1 **TITLE:**
2 Isolation of Enteric Glial Cells from the Submucosa and Lamina Propria of the Adult Mouse
3
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26 **KEYWORDS:**
27 duodenum, hGFAP-Cre, tdTomato, EDTA chelation, EGCs, Ca^{2+} flux, Sox10, GFAP
28

29 **SUMMARY:**
30 Here, we describe the isolation of enteric-glial cells from the intestinal-submucosa using
31 sequential EDTA incubations to chelate divalent cations and then incubation in non-enzymatic
32 cell recovery solution. Plating the resultant cell suspension on poly-D-lysine and laminin results
33 in a highly enriched culture of submucosal glial cells for functional analysis.
34

35 **ABSTRACT:**
36 The enteric nervous system (ENS) consists of neurons and enteric glial cells (EGCs) that reside
37 within the smooth muscle wall, submucosa and lamina propria. EGCs play important roles in gut
38 homeostasis through the release of various trophic factors and contribute to the integrity of the
39 epithelial barrier. Most studies of primary enteric glial cultures use cells isolated from the
40 myenteric plexus after enzymatic dissociation. Here, a non-enzymatic method to isolate and
41 culture EGCs from the intestinal submucosa and lamina propria is described. After manual
42 removal of the longitudinal muscle layer, EGCs were liberated from the lamina propria and
43 submucosa using sequential HEPES-buffered EDTA incubations followed by incubation in
44 commercially available non-enzymatic cell recovery solution. The EDTA incubations were

45 sufficient to strip most of the epithelial mucosa from the lamina propria, allowing the cell
46 recovery solution to liberate the submucosal EGCs. Any residual lamina propria and smooth
47 muscle was discarded along with the myenteric glia. EGCs were easily identified by their ability
48 to express glial fibrillary acidic protein (GFAP). Only about 50% of the cell suspension contained
49 GFAP+ cells after completing tissue incubations and prior to plating on the poly-D-
50 lysine/laminin substrate. However, after 3 days of culturing the cells in glial cell-derived
51 neurotrophic factor (GDNF)-containing culture media, the cell population adhering to the
52 substrate-coated plates comprised of >95% enteric glia. We created a hybrid mouse line by
53 breeding a hGFAP-Cre mouse to the ROSA-tdTomato reporter line to track the percentage of
54 GFAP+ cells using endogenous cell fluorescence. Thus, non-myenteric enteric glia can be
55 isolated by non-enzymatic methods and cultured for at least 5 days.
56
57 **INTRODUCTION:**
58 Interest in the function of enteric glial cells (EGCs) has steadily increased due to their recognized
59 roles in the gut integrity and homeostasis[1,2]. In addition, EGCs vary according to their location
60 along the length of the GI tract[3,4]. EGCs release various trophic factors including glial cell-
61 derived neurotrophic factor (GDNF), contribute to gut motility[1,5] and respond to microbial
62 byproducts[6,7]. Studies have indicated that the EGC population is heterogeneous and that their
63 function varies depending on whether they are submucosal or reside within the myenteric
64 plexus[1,7]. For example, EGCs within the submucosa contribute to tight junctions[8]. Differential
65 GFAP expression and phosphorylation in EGCs have been linked to Parkinson's Disease,
66 suggesting their possible link to the gut phenotype of this disorder[9]. Recently, it was observed
67 that the loss of the nuclear protein menin in isolated cultures of EGCs from the proximal
68 intestinal submucosa was sufficient to induce expression of the hormone gastrin[10]. As a result, it
69 was proposed that EGCs might be the origin of duodenal gastrinomas, a type of neuroendocrine
70 tumor[10]. Collectively, these examples underscore the relevance of studying the behavior and
71 function of isolated EGCs in neuropathic disorders and cancer[11].
72
73 The challenge in the field remains how to isolate and study either or both EGC populations in
74 vitro. Lineage trace experiments demonstrated that EGCs in the submucosa and lamina propria
75 originate from progenitor cells in the myenteric plexus[7]. Although there are several published
76 isolation protocols available to generate cultures of myenteric EGCs[12-19], none specifically
77 targets isolation of the submucosal/lamina propria EGC population. Existing protocols for EGC
78 isolation specifically use a combination of the mechanical separation or the microdissection of
79 the smooth muscle combined with enzymatic dissociation, eventually discarding the mucosal
80 cell layer.
81
82 The goal of this manuscript is to demonstrate the steps to non-enzymatically isolate primary
83 EGCs from the lamina propria for in vitro studies. Since there are no markers that specifically
84 distinguish myenteric EGCs from those in the submucosa, the spatial separation of the epithelial
85 mucosa from the smooth muscle was exploited to isolate submucosal EGCs. In addition, by
86 combining EDTA chelation with non-enzymatic dissociation, EGCs were isolated from the
87 submucosa in contrast to the smooth muscle, which was discarded along with the associated

