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SPARC_Duke_PelotGrill_OT2-OD025340_HumanVagusNerve_Claudin1IHC_Morphology V.4

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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.

Keywords: Vagus nerve, nerve morphology, human vagus nerve, claudin-1, endoneurium, perineurium, epineurium, fascicles, image segmentation

MATERIALS

- Microscope slides with paraffin slices
- Xylene
- Ethanol
- Deionized water
- HIER Buffer L (Thermo, TA-135-HBL)
- H₂O₂
- 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 50°C and then cool overnight.
- 2** Deparaffinize the slides and hydrate them to distilled water: xylene (2x 6 min), 100% ethanol (5 min), 95% ethanol (4 min), 70% ethanol (3 min), deionized water (2x 1 min).
- 3** Perform heat-induced epitope retrieval (HIER) at 120°C for 30 s followed by 90°C 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 (2x 2 min).
- 6 Block with 3% H₂O₂ diluted in deionized water for 10 min.
- 7 Rinse in deionized water (2x 2 min).
- 8 Rinse in Tris buffer (1x 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 4°C.
- 11 Rinse in Tris Tween buffer (2x 2 min).
- 12 Rinse in Tris buffer (1x 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 (2x 2 min).
- 15** Rinse in Tris buffer (1x 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 (2x 2 min).
- 18** Rinse in Tris buffer (1x 2 min).
- 19** Apply DAB chromogen (Thermo, TA-125-QHDX) for 1 min at room temperature.
- 20** Rinse in deionized water (2x 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.