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SPARC_Duke_PelotGrill_OT2-OD025340_PigVagusNerve_FibronectinIF_Morphology

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We use this protocol and it's working

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Disclaimer

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
- Triton X-100 (Fisher Scientific, BP151-500)
- Bovine serum albumin (BSA)
- Rabbit anti-fibronectin (Sigma, F3648)
- Goat polyclonal secondary antibody to rabbit IgG (H+L, FITC) (Abcam, ab97050)
- Fluoro-Gel II with DAPI (EMS, 17985-50)
- Microscope with fluorescence light source, FITC filter, and monochrome camera
- Nikon's NIS Elements
- Matlab

Immunofluorescence

1 Bake slides with sections of paraffin-embedded vagus nerve overnight at 50^{o}C 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 Incubate in 0.1% (v/v) Triton-X in PBS for 20 min.

5 Incubate in 7.5% (w/v) bovine serum albumin (BSA) in PBS for 1 hour.

6 Rinse with 1% (w/v) BSA in PBS.

7 Apply the primary antibody (rabbit anti-fibronectin, Sigma, F3648) diluted in 1% (w/v) BSA in PBS to a concentration of 1:50, and incubate overnight at 4^{o}C.

8 Rinse with PBS (3× 5 min).

9 Apply the secondary antibody (goat polyclonal secondary antibody to rabbit IgG (H+L, FITC) (Abcam, ab97050) diluted in 1% (v/v) BSA in PBS to a concentration of 1:100, and incubate for 1 hour at room temperature.

10 Rinse in PBS (3× 5 min).

11 Coverslip using Fluoro-Gel II with DAPI (EMS, 17985-50).

Microscopy

12 Each sample was imaged at 20x (Plan Apochromat Lambda, NA: 0.75) with a GFP/FITC/cy2 filter set (excitation: 466/40 nm (446-486 nm), emission: 525/50 nm

(500-550 nm), dichroic mirror: 495 nm; Nikon Instruments Inc.), a SOLA SE II 365 light engine (Lumencor, Beaverton, OR), and a Photometrics Prime 95B-25MM camera (Teledyne Photometrics, Tucson, AZ). We selected the best of four slices for each sample based on the quality of the slice (no tearing or fraying).

Image Segmentation

13 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.

14 We used Nikon's NIS Elements software (v5.02.01, Build 1270) to segment the inner and outer perineurium boundaries using the General Analysis tool. We only segmented fascicles with a single inner perineurium boundary for each outer boundary.

15 For each image, we selected preprocessing steps, such as smoothing and sharpening.

16 For each image, we selected fluorescent intensity values to identify the perineurium.

17 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.

18 We made manual adjustments as needed, including manual deletion of off-target regions and filling of target areas that had not been captured.

19 We converted the binary segmented image into “Graticule Masks”, binary images saved as TIFs.

Image Analysis

20 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.

21 We paired the interior and exterior perineurium traces for a given fascicle.

22 We scaled the pixel coordinates to microns using the segmented scale bar.

23 We calculated the effective diameter of each inner perineurium trace and outer perineurium traces using Matlab's polyarea, where effective diameter 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.