Establishment of Gastrointestinal Epithelial Organoids

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ABSTRACT

The intestinal epithelium constitutes a system of constant and rapid renewal triggered by proliferation of intestinal stem cells (ISCs), and is an ideal system for studying cell proliferation, migration, and differentiation. Primary cell cultures have proven to be promising for unraveling the mechanisms involved in epithelium homeostasis. In 2009, Sato et al. established a long-term primary culture to generate epithelial organoids (enteroids) with crypt- and villus-like epithelial domains representing the complete census of progenitors and differentiated cells. Similarly, isolated ISCs expressing Lgr5 (leucine-rich repeat-containing G protein–coupled receptor) can generate enteroids. Here, we describe methods to establish gastric, small intestinal, and colonic epithelial organoids and generate Lgr5^{+ve} single cell–derived epithelial organoids. We also describe the imaging techniques used to characterize those organoids. This in vitro model constitutes a powerful tool for studying stem cell biology and intestinal epithelial cell physiology throughout the digestive tract. *Curr. Protoc. Mouse Biol.* 3:217-240 © 2013 by John Wiley & Sons, Inc.

Keywords: gastrointestinal stem cells • 3-dimensional cell culture • organoids • Lgr5 cell sorting • imaging

INTRODUCTION

The intestine is organized into crypt-villus units lined with a monolayer of columnar epithelium that undergoes constant and rapid renewal. Proliferation within the epithelium is confined to the crypts, which contain intestinal stem cells (ISCs) near the crypt base. ISCs give rise to all intestinal epithelial lineages, i.e., enterocytes, enteroendocrine cells, and goblet cells, as well as Paneth cells in the small intestine (Noah et al., 2011). The different immature cell types differentiate progressively as they migrate out of the crypts toward the tips of the villi, to be finally extruded into the lumen, except Paneth cells, which stay in the crypt region. The colon is characterized by elongated glands and absence of villi. The colonic epithelium is composed mostly of absorptive cells (colonocytes) and goblet cells, with sparse enteroendocrine cells and no Paneth cells.

Various tissue culture technologies, primarily for transformed and cancer-derived intestinal epithelial cell lines, have proven to be important tools for the study of intestinal physiology and have been useful experimental systems to elucidate mechanisms of proliferation, barrier function, and epithelial nutrient and ion transport. However, none of these clonal cell cultures reflect the morphological and functional nature of the

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Table 1 Reagents Used in Establishment of Gastrointestinal Epithelial Organoids

Reagent name	Supplier	Cat. no.	Solvent	Stock solution	Final conc.
Minigut medium					
Advanced DMEM/F12	Invitrogen	12634-028	—	—	—
HEPES, 1 M	Invitrogen	15630-080	—	1 M	10 mM
GlutaMAX	Invitrogen	35050-061	—	100×	1×
Pen/Strep	Invitrogen	15140-148	—	100×	1×
N2 Supplement	Invitrogen	17502-048	—	100×	1×
B27 Supplement	Invitrogen	17504-044	—	50×	1×
N-Acetylcysteine	Sigma-Aldrich	A9165-5G	—	Powder	1 mM
BSA, Fraction V	Fischer	BP1600	—	Powder	1%
Growth factors, hormones, and inhibitors					
Matrigel, GFR, phenol free	BD Bioscience	356231	—	—	—
Human recombinant Noggin	R&D	6057-NG/CF	DPBS	100 μg/ml	100 ng/ml
Mouse recombinant R-Spondin	R&D	3474-RS	DPBS	1 mg/ml	1 μg/ml
Mouse recombinant Wnt3a	R&D	1324-WN/CF	DPBS	100 μg/ml	100 ng/ml
Human recombinant EGF	Sigma-Aldrich	E9644-.2MG	DPBS	500 μg/ml	50 ng/ml
Y-27632	Sigma-Aldrich	Y0503-1MG	H_2O	10 mM	10 μM
CHIR99021	Stemgent	04-0004	DMSO	10 mM	2.5 μM
Thiazovivin	Stemgent	04-0017	DMSO	10 mM	2.5 μM
[Leu15]-Gastrin I human	Sigma	G9145	DPBS	10 μM	10 nM
Human recombinant FGF10	PeproTech	100-26	DPBS	100 μg/ml	100 ng/ml
n-Acetylcysteine	Sigma	A7250	dH_2O	500 mM	1 mM
Crypt isolation reagents					
DPBS Ca^{2+}, Mg^{2+} free	Thermo Sci.	SH3002802	—	—	—
PBS	see recipe in Reagents and Solutions	—	—	—	—
EDTA	Sigma-Aldrich	431788	dH_2O	0.5 M	2 mM
FACS reagents					
Annexin V AlexaFluor 647 conjugate	Invitrogen	A23204	—	50×	1×
7-AAD	Invitrogen	A1310	DMSO	1 mg/ml	10 μg/ml
Immunofluorescence antibodies					
Rat anti–E-cadherin	Santa Cruz	sc-59778	PBS	200 μg/ml	2 μg/ml
Goat anti–rat IgG AlexaFluor 633 conjugate	Invitrogen	A21094	PBS	2 mg/ml	20 μg/ml
Goat F(ab′)2 anti–rabbit IgG AlexaFluor 488 conjugate	Invitrogen	A11070	PBS	2 mg/ml	20 μg/ml

