



Version 1

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Protocol for Pig Vagus Nerve Microdissection and Histology V.1

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ATTACHMENTS

[Protocol.docx](#)

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1 Protocol for Histological Analysis

Microdissection:

1. Following surgical experimental protocol and euthanasia, we further exposed either the right or left side vagus nerve.
2. We exposed the length of the vagus nerve from the pharyngeal branch to the recurrent laryngeal bifurcation using careful dissection, so as not to disturb any branches coming off the vagus nerve.
3. We obtained pictures of trajectories of the superior laryngeal (SL) and recurrent laryngeal (RL) and vagus nerve
 - a. We noted the presence and arrangement of the internal superior laryngeal (ISL) and external superior laryngeal (ESL) as well as their lengths from bifurcation to insertion into the muscle, and diameters approximately 1mm from the nodose ganglion.
4. We obtained standard measurements from each subject:
 - a. SL to carotid bifurcation (measured from the valley at the top of the bifurcation)
 - b. SL to the mandible (bend in the jaw)
 - c. SL to sternal notch
 - d. Distance to the most cranial LIFE electrode from SL
 - e. Distance to the most caudal LIFE electrode from SL
5. Determined the distance from LivaNova epineural stimulating cuff to the RL and SL branches:
 - a. Proximal contact of LivaNova cuff to SL
 - b. Distal contact of LivaNova cuff to SL
 - c. Proximal contact of LivaNova cuff to RL
 - d. Distal contact of LivaNova cuff to RL
6. Measured the length and diameter of SL and RL:
 - a. Length of SL (Length of SL to insertion in muscle):
 - i. Note the length of SL from nodose to bifurcation into external/internal branches
 - b. Diameter of SL 1mm from the nodose ganglion
 - c. Length of RL (bifurcation to insertion into muscle)
 - d. Diameter of the RL at the bifurcation
 - e. Distance from SL to recurrent bifurcation
7. Obtained vagus nerve measurements
 - a. Vagus nerve diameter at 1cm caudal to SL
 - b. Vagus nerve diameter at 6cm caudal to SL
 - c. Vagus nerve diameter at the RL bifurcation
8. Determined where each of the recurrent and superior laryngeal branches terminated with any associated branches.
9. Determined the distance from the stimulating electrode to the RL branch termination in the cricoarytenoid by using:
 - a. Distance from RL into termination from 6c
 - b. Distance from SL to RL bifurcation from 6e
 - c. Distance from SL to stimulating LivaNova cuff from 5a and 5b
10. Determined the distance from location of the stimulating electrode to the recurrent laryngeal insertion as it runs back up near the electrode along the esophagus.
11. Determined the location of the aortic depressor nerve (if found) and the sympathetic chain (running parallel or bundled).
12. Determined the location and trajectory of the pharyngeal branch and any other associated branches.
13. Remaining Experimental Measurements:
 - a. Measured the distance between the RL bifurcation to the experimental transection cut locations as well as to the insertion in the muscle.
 - b. Measured the distance between the RL bifurcation to the where the LIFE electrodes were located in the vagus trunk.

Histology:

1. Placed histological dye (Bradley Products, Inc., Davidson Marking System, Bloomington, MN) on the lateral and ventral edge to maintain orientation of nerve sample of interest.
2. Placed samples in neutral buffered formalin for ~24 hours at 4°C, and placed into histology tissue processor for 13-hour processing procedure [includes standard histological dehydration (ethanol, 50-100%), clearing (xylene), and infiltration (paraffin) steps].
3. Embedded each sample in paraffin wax and allowed to set (app. 24 hours)
4. Following the removal of excess wax (until samples were visible), placed samples in an ice water bath for approximately 1 hour before cutting 5 um sections using a Leica Biosystems Rotary Microtome (Buffalo Grove, Illinois).

5. Samples were floated in a 35-40°C water bath, and mounted on a glass slide.
6. Allowed slides to dry over night.

Staining Procedure for Gomori's Trichrome:

All staining procedures should be completed in an appropriate fume hood and SDS sheets consulted before working with chemicals:

1. Placed slides in xylene for 3 minutes (x2)
 - a. Sigma Aldrich, Product #: X54
2. Placed slides in 100% Ethanol for 2 minutes (x2)
 - a. Sigma Aldrich, Pure Ethyl Alcohol, Product #: 459844
3. Placed slides in 95% Ethanol for 2 minutes (x2)
 - a. Used pure Ethyl Alcohol to make 95% solution
4. Placed slides in 70% Ethanol for 2 minutes
 - a. Used pure Ethyl Alcohol to make 70% solution
5. Rinse slides in deionized water for 1 minute
6. Place slides in Bouin's Solution in a water bath at 60°C for 1 hour
 - a. Sigma Aldrich, Product #: HT10132
7. Rinse slides in running cold tap water until clear
8. Place slides in Weigert's Iron Hematoxylin for 10 minutes
 - a. Sigma Aldrich, Product #: HT1079
9. Place slides under running cold tap water for 5 minutes
10. Place slides in Gomori's Trichrome Stain for 20 minutes
 - a. Sigma Aldrich, Product #: HT10516
11. Place slides in 1% Glacial Acetic Acid solution for 1 minute
 - a. Sigma Aldrich, Product #: 695092
12. Place slides in 95% Ethanol for 1 minute
13. Place slides in 100% Ethanol for 1 minute (x2)
14. Place slides in xylene for 1 minute (x2)
15. Place a drop of mountant xylene substitute on the samples and coverslip.
 - a. ThermoFisher Scientific, Product #: 1900231
16. Allow slides to dry for approximately one hour.

Slide Scanning Procedure:

1. Following staining and coverslipping of slides, all images were collected at 20x using a Motic Slide Scanner (Motic North America, Richmond, British Columbia).
2. A preview scan was first conducted of the entire slide, and areas of interest were selected to be scanned at 20x using the EasyScan scanning application (Motic China Group Co., LTD).
3. Images were then exported to tiff format using the Motic VM conversion application (Version 3.0, Motic China Group Co., LTD).