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Video Article

Isolation of Enteric Glial Cells from the Submucosa and Lamina Propria of the Adult Mouse

Zhen Wang^{1,4}, Ramon Ocadiz-Ruiz¹, Sinju Sundaresan¹, Lin Ding¹, Michael Hayes¹, Nirakar Sahoo³, Haoxing Xu^{1,2}, Juanita L. Merchant^{1,2,5}¹Department of Internal Medicine-Gastroenterology, University of Michigan²Department of Molecular and Integrative Physiology, University of Michigan³Department of Molecular, Cellular and Developmental Biology, University of Michigan⁴Department of Gastrointestinal Surgery, The First Affiliated Hospital of Guangxi Medical University⁵Division of Gastroenterology, University of Arizona College of MedicineCorrespondence to: Juanita L. Merchant at jmerchant@deptofmed.arizona.eduURL: <https://www.jove.com/video/57629>DOI: [doi:10.3791/57629](https://doi.org/10.3791/57629)Keywords: Biology, Issue 138, duodenum, hGFAP-Cre, tdTomato, EDTA chelation, EGCs, Ca²⁺ flux, Sox10, GFAP

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Abstract

The enteric nervous system (ENS) consists of neurons and enteric glial cells (EGCs) that reside within the smooth muscle wall, submucosa and lamina propria. EGCs play important roles in gut homeostasis through the release of various trophic factors and contribute to the integrity of the epithelial barrier. Most studies of primary enteric glial cultures use cells isolated from the myenteric plexus after enzymatic dissociation. Here, a non-enzymatic method to isolate and culture EGCs from the intestinal submucosa and lamina propria is described. After manual removal of the longitudinal muscle layer, EGCs were liberated from the lamina propria and submucosa using sequential HEPES-buffered EDTA incubations followed by incubation in commercially available non-enzymatic cell recovery solution. The EDTA incubations were sufficient to strip most of the epithelial mucosa from the lamina propria, allowing the cell recovery solution to liberate the submucosal EGCs. Any residual lamina propria and smooth muscle was discarded along with the myenteric glia. EGCs were easily identified by their ability to express glial fibrillary acidic protein (GFAP). Only about 50% of the cell suspension contained GFAP+ cells after completing tissue incubations and prior to plating on the poly-D-lysine/laminin substrate. However, after 3 days of culturing the cells in glial cell-derived neurotrophic factor (GDNF)-containing culture media, the cell population adhering to the substrate-coated plates comprised of >95% enteric glia. We created a hybrid mouse line by breeding a hGFAP-Cre mouse to the ROSA-tdTomato reporter line to track the percentage of GFAP+ cells using endogenous cell fluorescence. Thus, non-myenteric enteric glia can be isolated by non-enzymatic methods and cultured for at least 5 days.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57629/>

Introduction

Interest in the function of enteric glial cells (EGCs) has steadily increased due to their recognized roles in the gut integrity and homeostasis^{1,2}. In addition, EGCs vary according to their location along the length of the GI tract^{3,4}. EGCs release various trophic factors including glial cell-derived neurotrophic factor (GDNF), contribute to gut motility^{1,5} and respond to microbial byproducts^{6,7}. Studies have indicated that the EGC population is heterogeneous and that their function varies depending on whether they are submucosal or reside within the myenteric plexus^{1,7}. For example, EGCs within the submucosa contribute to tight junctions⁸. Differential GFAP expression and phosphorylation in EGCs have been linked to Parkinson's Disease, suggesting their possible link to the gut phenotype of this disorder⁹. Recently, it was observed that the loss of the nuclear protein menin in isolated cultures of EGCs from the proximal intestinal submucosa was sufficient to induce expression of the hormone gastrin¹⁰. As a result, it was proposed that EGCs might be the origin of duodenal gastrinomas, a type of neuroendocrine tumor¹⁰. Collectively, these examples underscore the relevance of studying the behavior and function of isolated EGCs in neuropathic disorders and cancer¹¹.

The challenge in the field remains how to isolate and study either or both EGC populations *in vitro*. Lineage trace experiments demonstrated that EGCs in the submucosa and lamina propria originate from progenitor cells in the myenteric plexus⁷. Although there are several published isolation protocols available to generate cultures of myenteric EGCs^{12,13,14,15,16,17,18,19}, none specifically targets isolation of the submucosal/lamina propria EGC population. Existing protocols for EGC isolation specifically use a combination of the mechanical separation or the microdissection of the smooth muscle combined with enzymatic dissociation, eventually discarding the mucosal cell layer.

The goal of this manuscript is to demonstrate the steps to non-enzymatically isolate primary EGCs from the lamina propria for *in vitro* studies. Since there are no markers that specifically distinguish myenteric EGCs from those in the submucosa, the spatial separation of the epithelial mucosa from the smooth muscle was exploited to isolate submucosal EGCs. In addition, by combining EDTA chelation with non-enzymatic dissociation, EGCs were isolated from the submucosa in contrast to the smooth muscle, which was discarded along with the associated inter-

myenteric EGCs. Further separation of the submucosa and lamina propria EGCs occurred by culturing the cells on glial cell-friendly substrates, e.g., poly-D-lysine and laminin.

Protocol

All animal experiments described were approved by the University of Michigan's Committee on the Use and Care of Animals.

1. Preparation of Sterile Poly-D-lysine (PDL) and Laminin Solutions

- At least one day prior to cell isolation, prepare poly-D-lysine (PDL) and laminin coated plates.
NOTE: Both 6-well and 12-well plates were prepared depending on the experimental goals. Typically, 12-well plates were used for the quantitative analysis using western blots; whereas, 6-well plates were used to hold autoclaved (sterile) coverslips for immunohistochemistry. 24-well plates were used to culture EGCs for Ca^{2+} flux imaging.
- Dilute stock solutions with ultrapure tissue culture grade, DNase-free and RNase-free water in a tissue culture hood with HEPA-filtered air.
- Sterilizing glass coverslips**
 - Hold the coverslip with sterile forceps and immerse coverslips in a beaker of 100% ethanol for 15 min. Allow the ethanol to evaporate in the tissue culture hood and then place in the 6-well plates.
 - Alternatively, place several coverslips individually on 2 mm thick blotting paper in a plastic container and then autoclave.

2. Coating Plates

NOTE: perform these steps under a sterile tissue culture laminar flow hood.