intestinal epithelium. In contrast, primary cell cultures that allow maintenance of a more physiological environment for the epithelial cells have proven to be promising (Simon-Assmann et al., 2007).

Recently, Sato and colleagues have established long-term culture conditions under which single crypts or isolated stem cells from the stomach, small intestine, or colon grow to form crypt/glandular structures that expand via continual fission events, while continuously producing all of the differentiated cell types specific to the tissue of origin (Barker et al., 2010; Sato et al., 2009, 2011). These three-dimensional epithelial structures were originally called "organoids," but to avoid confusion among tissues and to distinguish these cultures from previous "organoids" composed of crypts and pericryptal myofibroblasts (Tait et al., 1994; Spence et al., 2011), we collectively term these three-dimensional structures epithelial organoids. More specifically, epithelial organoids from the stomach are gastroids, those from the small intestine are enteroids (Stelzner et al., 2012), and those from the colon are colonoids (Ramalingam et al., 2012; Stelzner et al., 2012). These experimental model systems constitute useful tools for studying the regulation of gastrointestinal stem cells as well as the proliferation and the differentiation of the intestinal epithelial cells throughout the digestive tract.

Here we describe methods to establish epithelial organoids from small intestine (Basic Protocol 1), stomach (Alternate Protocol 1), and colon (Alternate Protocol 2) crypts, as well as the generation of Lgr5^{+ve} single cell–derived epithelial organoids (Basic Protocol 2). In this methodological review, we also emphasize the imaging modalities that could be used to characterize this system (Basic Protocol 3) and the possible experimental strategies carried out by this model (see Commentary).

Refer to Table 1 for an alternate tabulation of key media, solutions, and reagents.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for care and use of laboratory animals.

DERIVATION OF ENTEROIDS FROM SMALL-INTESTINAL CRYPTS

BASIC PROTOCOL 1

In this section, we describe a protocol for the isolation and culture of primary small intestine crypts into three-dimensional units called enteroids. This method is the basis for other epithelial organoid cultures, which will be presented (see Fig. 1) as Alternate Protocol 1 (gastric) and Alternate Protocol 2 (colon). This basic protocol outlines the isolation process and culture of small intestinal crypts as well as the maintenance of the enteroids over time.

