0.53	0.41	0.76	0.96	0.97	0.99	1
	Weight	-0.55	-0.35	0.08	-0.45	-0.46	-0.46	-0.41

Table 3

		CSA		Longitudinal			
		LTA	MTA	Cartilages	Alar/SCA	LTA	MTA
CSA	LTA	1					
	MTA	0.9	1				
Longitudinal	Cartilages	0.21	0.33	1			
	Alar/SCA	0.05	0.07	0.73	1		
	LTA	-0.06	-0.04	0.64	0.96	1	
	MTA	-0.02	-0.02	0.6	0.79	0.84	1
	LP	-0.17	-0.15	0.52	0.76	0.85	0.91
	Weight	0.23	0.13	-0.24	-0.07	-0.15	-0.15

LP

1
-0.3

Name of Material/Equipment	Company	Catalog Number	Comments/Description
2-Methylbutane Certified	Fisher Chemical	35514	
Aluminum Foil	Fisherbrand	1213101	
Cryo Tongs SS	Thermo Scientific	11679123	
Cryostat	Leica Biosystems	CM3050	
Cryostat blades	C.L. Sturkey D554X50	22-210-045	
Disposable Base Molds 15mm x 15mm	Thermo Scientific	41-741	
Disposable Underpads	Medline	23-666-062	
Dissection kit	Thermo Scientific	9996969	
DPBS - Dulbecco's Phosphate-Buffered Saline	Gibco	14190136	
Frozen Section Medium	Fisher Healthcare	23-730-571	
Ice Bucket	Bel-Art	11999054	
Immunostain Moisture Chamber	Ted Pella Inc	NC9425474	
Needle holders	Assi	ASSI.B148	
Non-Woven Sponges, 4 Ply	Quick Medical	9023	
Orbital shaker	Troemner	02-217-987	
Pap pen			
Paraformaldehyde, 16% w/v aq. soln., methanol free	Alfa Aesar	50-00-0	
Premium Microcentrifuge Tubes	Fisherbrand	5408129	
Specimen Storage Bags	Fisherbrand	19240093	
Stainless Steel Graduated Measure 32 oz/100 mL	Polar Ware	114231B	
Superfrost Plus Microscope Slides	Fisherbrand	12-550-15	
Task wiper	Kimberly-Clark Professiona	06666A	
Timer	Fisherbrand	2261840	
Vannas Pattern Scissors	Assi	ASSI.SAS15RV	

NOTE: For all supplies, these are examples of equipment to purchase. The exact model is not necessary to complete our methods.

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Stratos Achlatis, M.D.

NYU Voice CENTER

March 3, 2020

Thank you for the review of our manuscript. We have addressed all comments and suggestions as detailed below.

We edited the manuscript as follows to address the editorial comments:

- The document was carefully proofread for spelling and grammatical issues.
- Six keywords were provided. An ethics statement was provided at the beginning of the protocol section.
- The age, sex, and strain of rat was specified. Labels were added to each panel of the figure and referenced in the manuscript.
- Table titles were removed from uploaded tables and include in manuscript text.
- Table of Materials was alphabetized. Surgical tools were specified throughout dissecting portion of the protocol.

We edited the manuscript as follows to address the reviewer comments:

- The shelf-life for storing sections in PBS at 4C before staining procedures was specified.
- The catalogue number and supplier for isopentane is located in the Table of Materials.
- Rather than including a figure of flash-freeze tissue with isopentane, we refer the reader to a detailed published JoVE article that thoroughly demonstrates fresh-freezing muscle tissue with isopentane.

Thank you again for considering our manuscript. We look forward to moving forward with the recording.

Sincerely,

Aaron M. Johnson

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