**Immunofluorescent staining for human enteric nervous system:**

The goal of our Pilot and Feasibility award from Diacomp was to perform intermittent H&E staining and immunostaining with antibodies against neuronal markers of serial FFPE (Formalin Fixed Paraffin Embedded) sections of human gastric tissues, such that by using AI-based methods, we would be able to create 3D reconstruction of the enteric nervous system and associated cells. This technique would allow for quantification of disease-related changes in the human gastric tissues in 3D as compared to in 2 dimensions of thin sections of tissue.

Our work that was made possible through this grant identified key issues with the current modalities of performing immunohistochemistry for ENS and solved them. We also performed a proof-of-concept CODA-based reconstruction of H&E images to show that our technique worked. However, we were unable to gain access to enough tissue sections from gastroparesis patients, despite our best efforts and continued approaches to GPCRC team members, which did not allow us to test our technique on gastroparesis tissues. Nevertheless, we made significant progress towards providing the first FFPE based immunofluorescence interrogation of human ENS tissue, from gastric, small intestinal, and colonic specimens. Our main results are mentioned below:

1. **Establishing the protocol for immunofluorescent staining of the human ENS in FFPE tissue sections.** We found out that currently there are no standardized protocols for interrogation of human ENS in FFPE tissue sections. Our work had previously performed immunostaining on free floating fixed full thickness human myenteric plexus layer, as well as on cryosections. However, unlike FFPE, these do not require antigen retrieval protocols. Current pathology for human ENS is based on non-specific markers such as S100, and not on markers that can label neuronal cell bodies, neural fibers, as well as identify important and novel cell populations within the ENS. For this, we performed various permutations and combinations of various antigen-retrival and immunostaining protocols for FFPE human tissues and finally we established a technique that works for most of the antibodies. The technique is detailed below:

a. Deparaffinize and rehydrate the slides containing the FFPE tissue sections by immersion through the following solutions: 1) 3 washes (5 minutes each) with Xylene; 2) 2 washes with 100% ethanol (10 minutes each); 3) 2 washes with 95% ethanol (10 minutes each); 4) 2 washes with 70% ethanol (10 minutes each); 5) 2 washes with 50% ethanol (10 minutes each); and finally 6) 2 washes with deionized water (5 minutes each).
b. Next, using pre-heated EDTA buffer (at 90°C, pH 9.0; 5 mL of 20x EDTA stock solution in 85 ml of deionized water), perform antigen retrieval for 30-minute antigen retrieval in a vegetable steamer for humidified conditions. Care should be taken not to allow the steam to wane or stop.
c. The slides were then cooled in room-temperature PBS. Slides were marked with a hydrophobic pen around the sections, and then blocked and permeabilized in blocking and permeabilization buffer (BPB; 5% normal goat serum, 0.5% triton X in PBS) for 1 hour.
d. Buffer was washed off and sections were incubated with the combination of requisite primary antibodies. Details of various antibodies that worked are provided in Table 1. Slides are next incubated for 24 h at 16°C in the dark.
e. Following incubation with primary antibody, the sections will be washed three times for 15-minutes each in PBS at room temperature in the dark.
f. The slides will then be incubated with the appropriate secondary antibodies (Concentrations of antibodies provided in Table 1) at room temperature for 1 hour.
g. The slides will again be washed three times in PBS at room temperature, counterstained with DAPI to stain the nuclei, overlaid with Prolong Antifade Gold mounting medium, cover-slipped, and imaged.