Materials

Mice: C57BL6/J strain (The Jackson laboratory) aged 6 to 8 weeks
70% ethanol
Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} (DPBS: Thermo Fisher Scientific, cat. no. SH3002802)
Crypt chelating buffer (see recipe)
Dissociation buffer (see recipe), cold
Matrigel, growth factor reduced (GFR), phenol red free (R&D Systems)
Murine recombinant R-spondin 1 (R&D Systems, 1000× stock; 1 mg/ml in sterile DPBS/0.1% BSA)
Murine recombinant Noggin (R&D Systems, 1000× stock; 100 μg/ml in sterile DPBS/0.1% BSA)
Human recombinant EGF (R&D Systems, 10,000× stock; 500 μg/ml in sterile DPBS/0.1% BSA)
Basal minigut medium (see recipe)

Complete minigut medium (see recipe)
Freezing medium (see recipe)
Isopropyl alcohol
Liquid N_2
Murine recombinant Wnt3a (R&D Systems, 1000× stock; 100 μg/ml in sterile DPBS /0.1% BSA)
Y27623 compound (Sigma-Aldrich, 10 mM in ultrapure H_2O, filter sterilized with 0.22-μm filter)

24-well plate
Tissue forceps
Surgical scissors
10-ml syringe with 18-G needle
Razor blades
15- and 50-ml conical polypropylene tubes
Orbital shaker
70-μm cell strainer
1-ml syringe with 27½-G needle (insulin syringe)
5-ml round-bottom tubes
Refrigerated centrifuge
Inverted microscope
Cryovials
Freezing container (e.g., Mr. Frosty from Thermo Scientific Nalgene)
Liquid N_2 storage container

Additional reagents and equipment for rodent euthanasia (Donovan and Brown, 2006)

Isolation of small intestinal crypts

1. Prepare all the reagents before the beginning of the experiment. Thaw the Matrigel on ice and pre-incubate a 24-well plate in a CO_2 incubator at 37°C.

2. Sacrifice mice using an authorized, legal method approved by the institution where the research is to be conducted.

   Euthanize mice with CO_2, immediately followed by cervical dislocation (Donovan and Brown, 2006).

3. Wet the abdomen of the mouse with 70% ethanol.

4. Make an incision into the abdominal cavity just cranial to the external genitalia. Extend the incision to the rib cage by cutting the abdominal musculature on both sides. Grasp the duodenum and cut the intestine from the stomach at the pyloric sphincter. Gently pull the intestine out of the abdominal cavity, cutting the mesentery with scissors as needed, and cut the distal segment at the ileocecal junction.

5. Flush the intestine with ice-cold DPBS using a 10-ml syringe mounted with an 18-G needle.

   The needle is placed into the lumen and the flushing proceeds until the DPBS becomes clear.

6. Cut the dissected intestine open lengthwise and chop with a razor blade into 2- to 4-cm pieces in ice-cold PBS. Place in a 15-ml conical tube filled with 10 ml ice-cold DPBS.