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

91 **PROTOCOL:**

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

96 **1. Preparation of sterile poly-D-lysine (PDL) and laminin solutions**

98 1.1. At least one day prior to cell isolation, prepare poly-D-lysine (PDL) and laminin coated
99 plates.

101 NOTE: Both 6-well and 12-well plates were prepared depending on the experimental goals.
102 Typically, 12-well plates were used for the quantitative analysis using western blots; whereas, 6-
103 well plates were used to hold autoclaved (sterile) coverslips for immunohistochemistry. 24-well
104 plates were used to culture EGCs for Ca^{2+} flux imaging.

106 1.2. Dilute stock solutions with ultrapure tissue culture grade, DNase-free and RNase-free water
107 in a tissue culture hood with HEPA-filtered air.

109 1.3. Sterilizing glass coverslips

111 1.3.1. Hold the coverslip with sterile forceps and immerse coverslips in a beaker of 100%
112 ethanol for 15 min. Allow the ethanol to evaporate in the tissue culture hood and then place in
113 the 6-well plates.

115 1.3.2. Alternatively, place several coverslips individually on 2 mm thick blotting paper in a plastic
116 container and then autoclave.

118 **2. Coating plates**

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

122 2.1. Thaw 1 mg/mL PDL stock and dilute 1:10 with sterile, tissue culture grade water so that the
123 final concentration is 100 µg/mL. Use 2 mL per well to coat 6-well plates, 1 mL to cover one well
124 of a 12-well plate and 0.5 mL for one well in the 24-well plates. Store the remaining diluted PDL
125 in 12 mL aliquots at -20 °C.

127 2.2. After allowing the PDL to coat the wells for at least 1 h, remove the PDL with a sterile
128 pipette. Allow the plates to air dry completely under a tissue culture laminar flow hood.

130 NOTE: After removing the PDL solution with a sterile pipette, it can be used twice more within 1
131 week after passing through a 0.22 µm filter and storing at 4 °C.

132

133   2.3. Thaw the laminin stock on ice to avoid gel formation.

134

135   NOTE: Undiluted laminin becomes solid when warmed above 8 °C.

136

137   2.4. Dilute the laminin stock (0.5 mg/mL) 1:50 with sterile Dulbecco's Phosphate Buffered Saline
138   (DPBS) to obtain a final concentration of 10 µg/mL. Store the diluted laminin in 12 mL aliquots
139   at -20 °C.

140

141   2.5. Add diluted laminin to the wells containing dried PDL. Use 1 mL to cover the 6-well surface
142   and 0.5 mL to cover the bottom of the 12-well plates. For coverslips, add 400 µL of diluted
143   laminin to the coverslips to achieve surface coverage.

144

145   2.6. Incubate the coated plates at 37 °C for 2 h, if the plate is used the same day (quick coating).
146   If not, seal the plates with plastic wrap, and store overnight at 4 °C (slow coating).

147

148   NOTE: Sealing is critical to avoid drying and contamination.

149

150   2.7. Remove the laminin solution carefully with a pipette to avoid scratching the surface. Gently
151   wash the wells three times with sterile DPBS.

152

153   2.8. Add complete glial growth media to each well and maintain the plates at 37 °C until ready
154   to add the EGC suspension.

155

156   NOTE: Poly-D lysine and laminin coated plates can be stored for 3 days at 4 °C. Coat the plates
157   several days ahead and then store at 4 °C, wash the plates with sterile DPBS three times. Add 2
158   mL of sterile DPBS to each well after the final wash. Seal the plate with parafilm and store at 4
159   °C. It is essential to ensure that the laminin does not dry out.