- Thaw 1 mg/mL PDL stock and dilute 1:10 with sterile, tissue culture grade water so that the final concentration is 100 μ g/mL. Use 2 mL per well to coat 6-well plates, 1 mL to cover one well of a 12-well plate and 0.5 mL for one well in the 24-well plates. Store the remaining diluted PDL in 12 mL aliquots at -20 °C.
- After allowing the PDL to coat the wells for at least 1 h, remove the PDL with a sterile pipette. Allow the plates to air dry completely under a tissue culture laminar flow hood.
NOTE: After removing the PDL solution with a sterile pipette, it can be used twice more within 1 week after passing through a 0.22 μ m filter and storing at 4 °C.
- Thaw the laminin stock on ice to avoid gel formation.
NOTE: Undiluted laminin becomes solid when warmed above 8 °C.
- Dilute the laminin stock (0.5 mg/mL) 1:50 with sterile Dulbecco's Phosphate Buffered Saline (DPBS) to obtain a final concentration of 10 μ g/mL. Store the diluted laminin in 12 mL aliquots at -20 °C.
- Add diluted laminin to the wells containing dried PDL. Use 1 mL to cover the 6-well surface and 0.5 mL to cover the bottom of the 12-well plates. For coverslips, add 400 μ L of diluted laminin to the coverslips to achieve surface coverage.
- Incubate the coated plates at 37 °C for 2 h, if the plate is used the same day (quick coating). If not, seal the plates with plastic wrap, and store overnight at 4 °C (slow coating).
NOTE: Sealing is critical to avoid drying and contamination.
- Remove the laminin solution carefully with a pipette to avoid scratching the surface. Gently wash the wells three times with sterile DPBS.
- Add complete glial growth media to each well and maintain the plates at 37 °C until ready to add the EGC suspension.
NOTE: Poly-D lysine and laminin coated plates can be stored for 3 days at 4 °C. Coat the plates several days ahead and then store at 4 °C, wash the plates with sterile DPBS three times. Add 2 mL of sterile DPBS to each well after the final wash. Seal the plate with parafilm and store at 4 °C. It is essential to ensure that the laminin does not dry out.

3. Preparation of Isolation Solutions

- Prepare the EDTA/HEPES/DPBS dissociation solution. For 500 mL of the solution, use sterile DPBS (without calcium and magnesium) to make a final solution of 10 mM HEPES and 5 mM EDTA. To 490 mL of the DPBS, add 5 mL of 1 M HEPES buffer and 5 mL of 0.5 M EDTA stock solution. Store at 4 °C until use.
- Prepare the glial cell resuspension media using the reagents listed in **Table 1**.
- Prepare glial growth media using the reagents listed in **Table 2**.
NOTE: Glial growth media containing GDNF can be stored for one week at 4 °C.

4. Removal of the Intestinal Segment

NOTE: Mice were allowed free access to the food and water prior to euthanization by the isoflurane drop method and removal of a vital organ. C57BL/6 mice greater than 8 weeks of age were used. Both genders were used without noticeable differences in the preparation.

- Euthanize the mouse with an overdose of isoflurane and place supine in a dissecting tray. Pin the extremities and sterilize the abdomen with 70% ethanol. Tent the skin with forceps and then open the abdomen with scissors.
- Lift the liver and identify the distal stomach/pylorus. Then remove 7 cm of the proximal intestine. Be careful not to tear the intestine.
- Hold the pylorus with forceps while snipping away the mesentery.**
 - Use blunt dissection with the back of the scissor to scrape away adherent pancreas.
 - Place the intestinal segment into ice-cold DPBS without Ca^{2+} or Mg^{2+} (**Figure 1A**).

NOTE: Other segments of the intestine or colon can also be removed using the same technique.

4. Use a 5 or 10 mL syringe attached to a blunt end 20 G needle to flush out the fecal contents with ice cold DPBS (**Figure 1B**).
5. Divide the intestine into 3 cm segments to remove the longitudinal muscle using the technique initially described by Smith *et al.*¹² and as modified in Sundaresan *et al.*¹⁰.
NOTE: Intestinal segments from up to two mice are used per 30 mL of the HEPES/EDTA/DPBS isolation solution.
6. Break off about 4 cm to separate the wooden stick from the cotton tip. Soak the wooden stick in DPBS (**Figure 1C**).
7. Slip one of the intestinal segments smoothly onto the wetted stick (**Figure 2A-C**).
8. Remove adherent mesentery still attached using needle-nose forceps (**Figure 2D**).
9. **Use a clean razor blade or scalpel to make two longitudinal nicks along the intestine where the mesentery was attached (Figure 2E).**
 1. Wet the cotton tip with DPBS. Rub the wetted cotton tip longitudinally along the muscle to loosen the longitudinal muscle/myenteric plexus (LMMP). Then move the cotton tip horizontally to tease away the LMMP from the circular muscle along the length of the intestinal segment during LMMP removal.
 2. Hold the tip of the stick between the thumb and forefinger while stabilizing the segment with the middle and fourth finger (**Figure 2F**). When complete, the LMMP will easily peel off the intestinal segment.
10. Discard the LMMP stripped from the intestinal tube and attached to the cotton swab unless harvesting EGCs from the myenteric plexus is desired.
11. Use a razor blade to flay open the intestinal segment longitudinally to remove from the wooden stick.
12. Store the stripped intestinal tissue (predominantly mucosa and submucosa plus circular muscle) in DPBS on ice. Repeat steps 4.4–4.11 until all intestinal segments are prepared.

5. Removal of the Epithelial Mucosa

1. Cut the intestines into smaller pieces of ~0.5 cm with fine scissors.
2. Place the tissue in a sterile 50 mL conical centrifuge tube containing 30 mL of ice-cold EDTA/HEPES/DPBS solution.
3. Rock the tissue-containing solution at 4 °C for 10 min at ~60 tilts per min.
4. Pre-moisten a 5 mL plastic pipette by pipetting the EDTA/HEPES/DPBS buffer up and down one time, and then use the pre-moistened pipette to pipette the tissue and buffer suspension up and down (trituration) 20 times to dislodge the epithelial mucosa.
5. Collect the tissue from the EDTA incubation by pouring the mixture through a 100 µm nylon cell strainer. Discard the turbid mucous-filled flow-through (**Figure 3**).
6. Place the tissue retained in the strainer into a new 50 mL conical centrifuge tube containing 30 mL of ice cold EDTA/HEPES/DPBS using needle-nose forceps.
7. Repeat the EDTA/HEPES/DPBS incubation a second time by rocking the tissue at 4 °C for 10 min at ~60 tilts per min to strip off additional epithelial mucosa .
8. Pre-moisten a 5 mL pipette with the buffer and then pipette the tissue suspension up and down 20 times to dislodge the mucous cells.
NOTE: The tissue will tend to stick to inside of the pipette as more of the epithelium is removed.
9. Collect the tissue after the second incubation by pouring the mixture through a 100 µm nylon cell strainer and discard the flow through. The second flow-through should be noticeably less turbid than the first (**Figure 3**).
10. Repeat the 10 min EDTA incubations until the solution is nearly clear (about 3 to 4 incubations depending on the amount of tissue and the effectiveness of the trituration) (**Figure 3**).

