Staining the Human Vagus Nerve with Osmium Tetroxide and Micro CT imaging V.2

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DISCLAIMER

ABSTRACT

In this protocol, we describe our steps to stain nerve fibers with Osmium Tetroxide for further visualization wtih microCT, 3D MUSE (Microscopy with Ultraviolet Surface Excitation), and electron microscopy.

Protocol status: Working
We use this protocol and it's working

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MATERIALS

Formaldehyde-fixed tissue sample
Deionized distilled water
1x Phosphate Buffered Saline (PBS) - Fisher Scientific
Osmium Tetroxide (2%) - Polysciences
Ethanol (95%) - Fisher Scientific
Glass beakers
Falcon Tubes (50ml) - Fisher Scientific
Forceps for tissue handling/transfers
Beaker filled with Corn oil for Osmium Tetroxide neutralization
Luer Lok Syringes (5 ml) with 20g Needles for Osmium Tetroxide transfer
Tray covered with disposable aluminum foil for cleanup

SAFETY WARNINGS

Formaldehyde is a known carcinogen; Osmium tetroxide is toxic, highly reactive, and a strong oxidizer. Great care should be used when handling these chemicals. Users should consult with their institution regarding any federal, state, and local guidelines for the handling, storage and disposal of these chemicals.

BEFORE START INSTRUCTIONS

Required PPE: All work must be done inside of the fume hood. Lab coat, Glasses, and DOUBLE NITRILE GLOVES need to be worn at all times when handling this stain. If any part of the top glove becomes black, remove both gloves immediately and replace with a fresh pair.

Corn oil should be available on-hand to neutralize Osmium Tetroxide residues on instruments, containers or any other surfaces contaminated.

Section 1: Harvesting Nerve Tissue

1 Nerve were harvested from embalmed cadavers and the orientation of the nerve was marked with sutures at the inferior/causal end of the nerve tissue and were stored in 50 ml falcon tubes containing formaldehyde.

1.1 Capture images of samples prior to staining to aid in post-image interpretation, documentation

2 Store formaldehyde-fixed nerve samples in 2-4°C fridge until staining.

3 Label the metrics of the sample (species, type of nerve, date of extraction, length, width, etc.)

Section 2: Staining

30m

4 Perform 3 washes of the nerve sample with 1X Phosphate-buffered saline letting the sample shake on an orbital shaker for 5 minutes at a time.

5 PREPERATION OF 1% OSMIUM TETROXIDE SOLUTION.
All the steps mentioned below must be performed INSIDE OF THE FUME HOOD
** waste should be placed in the OsO_4 container **
All diluted solutions of OsO_4 should be prepared fresh on the day of staining.

5.1 Take the glass container containing the 2% Osmium Tetroxide solution, and wrap the head of the bottle with two gloves and tissue rolls. Carefully snap the top glass cover of the bottle and dispose of the broken glass in biohazard sharps waste and dispose the gloves and tissue rolls in biohazard waste and set the osmium vial aside.

5.2 Prepare a 5ml syringe and needle, and using the syring extract all of the Osmium tetroxide into the syringe and transfer it into the falcon tube containing the nerve sample.

5.3 Add 5 ml of deionized water to the 2% osmium tetroxide solution to make it a 1% solution.

5.4 Wrap the head of the falcon tube containing the nerve sample with parafilm.

5.5 Place sample in OsO_4 solution on an orbital shaker and allow to shake and stain for 1 to 3 days (depending on the size of sample) in 2-4°C fridge.

5.6 All working solutions of OsO_4 can be placed into falcon tubes and other polypropylene containers. HOWEVER, if solution will be stored long term (>7days) sample and OsO_4 solution must be stored in a glass opaque container.

Section 3: Dehydration

3h

6 Prepare 70% Ethanol and 95 % ethanol solutions with deionized water as the solvent.

7 Pour the OsO_4 solution carefully into a designated glass container (and make sure this will be disposed off by appropriate authorities) noting the volume on the waste records.

8 Perform 2 quick rinses of the nerve sample with 70% ethanol.

9 Perform 3 washes of the stained nerve sample with 70% ethanol letting the sample shake on an orbital shaker for 30 minutes at a time.

10 Perform 3 washes of the stained nerve sample with 95 % ethanol letting the sample shake on an orbital shaker for 30 minutes at a time.