160

161   **3. Preparation of isolation solutions**

162

163   3.1. Prepare the EDTA/HEPES/DPBS dissociation solution. For 500 mL of the solution, use sterile
164   DPBS (without calcium and magnesium) to make a final solution of 10 mM HEPES and 5 mM
165   EDTA. To 490 mL of the DPBS, add 5 mL of 1 M HEPES buffer and 5 mL of 0.5 M EDTA stock
166   solution. Store at 4 °C until use.

167

168   3.2. Prepare the glial cell resuspension media using the reagents listed in **Table 1**.

169

170   3.3. Prepare glial growth media using the reagents listed in **Table 2**.

171

172   NOTE: Glial growth media containing GDNF can be stored for one week at 4 °C.

173

174   **4. Removal of the intestinal segment**

175

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.

4.1. 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.

4.2. Lift the liver and identify the distal stomach/pylorus. Then remove 7 cm of the proximal intestine. Be careful not to tear the intestine.

4.3. Hold the pylorus with forceps while snipping away the mesentery.

4.3.1. Use blunt dissection with the back of the scissor to scrape away adherent pancreas.

4.3.2. 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.

4.4. Use a 5 or 10 mL syringe attached to a blunt end 20-gauge needle to flush out the fecal contents with ice cold DPBS (**Figure 1B**).

4.5. 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.

4.6. Break off about 4 cm to separate the wooden stick from the cotton tip. Soak the wooden stick in DPBS (**Figure 1C**).

4.7. Slip one of the intestinal segments smoothly onto the wetted stick (**Figures 2A-2C**).

4.8. Remove adherent mesentery still attached using needle-nose forceps (**Figure 2D**).

4.9. Use a clean razor blade or scalpel to make two longitudinal nicks along the intestine where the mesentery was attached (**Figure 2E**).

4.9.1. 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.

219   4.9.2. Hold the tip of the stick between the thumb and forefinger while stabilizing the segment
220   with the middle and fourth finger (**Figure 2F**). When complete, the LMMP will easily peel off the
221   intestinal segment.
222
223   4.10. Discard the LMMP stripped from the intestinal tube and attached to the cotton swab
224   unless harvesting EGCs from the myenteric plexus is desired.
225
226   4.11. Use a razor blade to flay open the intestinal segment longitudinally to remove from the
227   wooden stick.
228
229   4.12. Store the stripped intestinal tissue (predominantly mucosa and submucosa plus circular
230   muscle) in DPBS on ice. Repeat steps 4.4-4.11 until all intestinal segments are prepared.
231
232   **5. Removal of the epithelial mucosa**
233
234   5.1. Cut the intestines into smaller pieces of ~0.5 cm with fine scissors.
235
236   5.2. Place the tissue in a sterile 50 mL conical centrifuge tube containing 30 mL of ice-cold
237   EDTA/HEPES/DPBS solution.
238
239   5.3. Rock the tissue-containing solution at 4 °C for 10 min at ~60 tilts per min.
240
241   5.4. Pre-moisten a 5 mL plastic pipette by pipetting the EDTA/HEPES/DPBS buffer up and down
242   one time, and then use the pre-moistened pipette to pipette the tissue and buffer suspension
243   up and down (trituration) 20 times to dislodge the epithelial mucosa.
244
245   5.5. Collect the tissue from the EDTA incubation by pouring the mixture through a 100 μm nylon
246   cell strainer. Discard the turbid mucous-filled flow-through (**Figure 3**).
247
248   5.6. Place the tissue retained in the strainer into a new 50 mL conical centrifuge tube containing
249   30 mL of ice cold EDTA/HEPES/DPBS using needle-nose forceps.
250
251   5.7. Repeat the EDTA/HEPES/DPBS incubation a second time by rocking the tissue at 4 °C for 10
252   min at ~60 tilts per min to strip off additional epithelial mucosa**.**
253
254   5.8. Pre-moisten a 5 mL pipette with the buffer and then pipette the tissue suspension up and
255   down 20 times to dislodge the mucous cells.
256
257   NOTE: The tissue will tend to stick to inside of the pipette as more of the epithelium is removed.
258
259   5.9. Collect the tissue after the second incubation by pouring the mixture through a 100 μm
260   nylon cell strainer and discard the flow through. The second flow-through should be noticeably
261   less turbid than the first (**Figure 3**).
262