6. Collection of Enteric Glial Cells from the Lamina Propria and Submucosa

1. Transfer the tissue from the nylon strainer to a 15 mL conical centrifuge tube containing 5 mL of the commercially available cell recovery solution and rock for 25–30 min at 4 °C.
2. **Triturate gently 10x to dissociate the enteric glial cells from the lamina propria.**
 1. Filter through a 40 µm filter and collect the filtrate in a clean 50 mL tube.
 2. Rinse the tissue on the nylon filter with 1 mL of DPBS.
 3. Discard the tissue and transfer the ~5–6 mL of filtrate to a clean 15 mL conical centrifuge tube.
 4. Spin the filtrate at 2,000 \times g in a swinging bucket centrifuge for 5 min at 4 °C.
3. Re-suspend the glial cell-containing pellet in at least 1 mL of resuspension buffer by gently pipetting the pellet up and down with a 500 µL pipette tip. Avoid introducing bubbles.
NOTE: Adjust the resuspension volume as needed for the number of wells and plates. For example, 1.2 mL for one 6-well plate; 2.4 mL for two 6-well plates; 2.4 mL for one 12-well plate.
4. Pipette 200 µL of the cell suspension into each well of the 6-well or 100 µL for a 12-well PDL-laminin coated plates containing glial growth media.
5. Do not disturb the dishes after adding the cells to the plates and placing in the 37 °C incubator to allow the maximum number of cells to adhere.
NOTE: A healthy preparation of cells will attach within 6–8 h but wait for at least 24 h before changing the media by gently pipetting off the media along with non-adherent cells.
6. Use the cells for experiments 3–4 days after they are placed into culture (**Figure 4**). Perform immunofluorescent analysis using antibodies of interest to specific protein markers (**Table 3**). For example, GFAP, S100b and p75NTR or Sox10 was used here. E-cadherin or alpha smooth muscle actin antibodies were used to assess the degree of contamination from other cell types.

7. Immunofluorescent Staining

1. Aspirate the media off the cultures and rinse twice with PBS.
2. Fix the cultures for 20 min at room temperature with 4% paraformaldehyde.
3. Remove the fixative and then rinse 3 times with PBS.
4. Permeabilize the cells with PBS containing 0.2% Triton X-100 at room temperature.
5. Incubate the permeabilized cells with blocking solutions comprised of anti-chicken IgY, anti-rabbit or anti-mouse IgG for 2 h at room temperature.
6. **Incubate the cells with primary antibodies overnight, e.g., GFAP (1:1,000).**
 1. Rinse the cells 3 times with PBS. If performing colocalization proceed to Step 7.7.
7. Incubate the next set of primary antibodies for at least 2 h at room temperature, but preferably overnight at 4 °C. Use a 1:1000 dilution of rabbit anti-S100 or a 1:500 dilution of mouse anti-p75NTR.
NOTE: All antibodies are diluted in PBS.
8. Rinse the cells 3 times with PBS to remove primary antibodies. Then incubate the rinsed cells with the appropriate fluorescently-tagged secondary antibodies for 2 h at room temperature (**Table 3**).
9. Rinse 3 times with PBS to remove the secondary antibodies.
10. Mount the coverslips onto slides using 1–2 drops of the mounting media with DAPI and view the cells under a fluorescent microscope.

8. Flow Cytometry

1. Remove adherent glial cells for flow cytometry by rinsing the cells once with DPBS and then add 0.25% trypsin-EDTA per manufacturer's protocol. Incubate the cells in the trypsin-EDTA solution at 37 °C for 3 min. Terminate the digestion by collecting the cells in complete glial cell growth media.
2. Collect the cells by centrifuging at 400 x g for 5 min, and then re-suspend the cells in PBS.
3. Fix the cells with 4% paraformaldehyde for 10 min and then permeabilize with 0.2% Triton X-100 in PBS as described in Step 7.4.
4. Block the cells with PBS containing 1% BSA and the tissue specific immunoglobulin for 20 min, e.g., donkey anti-chicken IgY (IgG) (H+L) (1:1,000) or donkey anti-goat IgG (H+L) (1:1,000).
5. Incubate cells with the primary antibodies GFAP (1:2,000), E-cadherin (1:400), α -smooth muscle actin (1:500), or Pgp9.5 (1:500) overnight at 4 °C.
6. **Wash away the antibody with PBS.**
 1. Incubate the antigen-antibody complex with fluorescently-tagged secondary antibodies incubated at room temperature for 30 min.
 2. Use a separate aliquot of cells incubated with secondary antibody to serve as the isotype control. Both populations of cells are washed and resuspended in PBS prior to flow cytometry.
 3. Analyze the two populations of cells on a flow cytometer by gating on the live cells.

9. Preparing Mouse Tissues from the hGFAP-Cre:tdTomato Mice

NOTE: The Lox-STOP-Lox-tdTomato cDNA is expressed from the ROSA locus^{22,23} (Jackson Labs, #007914) and generates endogenous fluorescence when bred to a mouse expressing the Cre recombinase (hGFAP-Cre). *In situ* analysis is performed by generating frozen tissue sections.

1. Fix intestinal tissue in 4% paraformaldehyde for 1 h and then dehydrate overnight in 1 M sucrose in PBS.
2. Embed the tissue in commercially purchased optimal cutting tissue (OCT) compound comprised of 10% polyvinyl alcohol and 4.2% polyethylene glycol, and then snap freeze in liquid nitrogen.
3. Prepare 5 μ m cryosections and then mount using an antifade mounting media with DAPI.
4. For flow cytometry, prepare EGCs from the hGFAP-Cre:tdTomato+ mice as described in Steps 4.1–6.5.
5. Trypsinize the cells from the plate after 3 days in cultures as in Step 8.1 and then fix for 10 min in 4% paraformaldehyde.
6. Analyze cells isolated from Cre Negative mice in parallel with cells isolated from the hGFAP-Cre:tdTomato+ mice on a flow cytometer.
NOTE: Gates were constructed to avoid dead cells and debris.

10. Ca²⁺ Flux Imaging

1. Incubate EGCs plated on a 24-well plate with 3 μ M Fluo-4-AM at 37 °C for 30 min.
2. Image the Ca²⁺ signal in commercially purchased Tyrode's solution using a spinning disk confocal microscope and an excitation wavelength of 480 nm (F480) after adding CCK or gastrin peptide. Image analysis was reported in Sundaresan *et al.*¹⁰.

