



# SPARC\_Duke\_PelotGrill\_OT2-OD025340\_PigVagusNerve\_FibronectinIF\_Morphology

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1 Works for me [dx.doi.org/10.17504/protocols.io.bfwjtjen](https://dx.doi.org/10.17504/protocols.io.bfwjtjen)

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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.

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## KEYWORDS

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

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## MATERIALS TEXT

- 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

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## Immunofluorescence

- 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 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°C.
- 8 Rinse with PBS (3x 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 (3x 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.