263 5.10. Repeat the 10 min EDTA incubations until the solution is nearly clear (about 3 to 4
264 incubations depending on the amount of tissue and the effectiveness of the trituration) (**Figure**
265 **3**).
266
267 **6. Collection of enteric glial cells from the lamina propria and submucosa**
268
269 6.1. Transfer the tissue from the nylon strainer to a 15 mL conical centrifuge tube containing 5
270 mL of the commercially available cell recovery solution and rock for 25-30 min at 4 °C.
271
272 6.2. Triturate gently 10x to dissociate the enteric glial cells from the lamina propria.
273
274 6.2.1. Filter through a 40 µm filter and collect the filtrate in a clean 50 mL tube.
275
276 6.2.2. Rinse the tissue on the nylon filter with 1 mL of DPBS.
277
278 6.2.3. Discard the tissue and transfer the ~5-6 mL of filtrate to a clean 15 mL conical centrifuge
279 tube.
280
281 6.2.4. Spin the filtrate at 2,000 x g in a swinging bucket centrifuge for 5 min at 4 °C.
282
283 6.3. Re-suspend the glial cell-containing pellet in at least 1 mL of resuspension buffer by gently
284 pipetting the pellet up and down with a 500 µL pipette tip. Avoid introducing bubbles.
285
286 NOTE: Adjust the resuspension volume as needed for the number of wells and plates. For
287 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.
288
289 6.4. Pipette 200 µL of the cell suspension into each well of the 6-well or 100 µL for a 12-well
290 PDL-laminin coated plates containing glial growth media.
291
292 6.5. Do not disturb the dishes after adding the cells to the plates and placing in the 37 °C
293 incubator to allow the maximum number of cells to adhere.
294
295 NOTE: A healthy preparation of cells will attach within 6-8 h but wait for at least 24 h before
296 changing the media by gently pipetting off the media along with non-adherent cells.
297
298 6.6. Use the cells for experiments 3-4 days after they are placed into culture (**Figure 4**). Perform
299 immunofluorescent analysis using antibodies of interest to specific protein markers (**Table 3**).
300 For example, GFAP, S100b and p75NTR or Sox10 was used here. E-cadherin or alpha smooth
301 muscle actin antibodies were used to assess the degree of contamination from other cell types.
302
303 **7. Immunofluorescent staining**
304
305 7.1. Aspirate the media off the cultures and rinse twice with PBS.
306

307   7.2. Fix the cultures for 20 min at room temperature with 4% paraformaldehyde.

308

309   7.3. Remove the fixative and then rinse 3 times with PBS.

310

311   7.4. Permeabilize the cells with PBS containing 0.2% Triton X-100 at room temperature.

312

313   7.5. Incubate the permeabilized cells with blocking solutions comprised of anti-chicken IgY, anti-
314   rabbit or anti-mouse IgG for 2 h at room temperature.

315

316   7.6. Incubate the cells with primary antibodies overnight (e.g., 1:1,000 GFAP).

317

318   7.6.1. Rinse the cells 3 times with PBS. If performing colocalization proceed to Step 7.7.

319

320   7.7. Incubate the next set of primary antibodies for at least 2 h at room temperature, but
321   preferably overnight at 4 °C. Use a 1:1000 dilution of rabbit anti-S100 or a 1:500 dilution of
322   mouse anti-p75NTR.

323

324   NOTE: All antibodies are diluted in PBS.

325

326   7.8. Rinse the cells 3 times with PBS to remove primary antibodies. Then incubate the rinsed
327   cells with the appropriate fluorescently-tagged secondary antibodies for 2 h at room
328   temperature (**Table 3**).

329

330   7.9. Rinse 3 times with PBS to remove the secondary antibodies.

331

332   7.10. Mount the coverslips onto slides using 1-2 drops of the mounting media with DAPI and
333   view the cells under a fluorescent microscope.

334

335   **8. Flow cytometry**

336

337   8.1. Remove adherent glial cells for flow cytometry by rinsing the cells once with DPBS and then
338   add 0.25% trypsin-EDTA per manufacturer's protocol. Incubate the cells in the trypsin-EDTA
339   solution at 37 °C for 3 min. Terminate the digestion by collecting the cells in complete glial cell
340   growth media.

341

342   8.2. Collect the cells by centrifuging at 400 × g for 5 min, and then re-suspend the cells in PBS.

343

344   8.3. Fix the cells with 4% paraformaldehyde for 10 min and then permeabilize with 0.2% Triton
345   X-100 in PBS as described in Step 7.4.