Representative Results

Preps were considered unsuccessful if GFAP+ cells did not adhere and spread within 24 h (**Figure 4A**). The number of glial cells could not be determined until after 24 h when the cells adhered and showed evidence of spreading into flat aggregates (**Figure 4B**). Cells at the edge of the clusters tended to extend long processes and expressed classic glial markers, e.g., GFAP, S100b and p75^{NTR} (**Figure 4C, 4D**)^{10,16}. By the 2nd day, glial cells tightly adhered to the surface allowing the non-adherent population to be rinsed away with PBS. Most of the floating cells were epithelial and lamina propria cells along with cell debris. The presence of these floating cell clumps reduced the yield of adherent glial cells underscoring the importance of performing the EDTA incubations until the flow-through is nearly clear, signaling that the epithelial cells have been removed from the lamina propria core. In addition, centrifugation at speeds lower than 1,500 x g after incubating in the cell recovery solution did not effectively collect all of the viable cells into a pellet leading to reduced yields. Typically, 40,000 to 100,000 cells morphologically consistent with EGCs adhered firmly by day 3 in culture.

Immunohistochemical analysis with glial-associated antibodies, indicated that the cell clusters that remained adherent by day 3 were GFAP, S100b, and Sox 10 positive^{10,16} (**Figure 5A**). In the current study, this same degree of purity was observed by permeabilizing the cells and analyzing by flow cytometry (**Figure 5B-F**). Immediately after isolating the submucosal/Lamina propria glial cells, flow cytometry revealed that about 51% of the cells were GFAP+ (**Figure 5B**), suggesting the presence of GFAP-negative cell types, e.g., epithelial, hematopoietic, endothelial, neuronal cells, myofibroblasts known to exist in the epithelium and lamina propria. After 3 days in culture, the number of GFAP+ cells represented over 95% of the cell population analyzed after removing the original media, gently rinsing with PBS and then adding fresh media (**Figure 5C**). Interestingly, flow analysis revealed high and low GFAP protein-expressing populations (**Figure 5C**). Indeed, immunofluorescent analysis of the cells revealed that the periphery of the clusters tended to express higher levels of GFAP (**Figure 4C, 4D**). Taken together, the two types of analysis might indicate differences in EGC maturity or differentiation status. Collectively, the percentage of α -SMA+ (myofibroblast cell marker), E-cadherin+ (epithelial cell marker), and Pgp 9.5+ (neuronal cell marker) cells was less than 5% (**Figure 5D-F**). These results were consistent with the prior study performed using immunofluorescent labeling of EGCs showing about 93% GFAP+ cells¹⁰. The flow analysis underscores the importance of allowing the cells to adhere to the plates since it allows removal of contaminating cell populations comprising approximately 5% of the cultures.

A goal in this study was to use a GFAP-activated reporter to facilitate flow cytometry of the cells for further analysis and to compare the degree of EGC enrichment using an endogenous fluorescent marker (**Figure 6**). The hGFAP-Cre mouse line was originally described by Messing and coworkers²⁰. In brief, the Cre recombinase was placed under the control of 2.2 kb of the human glial fibrillary acid protein (hGFAP) promoter. Thus, any cell transcribing this hGFAP promoter expressed the red fluorescent reporter tdTomato. The cell population labeled with the tdTomato reporter was visualized *in situ* using frozen sections of the intestine and colon (**Figure 6A, 6B**) before performing the EGC isolation procedures described above. After performing the EDTA/cell recovery isolation and culturing the cells for 3 days, EGCs from these mice were easily identified by their endogenous fluorescence (**Figure 6C**). Although GFAP+ cells arguably are more numerous in the proximal intestine^{4,21}, tdTomato+ EGCs were also observed in the colon (**Figure 6B**). Using the tdTomato⁺ fluorescent reporter activated by the hGFAP-Cre also revealed a bimodal population of hGFAP-tdTomato+ cells (**Figure 6D**) as observed using the immunofluorescent analysis of GFAP protein (**Figure 5C**). Although it has been reported that not all EGCs express GFAP protein⁴, this point might reflect differences in the level of GFAP expression. On average, about 66% of the cells analyzed exhibited the highest level of tdTomato fluorescence. Therefore, the EDTA/cell recovery protocol could be used to isolate subsets of EGCs throughout the small intestine and colon labeled by the reporter to examine differences in gene expression.

This protocol generated a sufficient number of relatively pure GFAP+ glial cells for biochemical studies and western blot analysis¹⁰. To demonstrate glial cell responsiveness, a 3-day glial cell prep was treated with the hormones cholecystokinin (CCK) or gastrin to induce Ca²⁺ flux (**Figure 7A-7B**)¹⁰. The results showed that these GFAP+ adherent cells responded to extracellular agonists demonstrating their ability to exhibit known enteric glial function.

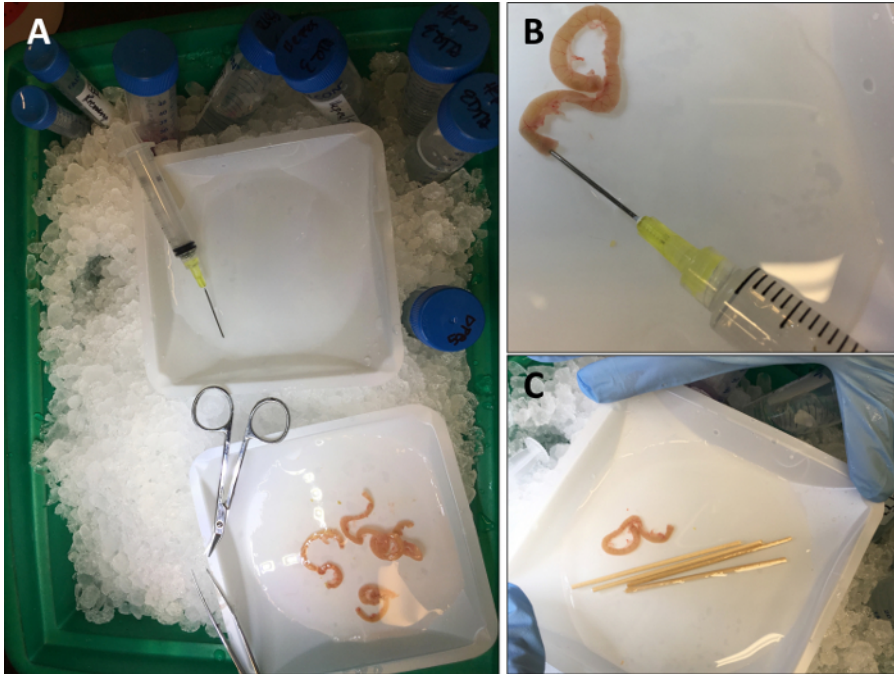


Figure 1: Set up and preparation of mouse intestines. (A) Picture of isolation set up on ice including extracted intestines. (B) Picture of 5 mL-syringe attached to a 20 G blunt end needle to flush out fecal contents. (C) Engineered end of a wooden cotton swab (~4 cm) soaking in the DPBS buffer. [Please click here to view a larger version of this figure.](#)