7. Gently invert the tube four times and discard the supernatant. Add 10 ml of ice-cold DPBS.

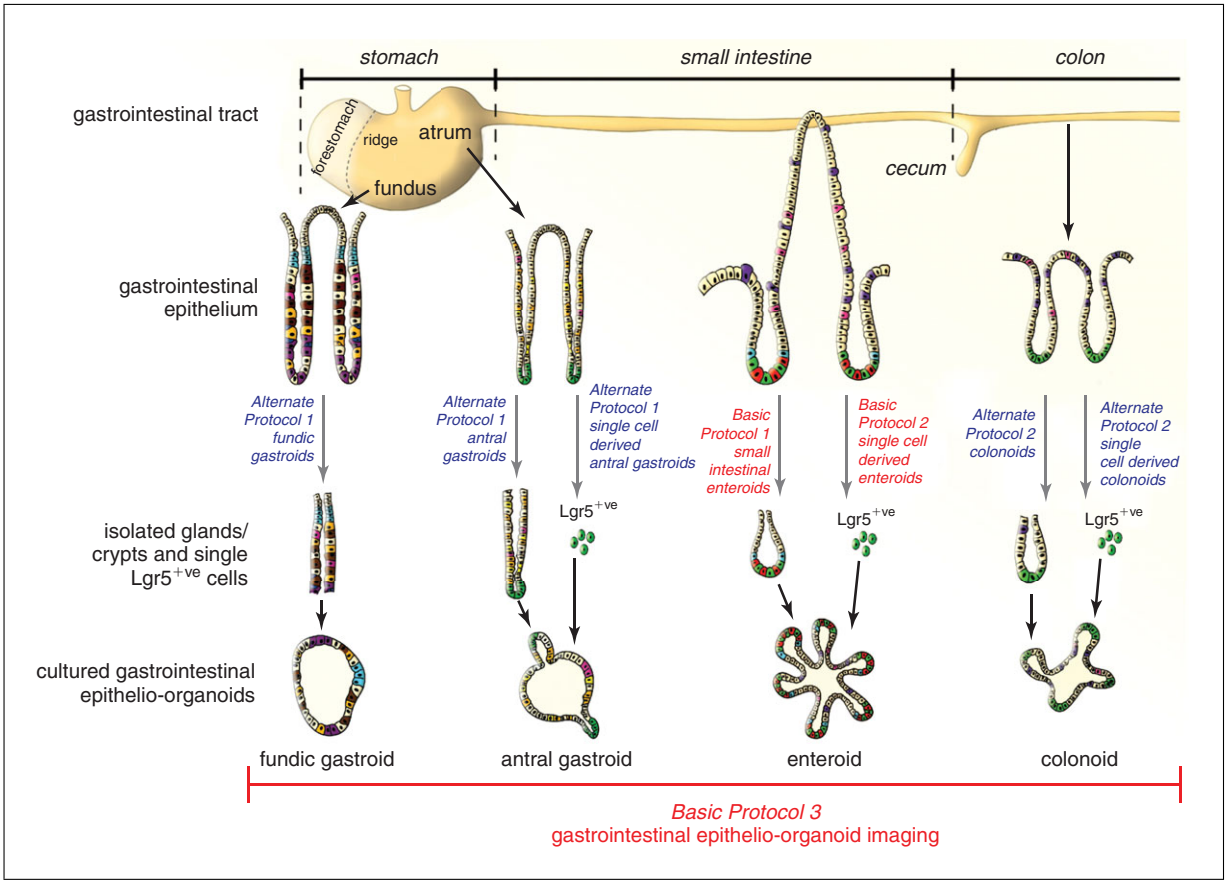

Figure 1 Workflow of gastric glands and intestinal crypts dissociation and generation of epithelial organoids in culture. Gastrointestinal tissues are processed differently according to their location. Cultured glands or crypts form epithelial organoids: fundic or antral gastroids for the stomach, enteroids for the small intestine, and colonoids for the colon. In addition to the gland/crypt culture, epithelial organoids can also be generated from single FACS-sorted stem cells.

8. Remove the tissue with forceps and cut into <5 mm pieces, then place it into a 15-ml conical tube containing with 5 ml of crypt chelating buffer.

9. Bury the tube on ice horizontally. Gently shake the tube for 30 min on an orbital shaker.

10. Gently invert the tube, allow the fragments to settle at bottom of tube, and discard the supernatant. Repeat the procedure twice. Add 5 ml cold dissociation buffer.

11. Shake the tube for 3 to 7 min depending on the tissue type—i.e., duodenum or ileum, respectively. With tube oriented perpendicular to the ground, shake by hand at 2 to 3 cycles per sec to dissociate epithelium from the basement membrane.

    Wrap the tube with paper towels or use an insulated glove to keep it cool.

12. Use forceps to remove any large remnant intestinal tissues, freed of crypts and villi (Fig. 2).

    The cell suspension can be observed under a microscope to check the crypts and villi enrichment.

13. Filter the solution through a 70-µm filter into a 50-ml conical tube to remove the villus fraction and collect the crypts fraction.

    The cell strainer can be washed with an additional 5 ml of dissociation buffer.

