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Tissue preparation, immunohistochemistry, Imaging, and Quantification

Bryan Yoo[1], Jessica Griffiths[1], Sarkis Mazmanian[1]

[1]California Institute of Technology

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Mazmanian Lab

Jessica Griffiths

Protocol for neuronal imaging used in Yoo et al 2021

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Tissue Preparation

1 100mg/kg of pentobarbital (Euthasol - Virbac, Carros, France) was administered intraperitoneally (IP), and tissues were perfused with 30mL of phosphate buffer solution (PBS) and next with cold 4% paraformaldehyde (PFA) in PBS.

2 GI tract was post-fixed in 4% PFA overnight at 4°C, and stored in PBS + 0.025% sodium azide.

immunohistochemistry

3 Tissues that underwent subsequent immunohistochemistry were made transparent by use of the passive CLARITY technique (PACT) (**Treweek et al., 2015**).

4 Briefly, perfused and fixed tissues were embedded with polymerized 4% (wt/vol) acrylamide, and lipids were eliminated using 8% (wt/vol) SDS solution.

5 Tissues were blocked in 3% donkey serum and permeabilized with PBS + 0.3% Triton (PBST).

6 Primary antibodies were incubated in PBST for 48 hours and washed with PBST for 24 hours (replacing wash 3 times). Primary antibodies used were rabbit PGP9.5 (1:300; Millipore Sigma AB1761-I) and chicken GFAP (1:500, BioLegend Cat #829401).

7 Tissues were next incubated in secondary antibodies (and DAPI) for 24 hours and washed in PBS for 48 hours, intermittently replacing the wash solution with fresh PBS. Secondary antibodies used were goat anti-chicken Alexa 647 (Life Technologies A-21450) and donkey anti-rabbit Alexa 568 (Life Technologies A10042).

Imaging

8 Tissues imaged just for virally expressed, endogenous fluorescence were made transparent using sorbitol-based optical clearing method, Sca*l*eS (**Hama et al., 2015**).

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9 Tissues were mounted in respective mounting mediums (RIMS and Scales S4), on a glass slide with a 0.5mm spacer (iSpacer, SunJin Lab Co.).

10 Images were acquired on Zeiss LSM 780 or 880, and microscope, laser settings, contrast, and gamma remained constant across images that were directly compared.

11 All confocal images were taken with the following objectives: Fluar 5× 0.25 M27 Plan-Apochromat 10× 0.45 M27 (working distance 2.0 mm) and Plan-Apochromat 25× 0.8 Imm Corr DIC M27 multiimmersion.

Quantification

12 Neurons in each ganglion were counted by counting cells that are of a distinct color. Large intestinal ganglia were defined as distinct if separated by a width of 3 or fewer neurons.

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