346

347   8.4. Block the cells with PBS containing 1% BSA and the tissue specific immunoglobulin for 20
348   min (e.g., donkey anti-chicken IgY (IgG) (H+L) (1:1,000) or donkey anti-goat IgG (H+L) (1:1,000)).

349

350 8.5. Incubate cells with the primary antibodies GFAP (1:2,000), E-cadherin (1:400), α-smooth
351 muscle actin (1:500), or Pgp9.5 (1:500) overnight at 4 °C.

353 8.6. Wash away the antibody with PBS.

355 8.6.1. Incubate the antigen-antibody complex with fluorescently-tagged secondary antibodies
356 incubated at room temperature for 30 min.

358 8.6.2. Use a separate aliquot of cells incubated with secondary antibody to serve as the isotype
359 control. Both populations of cells are washed and resuspended in PBS prior to flow cytometry.

361 8.6.3. Analyze the two populations of cells on a flow cytometer by gating on the live cells.

363 **9. Preparing mouse tissues from the hGFAP-Cre:tdTomato mice**

365 NOTE: The Lox-STOP-Lox-tdTomato cDNA is expressed from the ROSA locus[22,23] (Jackson Labs,
366 #007914) and generates endogenous fluorescence when bred to a mouse expressing the Cre
367 recombinase (hGFAP-Cre). In situ analysis is performed by generating frozen tissue sections.

369 9.1. Fix intestinal tissue in 4% paraformaldehyde for 1 h and then dehydrate overnight in 1 M
370 sucrose in PBS.

372 9.2. Embed the tissue in commercially purchased optimal cutting tissue (OCT) compound
373 comprised of 10% polyvinyl alcohol and 4.2% polyethylene glycol, and then snap freeze in liquid
374 nitrogen.

376 9.3. Prepare 5 µm cryosections and then mount using an antifade mounting media with DAPI.

378 9.4. For flow cytometry, prepare EGCs from the hGFAP-Cre:tdTomato+ mice as described in
379 Steps 4.1-6.5.

381 9.5. Trypsinize the cells from the plate after 3 days in cultures as in Step 8.1 and then fix for 10
382 min in 4% paraformaldehyde.

384 9.6. Analyze cells isolated from Cre Negative mice in parallel with cells isolated from the hGFAP-
385 Cre:tdTomato+ mice on a flow cytometer.

387 NOTE: Gates were constructed to avoid dead cells and debris.

389 **10. Ca^{2+} flux imaging**

391 10.1. Incubate EGCs plated on a 24-well plate with 3 µM Fluo-4-AM at 37 °C for 30 min.