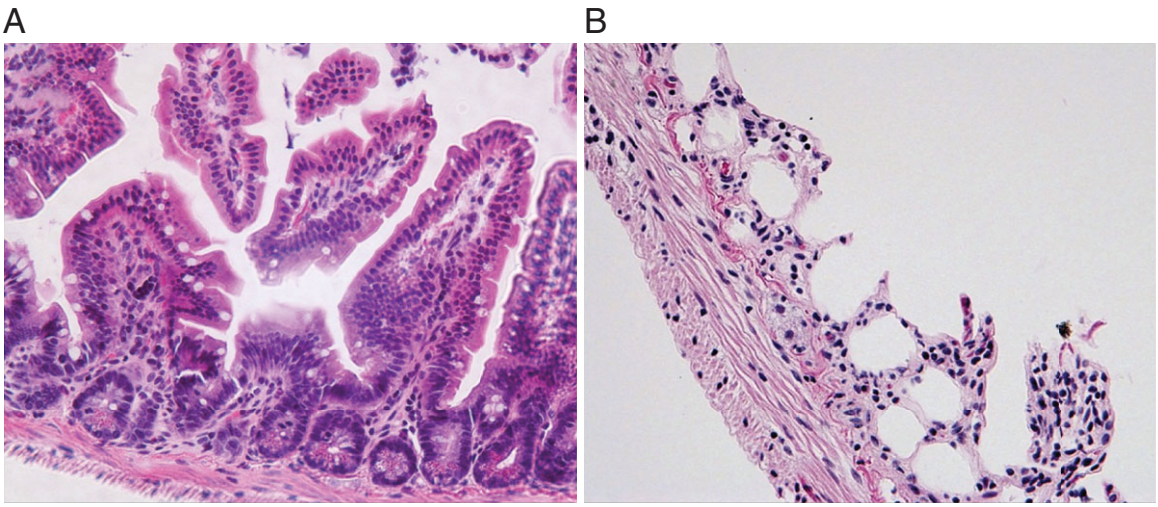

Figure 2 Hematoxylin-eosin sections of murine small intestine. (**A**) Intestinal tissue before crypts isolation by EDTA chelation. (**B**) Intestinal tissue, after EDTA chelation, freed of intestinal crypts and villi.

14. Centrifuge the crypts fraction 10 min at 150 × *g*, 4°C.

    The centrifugation in dissociation buffer allows the crypts to pellet but single cells remain in suspension.

15. Resuspend the pellet in 5 ml ice-cold DPBS.

16. Count the number of crypts per 10-µl drop from the crypts suspension under a microscope; the total number of crypts correspond to the number of counted crypts times 500. Take the corresponding volume out of the crypts suspension to plate 200 to 500 crypts per well, and transfer to a 5-ml round-bottom tube.

17. Centrifuge the crypts fraction 10 min at 150 × *g*, 4°C. Remove the supernatant.

Small intestinal crypt culture

18. Mix the Matrigel with the growth factors on ice. Per 50 µl of Matrigel, add 0.5 µl of 1 mg/ml R-spondin 1 (1 µg/ml final), 0.5 µl of 100 µg/ml Noggin (100 ng/ml final), and 0.05 µl of 500 µg/ml EGF (50 ng/ml final).

19. Using pre-chilled pipet tips, resuspend the crypts pellet (from step 17) in Matrigel supplemented with growth factors (200 to 500 crypts/50 µl Matrigel).

20. Apply 50 µl of crypts suspension in Matrigel per well on the pre-warmed plate. Slowly eject the Matrigel in the center of the well.

21. Place the 24-well plate in a 37°C, 5% CO_2 incubator for 20 min to allow a complete polymerization of the Matrigel.

22. Overlay the Matrigel with 500 µl of basal minigut medium.

23. Culture the plate in a 37°C, 5% CO_2 incubator (Fig. 3C).

24. Every 4 days, replace the medium with fresh complete minigut medium.

Passaging of enteroids culture

Enteroids can be passaged 7 to 10 days after seeding.

25. Prepare all the reagents before the beginning of the experiment. Thaw the Matrigel on ice and pre-incubate a 24-well plate in a CO_2 incubator at 37°C.