Some of these representative images are shown below:

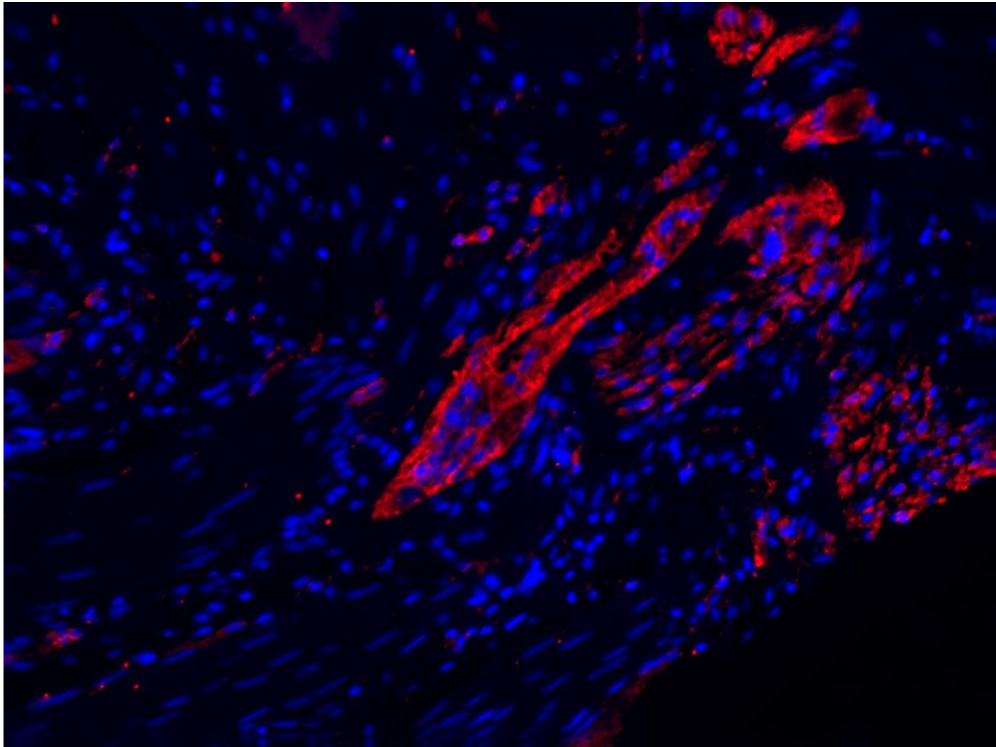


**Fig 1.** FFPE section of Human gastric tissue stained with antibodies against the pan-neuronal marker SNAP-25 (red), and counterstained with nuclear dye (DAPI), and imaged at 20X magnification using confocal microscope.