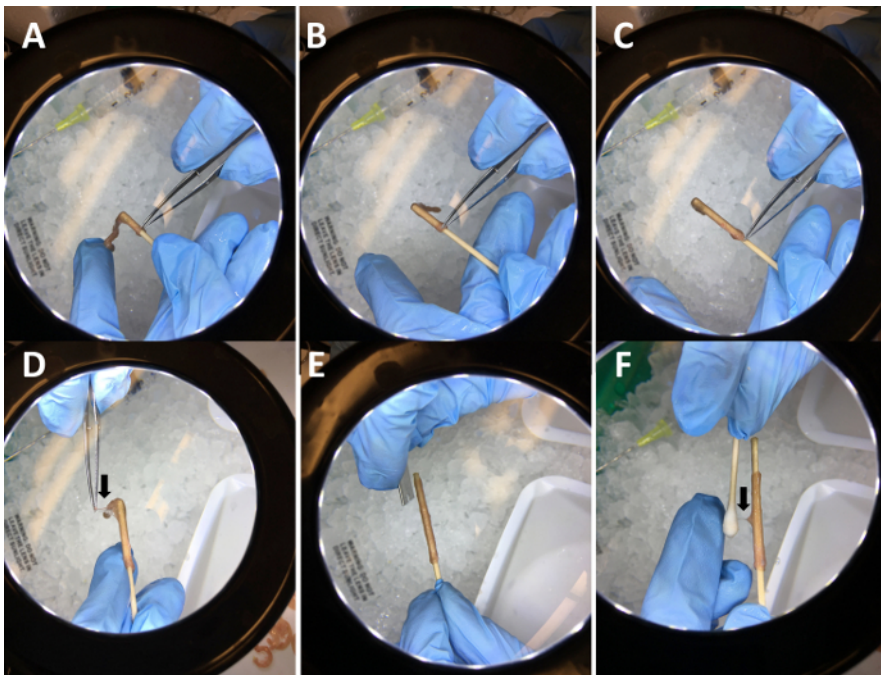


Figure 2: Steps used to clean intestines and remove the longitudinal muscle/myenteric plexus (LMMP). (A-C) Sliding an intestinal segment onto a wetted wooden stick. (D) Removal of adherent mesentery. (E) Nicking the intestine with a razor blade. (F) Removing the LMMP with a damp cotton swab. [Please click here to view a larger version of this figure.](#)

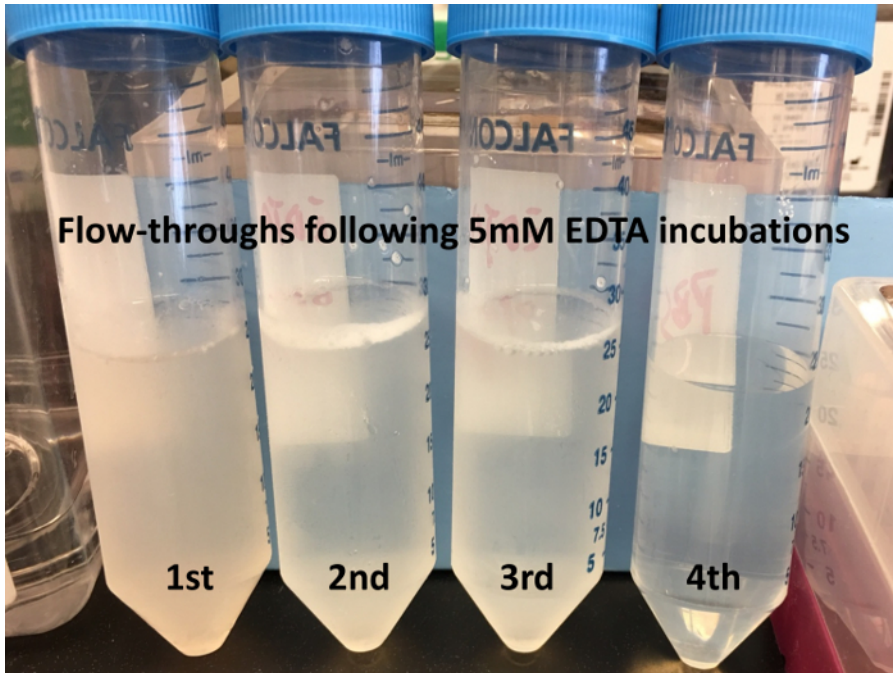


Figure 3: Sequential Flow-throughs after EDTA incubations. Shown are examples of the flow-throughs from 4 sequential incubations using three 7 cm intestinal segments incubated for 10 min with 5 mM EDTA/10mM HEPES in DPBS and then the triturated 20 times. Representative flow-throughs after pouring through a 100 μ m nylon mesh strainer are shown. [Please click here to view a larger version of this figure.](#)

