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**We use this protocol and it's working**

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## Abstract

The protocol describes immunohistochemistry with anti-claudin-1, imaging, image segmentation, and image analysis methods to quantify human vagus nerve morphology.

## Materials

- Microscope slides with paraffin slices
- Xylene
- Ethanol
- Deionized water
- HIER Buffer L (Thermo, TA-135-HBL)
- H<sub>2</sub>O<sub>2</sub>
- Tris buffer
- Tris Tween buffer
- DAKO Protein Block (X0909)
- Antibody Diluent OP Quanto (Thermo, TA-125-ADQ)
- Rabbit anti-claudin-1 (Abcam, ab15098)
- Biotinylated SP-conjugated Affinipure goat anti-rabbit IgG (H+L) (Jackson, 111-065-144)
- ABC Elite (Vector, PK-6100)
- DAB chromogen (Thermo, TA-125-QHDX)
- Harris hematoxylin (Thermo, 6765003)
- DPX mountant (Electron Microscopy Sciences, 13512)
- Microscope with color camera
- Nikon's NIS Elements
- Matlab

## Immunohistochemistry

- 1 Bake slides with sections of paraffin-embedded vagus nerve overnight at 50oC and then cool overnight.
- 2 Deparaffinize the slides and hydrate them to distilled water: xylene (2× 6 min), 100% ethanol (5 min), 95% ethanol (4 min), 70% ethanol (3 min), deionized water (2× 1 min).
- 3 Perform heat-induced epitope retrieval (HIER) at 120oC for 30 s followed by 90oC for 10 s, using a buffer with pH 6.0 (Thermo, TA-135-HBL).
- 4 Cool for 20 min at room temperature.
- 5 Rinse in deionized water (2× 2 min).
- 6 Block with 3% H2O2 diluted in deionized water for 10 min.
- 7 Rinse in deionized water (2× 2 min).
- 8 Rinse in Tris buffer (1× 2 min).
- 9 Block using DAKO Protein Block (X0909) for 10 min at room temperature.
- 10 Apply the primary antibody (rabbit anti-claudin-1, Abcam, ab15098) diluted in Thermo Antibody Diluent to a concentration of 1:50, and incubate overnight at 4oC.
- 11 Rinse in Tris Tween buffer (2× 2 min).
- 12 Rinse in Tris buffer (1× 2 min).



- 13 Apply the secondary antibody (biotinylated SP-conjugated Affinipure goat anti-rabbit IgG (H+L), Jackson, 111-065-144) diluted in Thermo Antibody Diluent to a concentration of 1:500, and incubate for 1 hour at room temperature.
- 14 Rinse in Tris Tween buffer (2× 2 min).
- 15 Rinse in Tris buffer (1× 2 min).
- 16 Apply ABC Elite (Vector, PK-6100) at a concentration of 1:50 for 30 min at room temperature.
- 17 Rinse in Tris Tween buffer (2× 2 min).
- 18 Rinse in Tris buffer (1× 2 min).
- 19 Apply DAB chromogen (Thermo, TA-125-QHDX) for 1 min at room temperature.
- 20 Rinse in deionized water (2× 2 min).
- 21 Counterstain using hematoxylin.
- 22 Dehydrate, clear, and coverslip using DPX mountant.

## Microscopy

- 23 Each sample was imaged at 10x using a Nikon Ti2 microscope with a Photometrics Prime 95B-25MM camera (Nikon Instruments Inc.). We selected the best of four slices for each sample based on the quality of the slice (no tearing or fraying).

## Image Segmentation

- 24 We used Nikon's NIS Elements software (v5.02.01, Build 1270) to segment human vagus nerve immunohistochemical micrographs (anti-claudin-1) using the General Analysis RGB

tool.

- 25 For each image, we selected preprocessing steps, such as smoothing and sharpening.
- 26 For each image, we selected ranges of hues, saturations, and intensities to values that identify the perineurium and different values to identify the entirety of the nerve.
- 27 For each image, we selected postprocessing steps, such as setting a minimum size criterion (eliminate small off-target regions), smoothing, cleaning, closing, and filling holes.
- 28 We made manual adjustments as needed, including manual deletion of off-target regions and filling of target areas that had not been captured.
- 29 We converted the binary segmented image into "Graticule Masks", binary images saved as TIFs.

## Image Analysis

- 30 We imported the TIFs into Matlab and generated a data structure of the x and y coordinates of the pixels for each closed boundary of the loaded binary images using the *bwboundaries* function.
- 31 We stored the pixel coordinates with indexing that assigns a fascicle number, which were then checked so that both the interior and exterior perineurium trace relate to the same fascicle.
- 32 We scaled the pixel coordinates to microns using the segmented scale bar.
- 33 We calculated cross-sectional area of each fascicle (inner perineurium and outer perineurium traces) and nerve using Matlab's *polyarea*. Effective diameter (for a nerve or fascicle) is the diameter of the circle that has the same cross-sectional area as the raw trace. The perineurium thickness is half of the difference in effective diameters of the inner and outer perineurium traces.