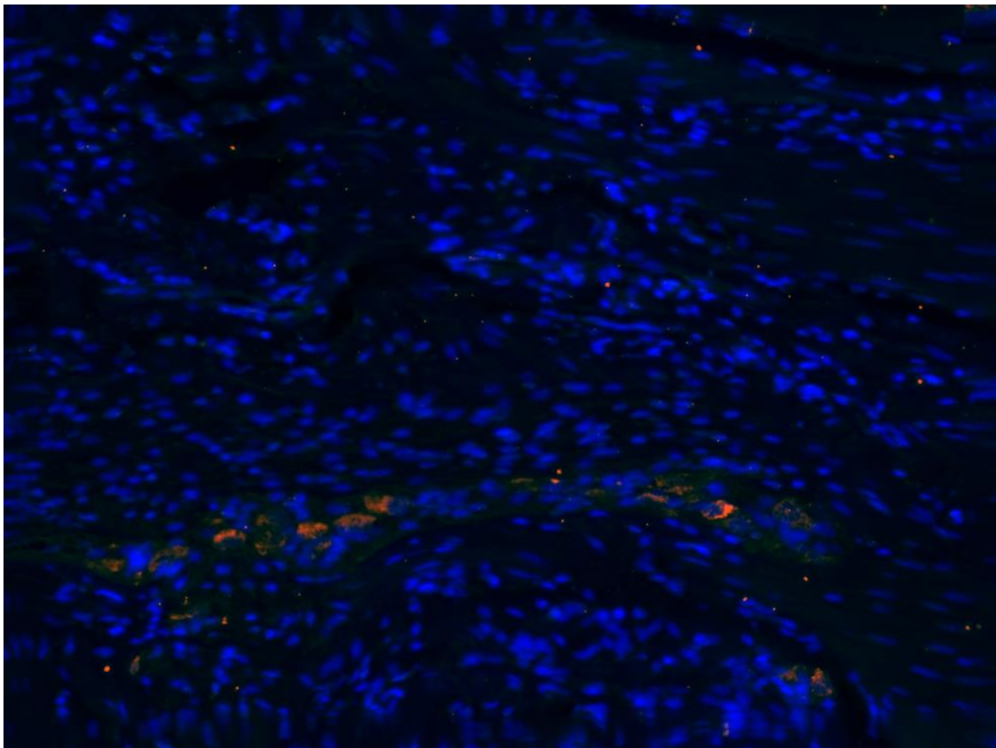


**Fig 2.** FFPE section of Human gastric tissue stained with antibodies against the pan-neuronal marker SLC17A9 (red), and counterstained with nuclear dye (DAPI), and imaged at 20X magnification using confocal microscope.

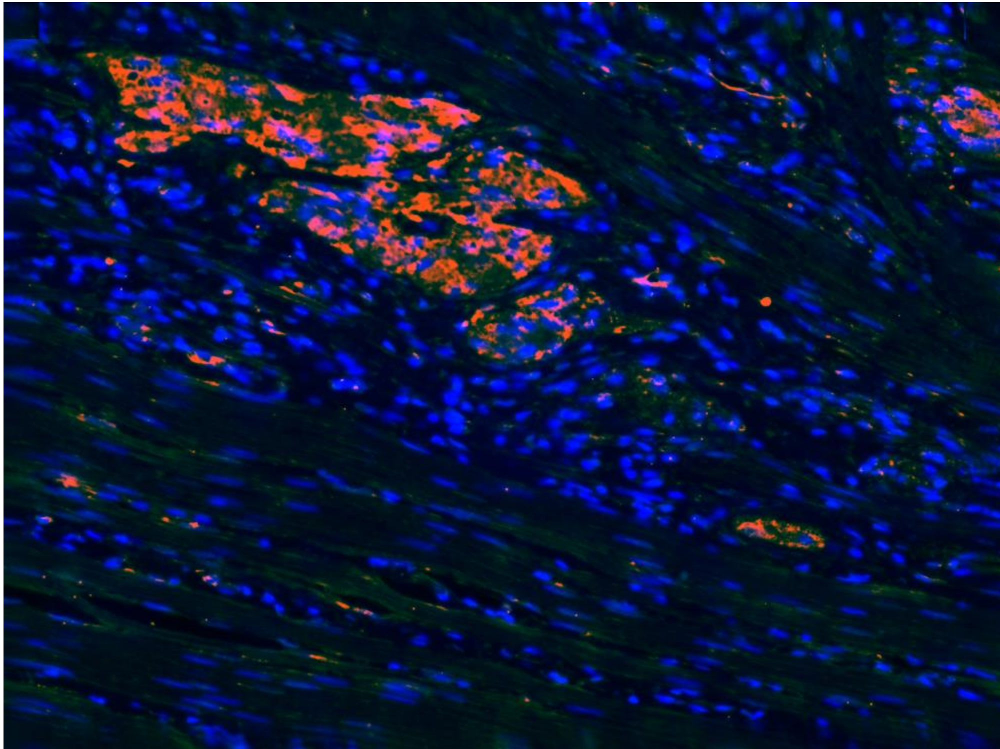


**Fig 3.** FFPE section of Human gastric tissue stained with antibodies against the pan-neuronal marker PDE10A (red), and counterstained with nuclear dye (DAPI), and imaged at 20X magnification using confocal microscope.