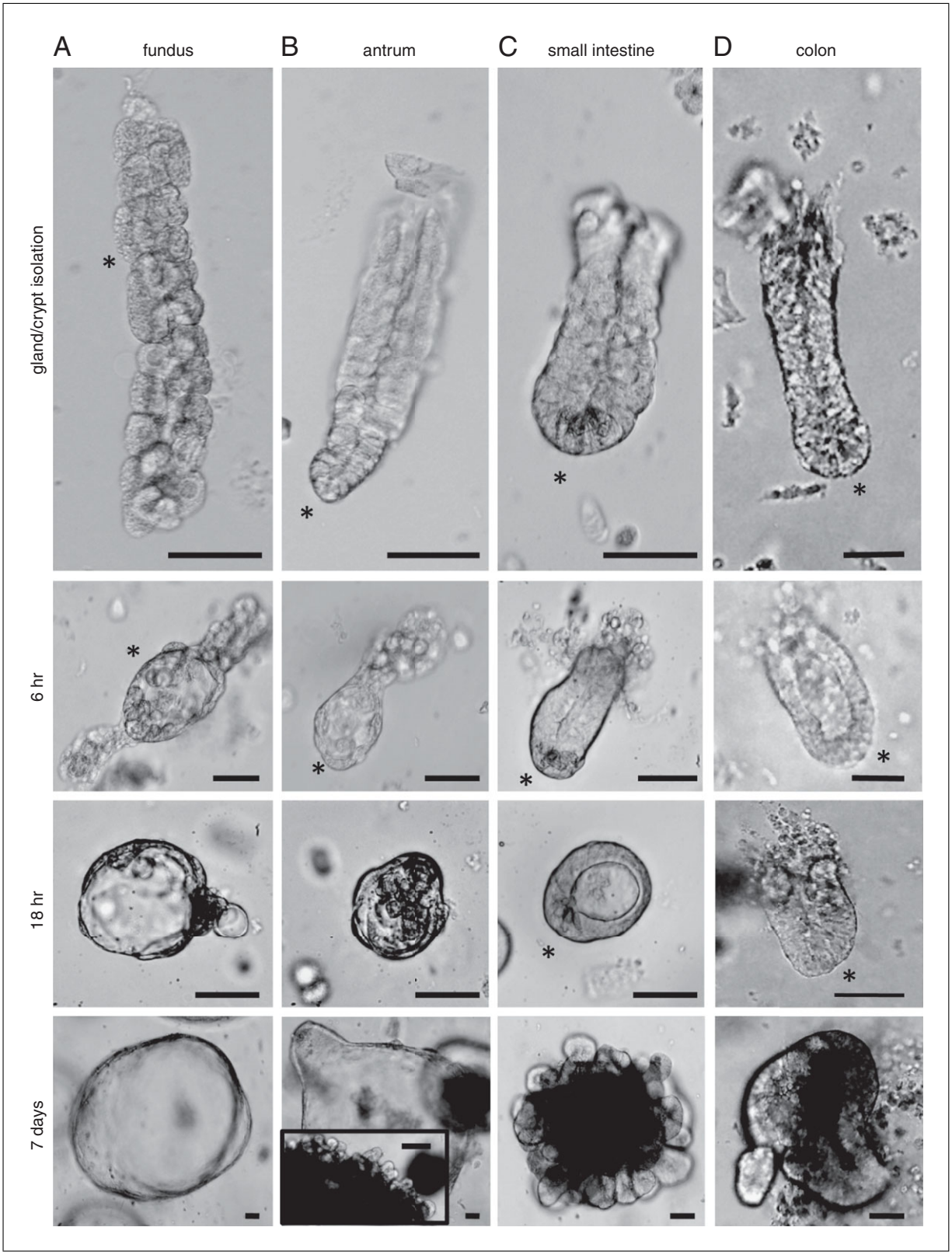

Figure 3 Crypt/gland culture and gastrointestinal epithelial organoid generation. (**A**) Fundic glands plated in Matrigel after isolation. The gland is closing up after 6 hr and starts to balloon up beyond this time. At 7 days, the fundic gastroid is formed. (**B**) After isolation and culture, antral glands behave like the fundic glands and form a gastroid. (**C**) Small-intestine crypts are plated in Matrigel after isolation and close up 6 hr later. The closing crypt forms an enterosphere that undergoes extensive budding by 7 days. (**D**) Colonic crypts are plated in Matrigel after isolation. The crypt closes and forms a colonoid after 7 days. Scale bar = 50 μm.

