Keywords: myenteric plexus, submucosal plexus, immunostain, immunofluorescence, enteric neuron, ileum, macrophage, T cell

Note: The plexuses are fragile. At each wash, use a micropipette and a dissecting microscope to carefully remove the solution from the well not suck up the plexus.

1. Cut out two small regions of each plexus sheet in PBS at 4 degrees
2. Add each tissue section to separate wells in a 96 well dish.
3. Add 10% normal donkey serum (Jackson Immunoresearch, Cat #017-000-121; West Grove, PA) and 1% Triton-X in PBS. Leave in blocking solution for 1 hour at room temperature on a rotator.
4. Incubate the tissue overnight at room temperature on a rotator with primary antibodies in 10% normal donkey serum, 1% Triton-X in PBS.
    a. Sections were stained with antibodies against tyrosine hydroxylase (TH) (1:500, Millipore-Sigma, Cat # AB152; Burlington, MA) and CD3 (1:400, Bio-Rad Laboratories, Cat #MCA2690; Hercules, CA), ANNA1 (1:32,000; kind gift by the Gershon laboratory (Margolis et al., 2016)), Iba1 (1:500, WAKO, Cat # 019-19741; Richmond, VA), CD68 (1:1000, Abcam, Cat # ab53444; Waltham, MA).
5. On Day 2, remove the primary antibody solution from each well and wash three times with PBS-Tween (0.1%) for 10 minutes each.
6. Incubate in secondary antibody (1:1000) in blocking solution for two hours at room temperature, covered on a rotator.
7. Wash again three times for 10 minutes each in 0.1% PBS-Tween
8. Mount on slide with vectashield medium with DAPI
9. Imaging:

a. For imaging enteric neurons, for each plexus collect 2-3, 2x2 tile z-stack 640.17x 640.17 μm confocal images at 20x magnification
b. For imaging macrophages, for each plexus collect 2-3, 2x2 tile z-stack 390.09 x 390.09 μm confocal images at 20x magnification

10. Analysis:
    a. Count the number of ANNA1$^+$, TH$^+$ cells, and IBA1$^+$ cells were counted for each stacked image using Fiji
    b. Within the SP, threshold the TH$^+$ signal, then analyze mean fluorescent intensity (MFI) and the area of the TH signal. Keep the thresholding consistent across each image, animal, and condition within each experiment
    c. Within each animal, sum the the number of ANNA1$^+$, TH$^+$ cells, and IBA1$^+$ cells across all images separately then divide by the acquisition area.
    d. For each experiment, normalize to the CFA only condition.