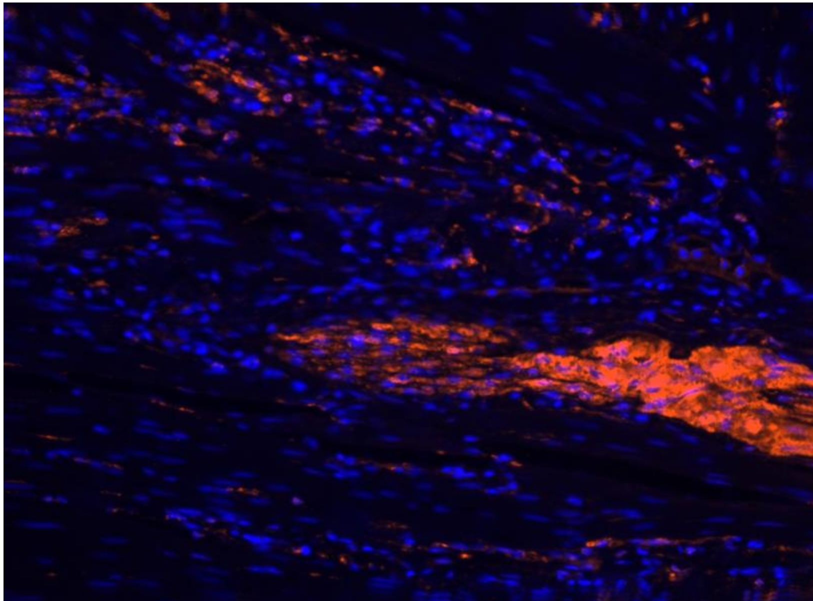


**Fig 4.** FFPE section of Human gastric tissue stained with antibodies against the pan-neuronal marker PGP9.5 (red), and counterstained with nuclear dye (DAPI), and imaged at 20X magnification using confocal microscope.

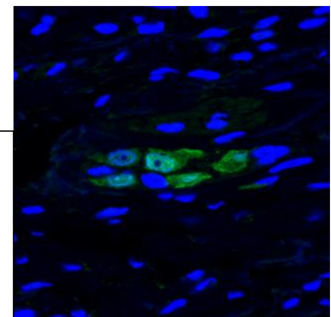
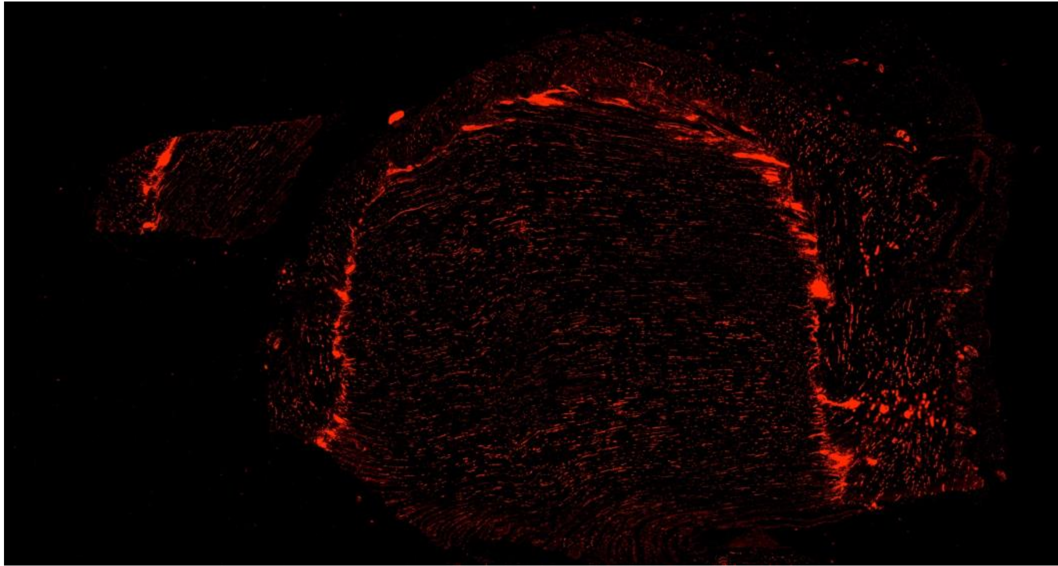
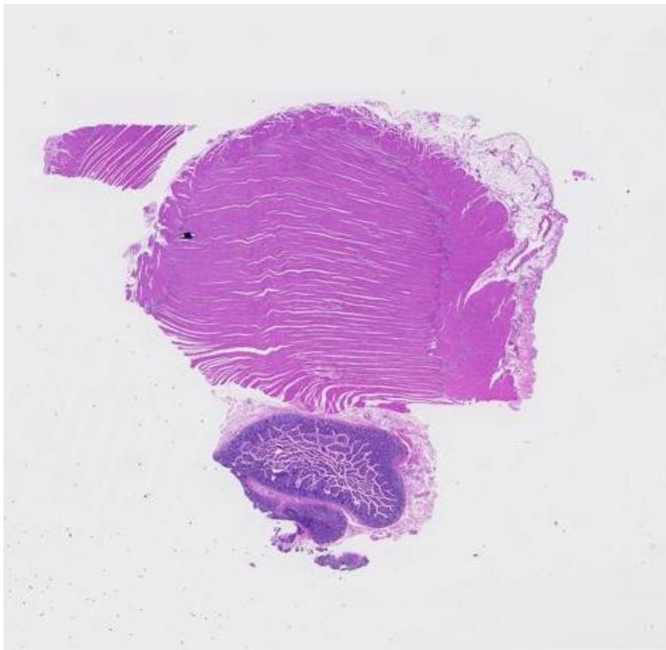