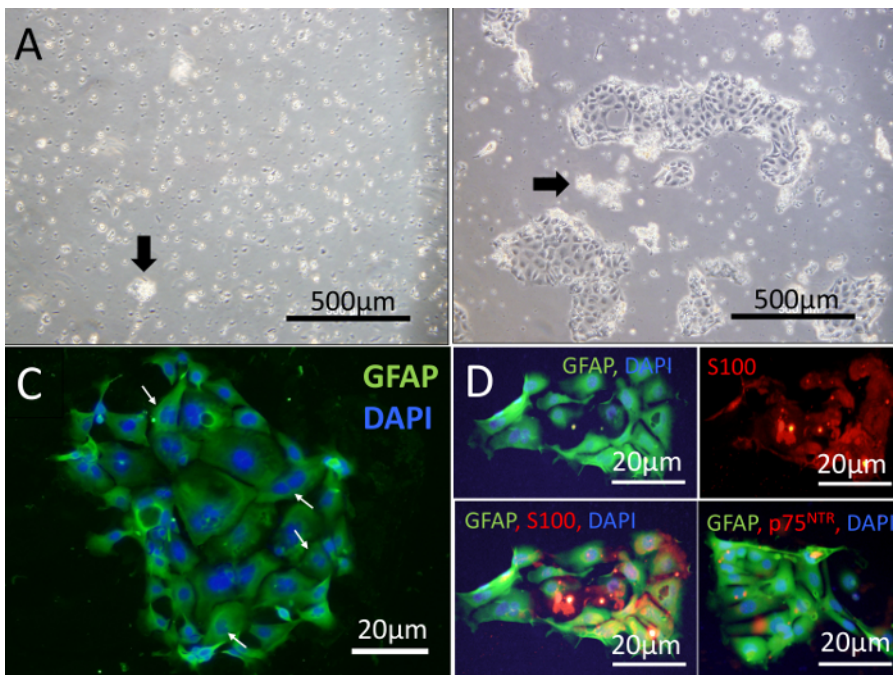


Figure 4: EGC images 24 h after plating. Light microscopic examination of resuspended cells 24 h after plating. (A) Shown is a representative example of a poor prep showing floating epithelial cell clumps (arrow) and debris. No adherent EGCs were observed after 24 h. (B) Shown is a representative example of an excellent prep 24 h after the cell isolation and resuspension in which patches of EGCs adhered to the PDL/Laminin coated plates. Images were captured on an inverted fluorescent microscope with a digital camera. Scale bars = 500 μ m (A-B). (C) High power view of a patch of EGC cells stained with GFAP antibody (green) showing cells at the periphery with a higher intensity of labeling. Several of the cells showed double nuclei (arrowhead, DAPI, blue). (D) Colocalization of GFAP (green) with S100 (red) or p75^{NTR} (red). Scale bars = 20 μ m (C-D). The cells were mounted with an antifade reagent and DAPI (blue nuclei). [Please click here to view a larger version of this figure.](#)

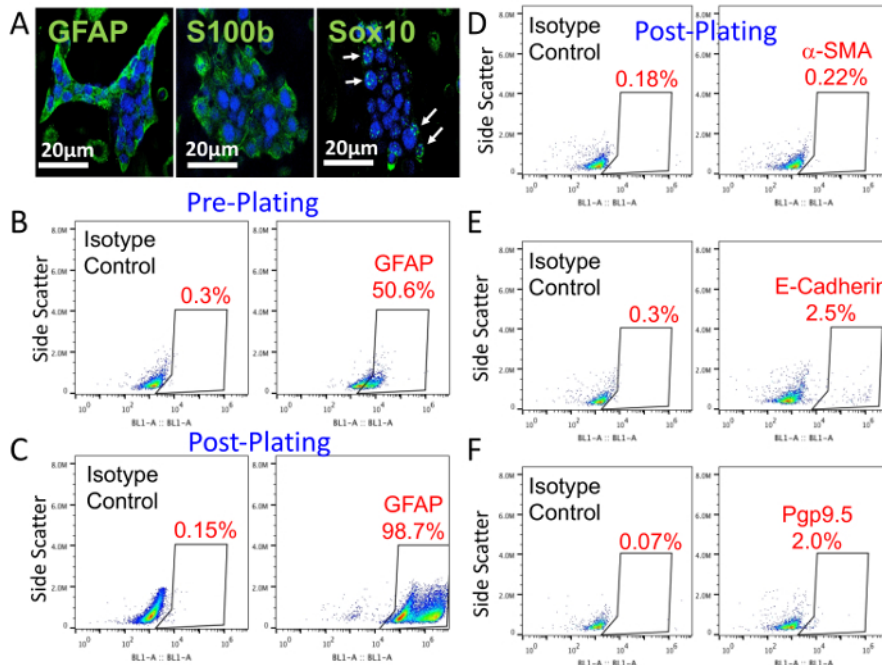


Figure 5: Flow cytometry of enteric glial cells isolated from the duodenal lamina propria of the adult mouse. (A) Immunofluorescence of GFAP, S100B and Sox10 on EGC cultures after 3 days. Arrows indicate Sox 10 positive nuclei. Scale bars = 20 μm (used with permission from Sundaresan *et al.*¹⁰). (B) Percentage of GFAP positive cells before plating on coated plates (fluorescence intensity (or forward scatter, X-axis) versus side scatter (Y-axis)). (C) Percentage of GFAP positive (D) α-SMA positive (E) E-cadherin positive (F) and Pgp 9.5 positive cells 3 days after plating. Gates were constructed to avoid dead cells and debris. Shown is the mean percentage of the fluorescent population relative to the number of cells (side scatter). [Please click here to view a larger version of this figure.](#)

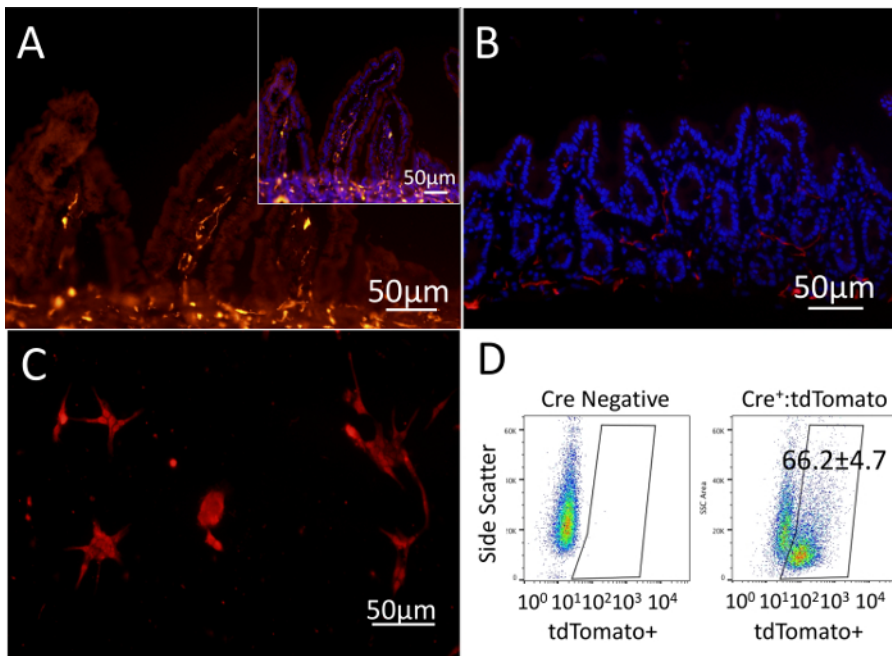


Figure 6: Identification of enteric glial cells isolated from the duodenal lamina propria of the hGFAP-Cre⁺:tdTomato⁺ adult mouse. Endogenous tdTomato fluorescent (red) images were captured on a phase contrast fluorescent microscope with a digital camera in the (A) Intestine. (inset) Same as A except merged with DAPI image (blue nuclei). (B) Colon merged with DAPI. (C) Enteric glial cells (red) isolated from hGFAP-Cre:tdTomato⁺ mice and cultured for 3 days on PDL/Laminin substrate. Scale bars = 50 μm. (D) Flow cytometric analysis of tdTomato cells. Shown is the mean % of GFAP+ cells ± SEM for 3 different preparations (3 mice per prep). [Please click here to view a larger version of this figure.](#)