11 Store the sample in 70% ethanol in 2-4°C fridge.

Section 4: Preparing of sample and Micro CT imaging

12 Nerve tissue samples after dehydration were submitted to the Histology core for paraffin embedding in a custom designed mold. The sample was processed in the following the following chemicals for duration mentioned:

12.1 1. 70 % Ethanol for 1 hour

12.2 80 % Ethanol for 1 hour

12.3 95 % Ethanol for 1 hour

12.4 100 % Ethanol for 1 hour

12.5 100 % Ethanol for 1 hour

12.6 100 % Ethanol for 1 hour

12.7 Xylene for 1 hour

12.8 Xylene for 2 hour

12.9 Xylene for 2 hour

12.10 Paraffin for 2 hours

12.11 Paraffin for 1.5 hours

13 Once the tissue was processed, the nerve was placed in a mold and paraffin was poured into the mold. Once the paraffin was hardened the mold is now ready to be placed into the micro CT machine

Section 5: Setting up the scan parameters

14 Turn on the Micro CT machine and set the bed to the deepest point along the machine.

15 Make sure the bore placed is 72 microns. Set the filter to Aluminum and copper, set the tube voltage to 90Kv and the current to 80 micro amps.

16 The scan time is set to high resolution and 14 minutes.

17 Create a new database for each sample that will be scanned.

Section 6: Imaging the nerve

18 Place the mold in which the nerve is placed into the micro-CT tray.

19 Enable the live mode on the machine and navigate the bed to reach the most rostral/superior point of the nerve from where you will begin the scan.

20 Rotate the bed to 270^0 view and make sure that the region of interest to be scanned stays within the micro-CT view box.

21 Click the CT scan button and begin the scan. This step being the first scan- the machine will prompt you asking for a warmup scan of 57 minutes. Click YES.

22 Once the scan is complete, move the bed of the micro-CT 1.5 cm in the caudal direction using the navigation buttons. Create a new study and label it as you find it appropriate. Start the second scan of the nerve.

23 Repeat step 21 along the entire nerve moving 1.5 cm down/rostral/inferior (Using 1.5 cm will ensure that there is an overlap of 0.3 cm between two adjacent scans) until the whole nerve scan is complete.

Section 7: Reconstruction

24 Once all the scans are complete, open each scan in the native Quantum GX 2 software viewer window. Select the option “Sub” and choose the resolution you would like to reconstruct the sample at, and this will generate a blue box. Click and drag this box such that the nerve fits into the box. (Note: Make sure that the nerve is in the blue box in all the three different views, otherwise stitching will fail in the next step).

25 When doing adjacent blocks of reconstruction make sure that the two blocks have an overlap of 20% based on the resolution of the reconstruction that is done.

26 Continue this along the length of the nerve within the sample and across all the samples to reconstruct the entire length of the nerve.

Section 8: Image Stitching

27 Using the export feature in quantum GX2, export all reconstructions into a folder in a series (use any naming convention convenient) as dicom files.

28 The multiple blocks must be stitched together using the pairwise registration function on ImageJ/Fiji (Plugins>Stitiching>Pairwise Stitching). This should generate a completely stitched scan of the entire nerve.

Section 9: Fascicle Morphometry and image analysis

29 We will import the dicom images into image J

29.1 Open and run Fiji (ImageJ).

29.2 Click on File>Import, this will open an explorer window and select the file you want to import.

30 Using ImageJ we will analyze fascicle morphometry. The first step after import will be to set the scale for the image. We will define the size for each pixel of the image.

30.1 Click on Analyze> Set Scale

30.2 Now set the value of pixel to “1” and set the known distance to 10 microns (This will depend on the reconstruction window) and set the measurement unit to microns.

31 Image J window will have multiple tools for ROI measurement, right click on the oval selection and choose “Elliptical”

32 Click Analyze>Set Measurements. This opens a tab to choose all the measurements you would like to make of the fascicle. It is recommended to select Area, Fit ellipse, Feret’s diameter and centroid.

33 Click Analyze>tools>ROI manager. This will open a window for selecting your regions of interests.

34 Now using the cursor make an elliptical shape around the fascicle in the best fit possible. One you have selected your ROIs, click on “Add” which will save the ROIs. You can select multiple ROIs on one single image to measure all the fascicles in frame.

35 Now click on the “Measure” tab to get the various measurement values, which will open up on a new window.

36 From the new window with all the measurements recorded copy and paste (to export) it into an excel file.