**Fig 5.** FFPE section of Human gastric tissue stained with antibodies against the human ANNA-1 antisera (green), and counterstained with nuclear dye (DAPI), and imaged at 20X magnification using confocal microscope.

2. **Imaging the immunostained tissue section:** The next part of our protocol required us to perform section wide imaging to ensure that the entire stained section is imaged at a 20X magnification, which would allow for assessment of an individual neuron in the tissue, as well as the ability to stitch all the component images into one super image – which can then be fed into the CODA software for 3D reconstruction. Here, we show a stitched image, generated from ~550 individual images of contiguous 20X fields of human gastric tissue, stained with antibodies against the pan-neuronal marker PGP9.5 (red).





3. **Performing CODA-based 3D reconstruction of human gut tissues** We next performed CODA based alignment of H&E stained gut tissues to further show that our technique of serial section-based 2D-to-3D reconstruction works. We show here a video of this process.

Unfortunately, due to a paucity of available tissues through the GPCRC, we were not able to complete the last

step of our goals. However, these results show that we have made the first foray into establishing important antibodies for immunostaining of the human gut tissues, and have used CODA to perform 3D reconstruction of thin 2D sections of human gut.

***Table 1.***

| Antibody | Best concentration | Worked? | Company |
|---|---|---|---|
| ANNA-1 antisera | 1:500 | Yes | Human antisera |
| Anti-Hu | 1:500 | Yes | Abcam |
| Anti-phosphor-Histone H3 | 1:250 | Yes | Sigma |
| Anti-Cleaved Caspase 3 | 1:250 | Yes | Cell Signalling |
| Anti-Decorin | 1:100 | Yes | DSHB |
| Anti-Slc17a9 | 1:100 | Yes | Proteintech |
| Anti-Gephyrin | 1:100 | Yes | Proteintech |
| Anti-Pde10a | 1:100 | Yes | Invitrogen |
| Anti-SNAP25 | 1:100 | Yes | Proteintech |
| Anti-PGP9.5 | 1:250 | Yes | Abcam |
| Anti-Cadherin 3 | 1:250 | Yes | Abcam |
| Anti-NOS1 | N/A | No | Invitrogen |
| Anti-Rabbit 488 | 1:500 | Yes | Invitrogen |
| Anti-human 647 | 1:500 | Yes | Invitrogen |
| Anti-mouse 647 | 1:500 | Yes | Invitrogen |

For this, we purchased the Life Technologies M7000 microscope with a motorized stage and an inbuilt software, which allows for ‘stitching’ individual images of contiguous fields of tissue. We used the microscope to image contiguous 20X fields of the stained section, and then stitched