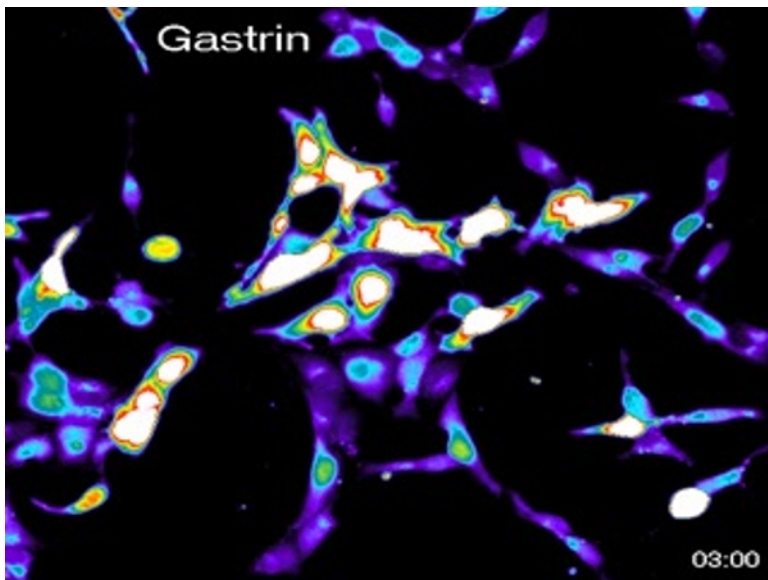


Figure 7: Video of Ca²⁺ fluxes after hormone treatment. EGCs plated on 24-well PDL and laminin coated plates were cultured in the glial growth media for 3 days. The cells were pre-loaded with 3 μ M of Fura-2-AM for 20 min at 37 °C and then were treated with (A) 100 nM cholecystokinin (CCK) or (B) 100 nM gastrin. [Please click here to download this file.](#)

Components	Volume to be added	Final Concentration
DMEM/F12	44.5 mL	–
Fetal Bovine Serum (FBS)	5 mL	10%
Penicillin-Streptomycin	500 μ L	100 IU/mL Pen
Streptomycin		100 μ g/mL Strep
Gentamicin (50 mg/mL stock)	20 μ L	20 μ g/mL

Table 1: Composition of Glial Cell Resuspension Media.

Components	Volume to be added	Final Concentration
DMEM/F12	44 mL	–
Fetal Bovine Serum (FBS)	5 mL	10%
Penicillin-Streptomycin (100x)	500 μ L	100 IU/mL (Pen)
		100 μ g/mL (Strep)
Gentamicin (50 mg/mL stock)	20 μ L	20 μ g/mL
GDNF (10 μ g/mL stock)	50 μ L	10 ng/mL
L-Glutamine (200 mM stock)	500 μ L	2 mM

Table 2: Composition of Glial Cell Growth Media.

Components	Immunofluorescence Dilution	Flow Cytometry Dilution
Chicken anti-GFAP	1 to 500	1 to 2000
Rabbit anti-S100	1 to 500	1 to 500
Mouse anti-p75 NTR	1 to 500	1 to 500
Goat anti-E-cadherin		1 to 400
Mouse anti-Pgp9.5		1 to 500
Goat anti- α -Smooth Muscle Actin		1 to 500
Alexa Fluor 488 Goat Anti-Chicken IgY	1 to 1000	1 to 1000
Alexa Fluor 568 Goat Anti-Mouse IgG	1 to 1000	1 to 1000
Alexa Fluor 594 Donkey Anti-Rabbit IgG	1 to 1000	
Alexa Fluor 488 Donkey Anti-Goat IgG		1 to 1000

Table 3: List of Antibodies

Discussion

EGCs play important roles in gut homeostasis, and it is essential to isolate and study them *in vitro*. In this protocol, a simple method for isolating EGCs from the lamina propria of the adult mouse intestine was introduced to study enteric glial function.

Removing the adherent mesentery and LMMP with a cotton swab removes some of the inter-myenteric glia residing between the longitudinal and circular muscle, increases the accessibility of buffers to the submucosal surface and removes much of the larger capillaries. The latter reduces the number of red blood cells contaminating the final cultures. The series of EDTA incubations strip away the epithelial mucosa exposing the lamina propria and submucosal glia, which can be fragile after exposure to the chelating solutions. Agitation either by shaking, vortexing or triturating (repetitive up and down pipetting) must be performed carefully and timed to avoid excessive damage to the cells. Trituration was used here because the technique resulted in a more consistent yield of adherent, viable glial cells. Shaking tended to introduce bubbles and reduce cell viability assessed morphologically by cell adherence to the coated plates. The tissue must be cut into pieces <0.5 cm to effectively triturate the tissue with the 5 mL pipette.

Generally, 7 cm segments from at least 3 mice is sufficient to generate enough EGCs to cover one 6-well plate at about 20–30% confluency, which can then be used for immunocytochemistry and qPCR. However, more cells might be required for biochemical methods such as western blots depending on the expression level of the gene studied. Although, type 1 collagen or Matrigel was briefly examined as an alternative to the poly-D-lysine/Laminin substrate, greater adherence of the epithelial cell population was observed, ultimately increasing contamination of the EGC cultures with other cell types. In addition, type 1 collagen did not adhere firmly to the plates and was easily dislodged from the surface when changing the media. Fibronectin is another substrate that has been used²⁴, but was not tested here. Laminin apparently enhances the binding and differentiation of neural-crest derived cells²⁵. However, lots of laminin can vary, impacting cell adherence and yields. Microbial contamination of the cultures occurred about 5% of the time and was kept to a minimum by the use of sterile reagents, technique and antibiotics. Use of the antifungal reagent was optional.

The major limitation of the protocol here is standardization of the agitation to remove the epithelium without damaging the underlying EGCs. Moreover, assessment of the preparation cannot be made immediately since it depends on cell adherence to the coated plates. Three areas to focus on when troubleshooting include: 1) Use of fresh PDL and laminin to coat the plates; 2) Minimize the time from dissection to beginning the EDTA incubations by increasing the number of assistants; 3) Quantify the time and method used to agitate the tissue to effectively remove the epithelium and then the EGCs. Extending the time in either of these steps increases EGC fragility and the likelihood that they will not recover when plated in growth media. Although the trituration method to dissociate the epithelium was used here, shaking and vortexing were also tested with inconsistent results perhaps because it is more operator dependent and difficult to quantify. Once plated the yield of adherent cells was greater if the cells were not disturbed for 16–24 h.