393   10.2. Image the Ca^{2+} signal in commercially purchased Tyrode's solution using a spinning disk
394   confocal microscope and an excitation wavelength of 480 nm (F480) after adding CCK or gastrin
395   peptide. Image analysis was reported in Sundaresan et al.[10] .
396
397   **REPRESENTATIVE RESULTS:**
398   Preps were considered unsuccessful if GFAP+ cells did not adhere and spread within 24 h
399   (**Figure 4A**). The number of glial cells could not be determined until after 24 h when the cells
400   adhered and showed evidence of spreading into flat aggregates (**Figure 4B**). Cells at the edge of
401   the clusters tended to extend long processes and expressed classic glial markers (e.g., GFAP,
402   S100b and p75^{NTR} (**Figure 4C,D**)[10,16]). By the 2nd day, glial cells tightly adhered to the surface
403   allowing the non-adherent population to be rinsed away with PBS. Most of the floating cells
404   were epithelial and lamina propria cells along with cell debris. The presence of these floating
405   cell clumps reduced the yield of adherent glial cells underscoring the importance of performing
406   the EDTA incubations until the flow-through is nearly clear, signaling that the epithelial cells
407   have been removed from the lamina propria core. In addition, centrifugation at speeds lower
408   than 1,500 x *g* after incubating in the cell recovery solution did not effectively collect all of the
409   viable cells into a pellet leading to reduced yields. Typically, 40,000 to 100,000 cells
410   morphologically consistent with EGCs adhered firmly by day 3 in culture.
411
412   Immunohistochemical analysis with glial-associated antibodies, indicated that the cell clusters
413   that remained adherent by day 3 were GFAP, S100b, and Sox 10 positive[10,16] (**Figure 5A**). In the
414   current study, this same degree of purity was observed by permeabilizing the cells and analyzing
415   by flow cytometry (**Figure 5B-F**). Immediately after isolating the submucosal/Lamina propria
416   glial cells, flow cytometry revealed that about 51% of the cells were GFAP+ (**Figure 5B**),
417   suggesting the presence of GFAP-negative cell types, e.g., epithelial, hematopoietic, endothelial,
418   neuronal cells, myofibroblasts known to exist in the epithelium and lamina propria. After 3 days
419   in culture, the number of GFAP+ cells represented over 95% of the cell population analyzed
420   after removing the original media, gently rinsing with PBS and then adding fresh media (**Figure
421   5C**). Interestingly, flow analysis revealed high and low GFAP protein-expressing populations
422   (**Figure 5C**). Indeed, immunofluorescent analysis of the cells revealed that the periphery of the
423   clusters tended to express higher levels of GFAP (**Figure 4C,D**). Taken together, the two types of
424   analysis might indicate differences in EGC maturity or differentiation status. Collectively, the
425   percentage of α-SMA+ (myofibroblast cell marker), E-cadherin+ (epithelial cell marker), and Pgp
426   9.5+ (neuronal cell marker) cells was less than 5% (**Figure 5D,E,F**). These results were consistent
427   with the prior study performed using immunofluorescent labeling of EGCs showing about 93%
428   GFAP+ cells[10]. The flow analysis underscores the importance of allowing the cells to adhere to
429   the plates since it allows removal of contaminating cell populations comprising approximately
430   5% of the cultures.
431
432   A goal in this study was to use a GFAP-activated reporter to facilitate flow cytometry of the cells
433   for further analysis and to compare the degree of EGC enrichment using an endogenous
434   fluorescent marker (**Figure 6**). The hGFAP-Cre mouse line was originally described by Messing
435   and coworkers [20]. In brief, the Cre recombinase was placed under the control of 2.2 kb of the
436   human glial fibrillary acid protein (hGFAP) promoter. Thus, any cell transcribing this hGFAP

437   promoter expressed the red fluorescent reporter tdTomato. The cell population labeled with the
438   tdTomato reporter was visualized *in situ* using frozen sections of the intestine and colon (**Figure**
439   **6A,B**) before performing the EGC isolation procedures described above. After performing the
440   EDTA/cell recovery isolation and culturing the cells for 3 days, EGCs from these mice were easily
441   identified by their endogenous fluorescence (**Figure 6C**). Although GFAP+ cells arguably are
442   more numerous in the proximal intestine[4,21], tdTomato+ EGCs were also observed in the colon
443   (**Figure 6B**). Using the tdTomato[+] fluorescent reporter activated by the hGFAP-Cre also revealed
444   a bimodal population of hGFAP-tdTomato+ cells (**Figure 6D**) as observed using the
445   immunofluorescent analysis of GFAP protein (**Figure 5C**). Although it has been reported that not
446   all EGCs express GFAP protein[4], this point might reflect differences in the level of GFAP
447   expression. On average, about 66% of the cells analyzed exhibited the highest level of tdTomato
448   fluorescence. Therefore, the EDTA/cell recovery protocol could be used to isolate subsets of
449   EGCs throughout the small intestine and colon labeled by the reporter to examine differences in
450   gene expression.
451
452   This protocol generated a sufficient number of relatively pure GFAP+ glial cells for biochemical
453   studies and western blot analysis[10]. To demonstrate glial cell responsiveness, a 3-day glial cell
454   prep was treated with the hormones cholecystokinin (CCK) or gastrin to induce Ca^{2+} flux
455   (**Figures 7A-7B**)[10]. The results showed that these GFAP+ adherent cells responded to
456   extracellular agonists demonstrating their ability to exhibit known enteric glial function.
457
458   **FIGURE AND TABLE LEGENDS:**
459   **Figure 1: Set up and preparation of mouse intestines** (**A**) Picture of isolation set up on ice
460   including extracted intestines. (**B**) Picture of 5 mL-syringe attached to a 20-gauge blunt end
461   needle to flush out fecal contents. (**C**) Engineered end of a wooden cotton swab (~4 cm) soaking
462   in the DPBS buffer.
463
464   **Figure 2: Steps used to clean intestines and remove the longitudinal muscle/myenteric plexus**
465   **(LMMP). (A-C)** Sliding an intestinal segment onto a wetted wooden stick. **(D)** Removal of
466   adherent mesentery. **(E)** Nicking the intestine with a razor blade. **(F)** Removing the LMMP with
467   a damp cotton swab.
468
469   **Figure 3: Sequential Flow-throughs after EDTA incubation**s. Shown are examples of the flow-
470   throughs from 4 sequential incubations using three 7 cm intestinal segments incubated for 10
471   min with 5 mM EDTA/10mM HEPES in DPBS and then the triturated 20 times. Representative
472   flow-throughs after pouring through a 100 µm nylon mesh strainer are shown.
473
474   **Figure 4: EGC images 24 h after plating.** Light microscopic examination of resuspended cells 24
475   h after plating. (**A**) Shown is a representative example of a poor prep showing floating epithelial
476   cell clumps (arrow) and debris. No adherent EGCs were observed after 24 h. **(B)** Shown is a
477   representative example of an excellent prep 24 h after the cell isolation and resuspension in
478   which patches of EGCs adhered to the PDL/Laminin coated plates. Images were captured on an
479   inverted fluorescent microscope with a digital camera. Scale bars for A and B are 500 µm. **(C)**
480   High power view of a patch of EGC cells stained with GFAP antibody (green) showing cells at the