The method presented here was modified according to the original study by Smith *et al.*¹², which focused on isolating the LMMP. The Smith approach was used here only to remove and discard the LMMP so that most of the glial cells isolated would originate from the submucosa and lamina propria. In addition, without enzymatic digestion, the myenteric EGCs are not readily liberated¹². Nevertheless, since there are no specific markers for myenteric versus submucosal glia, the presence of glial cells from the inter-myenteric plexus cannot be excluded. Rosenbaum *et al.* reported flow cytometric analysis of EGCs expressing enhanced green fluorescent protein (EGFP) from the hGFAP promoter. They used very young mice (postnatal day 7) and isolated EGCs from the whole intestine by enzymatic digestion¹⁹. In addition, flow cytometric analysis was performed after two weeks of maintaining conditions that promoted free-floating neurosphere formation. Although the authors reported that the neurospheres highly expressed both GFAP and S100b, the floating cell aggregates also strongly expressed α -SMA, suggesting that expansion of the myofibroblast cell population encouraged gliosphere development in contrast to the flat sheet-like morphology described here. Therefore, while interesting, the study is not directly comparable to this protocol. By contrast, the morphology of the cells coupled with significantly less α -SMA+ cells suggest that the EGCs described in this protocol do not exhibit the 3-dimensional morphology during the 3 to 5-day culture period. Nevertheless, both the protocol here and the Rosenbaum method use a fluorescent reporter to identify and isolate GFAP+ cells that will improve investigator's ability to do live cell profiling.

In conclusion, the current protocol describes the isolation of enteric glial cells from the submucosa using a non-enzymatic approach. The entire protocol takes about 3 h from mouse dissection to initial plating on the pre-coated tissue culture plates. The most time intensive step in the protocol is the removal and preparation of the intestines for the first EDTA incubation. Preparing the plates and storing in DPBS is strongly

recommended. The success of the preparation depends upon the efficient removal of the epithelium without damaging the underlying EGCs and adherence to the PDL/Laminin coated plates within 24 h. Primary EGCs are useful for *in vitro* studies such as biochemical analysis, Ca²⁺ fluxes and adenoviral transfections¹⁰. It is anticipated that the ability to isolate EGCs from either the submucosa or the LMMP will permit further studies to define the differences in EGC growth properties, differentiation and morphology using whole genome approaches.

Disclosures

The authors have nothing to disclose.

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References

1. Grubisic, V., & Gulbransen, B. D. Enteric glia: the most alimentary of all glia. *Journal of Physiology*. **595** (2), 557-570 (2017).
2. Gulbransen, B. D., & Sharkey, K. A. Novel functional roles for enteric glia in the gastrointestinal tract. *Nature Reviews Gastroenterology & Hepatology*. **9** (11), 625-632 (2012).
3. Rao, M. *et al.* Enteric Glia Regulate Gastrointestinal Motility but Are Not Required for Maintenance of the Epithelium in Mice. *Gastroenterology*. **153** (4), 1068-1081 e1067 (2017).
4. Rao, M. *et al.* Enteric glia express proteolipid protein 1 and are a transcriptionally unique population of glia in the mammalian nervous system. *Glia*. (2015).
5. McClain, J. *et al.* Ca²⁺ responses in enteric glia are mediated by connexin-43 hemichannels and modulate colonic transit in mice. *Gastroenterology*. **146** (2), 497-507 e491 (2014).
6. Cunha Franceschi, R. *et al.* Enteric glial reactivity to systemic LPS administration: Changes in GFAP and S100B protein. *Neuroscience Research*. **119** 15-23 (2017).
7. Kabouridis, P. S. *et al.* Microbiota controls the homeostasis of glial cells in the gut lamina propria. *Neuron*. **85** (2), 289-295 (2015).
8. Yu, Y. B., & Li, Y. Q. Enteric glial cells and their role in the intestinal epithelial barrier. *World Journal of Gastroenterology*. **20** (32), 11273-11280 (2014).
9. Clairembault, T. *et al.* Enteric GFAP expression and phosphorylation in Parkinson's disease. *Journal of Neurochemistry*. **130** (6), 805-815 (2014).
10. Sundaresan, S. *et al.* Gastrin Induces Nuclear Export and Proteasome Degradation of Menin in Enteric Glial Cells. *Gastroenterology*. **153** 1555-1567 (2017).
11. Gulbransen, B. D. Enteric Glia: The Origin of Duodenal Gastrinomas? *Gastroenterology*. (2017).
12. Smith, T. H., Ngwainmbi, J., Grider, J. R., Dewey, W. L., & Akbarali, H. I. An *in vitro* preparation of isolated enteric neurons and glia from the myenteric plexus of the adult mouse. *Journal of Visualized Experiments*. (78) (2013).
13. Fried, D. E., & Gulbransen, B. D. In situ Ca²⁺ imaging of the enteric nervous system. *Journal of Visualized Experiments*. (95) (2015).
14. Bernstein, C. N., & Vidrich, A. Isolation, identification, and culture of normal mouse colonic glia. *Glia*. **12** (2), 108-116 (1994).
15. Jaeger, C. B. Isolation of enteric ganglia from the myenteric plexus of adult rats. *Journal of Neural Transplantation & Plasticity*. **5** (4), 223-232 (1995).
16. Almond, S., Lindley, R. M., Kenny, S. E., Connell, M. G., & Edgar, D. H. Characterisation and transplantation of enteric nervous system progenitor cells. *Gut*. **56** (4), 489-496 (2007).
17. Ruhl, A., Trotter, J., & Stremmel, W. Isolation of enteric glia and establishment of transformed enteroglia cell lines from the myenteric plexus of adult rat. *Neurogastroenterology & Motility*. **13** (1), 95-106 (2001).
18. Boyen, G. B. *et al.* Distribution of enteric glia and GDNF during gut inflammation. *BMC Gastroenterology*. **11**, 3 (2011).
19. Rosenbaum, C. *et al.* Activation of Myenteric Glia during Acute Inflammation In Vitro and In Vivo. *Public Library of Science One*. **11** (3), e0151335 (2016).
20. Zhuo, L. *et al.* hGFAP-cre transgenic mice for manipulation of glial and neuronal function *in vivo*. *Genesis*. **31** (2), 85-94 (2001).
21. Boesmans, W., Lasrado, R., Vanden Berghe, P., & Pachnis, V. Heterogeneity and phenotypic plasticity of glial cells in the mammalian enteric nervous system. *Glia*. **63** (2), 229-241 (2015).
22. Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nature Biotechnology*. **22** (12), 1567-1572 (2004).
23. Madisen, L. *et al.* A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nature Neuroscience*. **13** (1), 133-140 (2010).
24. Kabouridis, P. S. *et al.* The gut microbiota keeps enteric glial cells on the move; prospective roles of the gut epithelium and immune system. *Gut Microbes*. **6** (6), 398-403 (2015).
25. Pomeranz, H. D., Sherman, D. L., Smalheiser, N. R., Tennyson, V. M., & Gershon, M. D. Expression of a neurally related laminin binding protein by neural crest-derived cells that colonize the gut: relationship to the formation of enteric ganglia. *The Journal of Comparative Neurology*. **313** (4), 625-642 (1991).