481   periphery with a higher intensity of labeling. Several of the cells showed double nuclei
482   (arrowhead, DAPI, blue). (**D**) Colocalization of GFAP (green) with S100 (red) or p75NTR (red).
483   Scale bars for C and D are 20 μm. The cells were mounted with an antifade reagent and DAPI
484   (blue nuclei).
485
486   **Figure 5: Flow cytometry of enteric glial cells isolated from the duodenal lamina propria of**
487   **the adult mouse. (A)** Immunofluorescence of GFAP, S100B and Sox10 on EGC cultures after 3
488   days. Arrows indicate Sox 10 positive nuclei. Scale bars are 20 μm (used with permission from
489   Sundaresan et al.[10]). (**B**) Percentage of GFAP positive cells before plating on coated plates
490   (fluorescence intensity (or forward scatter, X-axis) versus side scatter (Y-axis). (**C**) Percentage of
491   GFAP positive (**D**) α-SMA positive (**E**) E-cadherin positive (**F**) and Pgp 9.5 positive cells 3 days
492   after plating. Gates were constructed to avoid dead cells and debris. Shown is the mean
493   percentage of the fluorescent population relative to the number of cells (side scatter).
494
495   **Figure 6: Identification of enteric glial cells isolated from the duodenal lamina propria of the**
496   **hGFAP-Cre⁺:tdTomato⁺ adult mouse.** Endogenous tdTomato fluorescent (red) images were
497   captured on a phase contrast fluorescent microscope with a digital camera in the (**A**) Intestine.
498   **(inset)** Same as A except merged with DAPI image (blue nuclei). (**B**) Colon merged with DAPI. (**C**)
499   Enteric glial cells (red) isolated from hGFAP-Cre:dtTomato⁺ mice and cultured for 3 days on
500   PDL/Laminin substrate. Scale bars = 50 μm. (**D**) Flow cytometric analysis of tdTomato cells.
501   Shown is the mean % of GFAP+ cells ± SEM for 3 different preparations (3 mice per prep).
502
503   **Figure 7: Video of Ca²⁺ fluxes after hormone treatment. EGCs** plated on 24-well PDL and
504   laminin coated plates were cultured in the glial growth media for 3 days. The cells were pre-
505   loaded with 3 μM of Fura-2-AM for 20 min at 37 °C and then were treated with (**A**) 100 nM
506   cholecystokinin (CCK) or (**B**) 100 nM gastrin.
507
508   **Table 1: Composition of Glial Cell Resuspension Media**
509
510   **Table 2: Composition of Glial Cell Growth Media**
511
512   **Table 3: List of Antibodies**
513
514   **DISCUSSION:**
515   EGCs play important roles in gut homeostasis, and it is essential to isolate and study them in
516   vitro. In this protocol, a simple method for isolating EGCs from the lamina propria of the adult
517   mouse intestine was introduced to study enteric glial function.
518
519   Removing the adherent mesentery and LMMP with a cotton swab removes some of the inter-
520   myenteric glia residing between the longitudinal and circular muscle, increases the accessibility
521   of buffers to the submucosal surface and removes much of the larger capillaries. The latter
522   reduces the number of red blood cells contaminating the final cultures. The series of EDTA
523   incubations strip away the epithelial mucosa exposing the lamina propria and submucosal glia,
524   which can be fragile after exposure to the chelating solutions. Agitation either by shaking,

525 vortexing or triturating (repetitive up and down pipetting) must be performed carefully and
526 timed to avoid excessive damage to the cells. Trituration was used here because the technique
527 resulted in a more consistent yield of adherent, viable glial cells. Shaking tended to introduce
528 bubbles and reduce cell viability assessed morphologically by cell adherence to the coated
529 plates. The tissue must be cut into pieces <0.5 cm to effectively triturate the tissue with the 5
530 mL pipette.

532 Generally, 7 cm segments from at least 3 mice is sufficient to generate enough EGCs to cover
533 one 6-well plate at about 20-30% confluency, which can then be used for immunocytochemistry
534 and qPCR. However, more cells might be required for biochemical methods such as western
535 blots depending on the expression level of the gene studied. Although, type 1 collagen or
536 Matrigel was briefly examined as an alternative to the poly-D-lysine/Laminin substrate, greater
537 adherence of the epithelial cell population was observed, ultimately increasing contamination
538 of the EGC cultures with other cell types. In addition, type 1 collagen did not adhere firmly to
539 the plates and was easily dislodged from the surface when changing the media. Fibronectin is
540 another substrate that has been used[24], but was not tested here. Laminin apparently enhances
541 the binding and differentiation of neural-crest derived cells[25]. However, lots of laminin can vary,
542 impacting cell adherence and yields. Microbial contamination of the cultures occurred about 5%
543 of the time and was kept to a minimum by the use of sterile reagents, technique and antibiotics.
544 Use of the antifungal reagent was optional.

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

559 The method presented here was modified according to the original study by Smith et al.[12],
560 which focused on isolating the LMMP. The Smith approach was used here only to remove and
561 discard the LMMP so that most of the glial cells isolated would originate from the submucosa
562 and lamina propria. In addition, without enzymatic digestion, the myenteric EGCs are not
563 readily liberated[12]. Nevertheless, since there are no specific markers for myenteric versus
564 submucosal glia, the presence of glial cells from the inter-myenteric plexus cannot be excluded.
565 Rosenbaum et al. reported flow cytometric analysis of EGCs expressing enhanced green
566 fluorescent protein (EGFP) from the hGFAP promoter. They used very young mice (postnatal day
567 7) and isolated EGCs from the whole intestine by enzymatic digestion[19]. In addition, flow
568 cytometric analysis was performed after two weeks of maintaining conditions that promoted

569 free-floating neurosphere formation. Although the authors reported that the neurospheres
570 highly expressed both GFAP and S100b, the floating cell aggregates also strongly expressed α-
571 SMA, suggesting that expansion of the myofibroblast cell population encouraged gliosphere
572 development in contrast to the flat sheet-like morphology described here. Therefore, while
573 interesting, the study is not directly comparable to this protocol. By contrast, the morphology of
574 the cells coupled with significantly less α-SMA+ cells suggest that the EGCs described in this
575 protocol do not exhibit the 3-dimensional morphology during the 3 to 5-day culture period.
576 Nevertheless, both the protocol here and the Rosenbaum method use a fluorescent reporter to
577 identify and isolate GFAP+ cells that will improve investigator's ability to do live cell profiling.
578
579 In conclusion, the current protocol describes the isolation of enteric glial cells from the
580 submucosa using a non-enzymatic approach. The entire protocol takes about 3 h from mouse
581 dissection to initial plating on the pre-coated tissue culture plates. The most time intensive step
582 in the protocol is the removal and preparation of the intestines for the first EDTA incubation.
583 Preparing the plates and storing in DPBS is strongly recommended. The success of the
584 preparation depends upon the efficient removal of the epithelium without damaging the
585 underlying EGCs and adherence to the PDL/Laminin coated plates within 24 h. Primary EGCs are
586 useful for in vitro studies such as biochemical analysis, Ca^{2+} fluxes and adenoviral
587 transfections[10]. It is anticipated that the ability to isolate EGCs from either the submucosa or the
588 LMMP will permit further studies to define the differences in EGC growth properties,
589 differentiation and morphology using whole genome approaches.
590

594

595 **DISCLOSURES:**
596 The authors have nothing to disclose.
597

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