26. Remove medium and add 1 ml of ice-cold DPBS to each well. Break up the Matrigel by pipetting back and forth several times with 1000-µl (P-1000) tips.

27. Remove the Matrigel suspension with a 1-ml syringe equipped with a 27½-G needle. Pass the total volume through the needle by forcefully syringing one time.

28. Transfer the suspension into a 5-ml round-bottom tube filled with ice-cold DPBS.

29. Centrifuge and resuspend the dissociated enteroids in Matrigel as in steps 18 to 24.

    Usually, one well of enteroids can be split into three to four wells.

Freezing the enteroids

Enteroids can be frozen 2 to 3 days after passaging.

30. Remove the medium and add 1 ml of ice-cold DPBS to each well. Break up the Matrigel by pipetting back and forth several times with 1000-µl (P-1000) tips.

31. Transfer the suspension from two to three wells into a 5-ml round-bottom tube filled with ice-cold DPBS.

32. Centrifuge 10 min at 150 × *g*, 4°C, and resuspend enteroids in freezing medium using 1 ml of freezing medium per three collected wells.

33. Place 1 ml of enteroids in freezing medium in a labeled cryovial. Place the cryovial in a freezing container containing 500 ml of isopropyl alcohol.

34. Transfer the freezing container to a −80°C freezer for 24 hr then, transfer cryovial to liquid nitrogen storage.

    The enteroids can be stored at least for 1 year.

Thawing the enteroids

35. Thaw the Matrigel on ice and pre-incubate a 24-well plate in a CO_2 incubator at 37°C.

36. Thaw the cryovial at 37°C in a water bath.

    The thawing is complete when the medium becomes liquid. Do not let the medium warm up, as this could affect the efficiency of the culture.

37. Aspirate the solution out of the cryovial and transfer it into a 15-ml conical tube containing 5 ml ice-cold basal minigut medium without growth factors.

38. Centrifuge and resuspend the enteroids in Matrigel as in steps 18 to 21.

    Usually, one cryovial of enteroids can be split into two wells.

39. Overlay the Matrigel with 500 µl of basal minigut medium supplemented with 100 ng/ml Wnt3a (1:1000 stock dilution) and 10 µM Y27623 compound at a 1:1000 stock dilution.

    Wnt3a and Y27623 compound are only added after the seeding.

40. Culture the plate in a 37°C, 5% CO_2 incubator.

ALTERNATE PROTOCOL 1

PRIMARY GASTRIC EPITHELIAL CULTURE FROM THE FUNDUS OR ANTRUM

This protocol will describe the isolation and culture of gastric epithelial organoids (gastroids) isolated from the fundus or antrum. Based on Basic Protocol 1, we delineate the steps specific to the fundic or antral tissue isolation and the culture of gastroids from this area.

Additional Materials *(also see Basic Protocol 1)*

Mice: C57BL6/J strain (The Jackson laboratory) aged at least 6 weeks
Gastric gland chelating buffer: 5 mM EDTA in DPBS
Human recombinant FGF10 (PeproTech, 1000× stock; 100 μg/ml in sterile DPBS/0.1% BSA)
Human [Leu15]-Gastrin I (Sigma-Aldrich, 1000× stock; 10 μM in sterile DPBS/0.1% BSA)
N-Acetylcysteine (Sigma-Aldrich, 500× stock; 500 mM in ultrapure H_2O, filter sterilized with 0.22-μm filter)

Silicone-coated dish: silicone made in glass dish using SYLGARD 184 Silicone Elastomer kit according to manufacturer's instructions (Dow Corning, cat. no. 3097358-1004)
Dissecting microscope
Micro-dissecting curved scissors
Two pairs of #7 fine point curved forceps

Isolation of fundic and antral gland

1. Perform steps 1 to 3 of Basic Protocol 1.

2. Make an incision into the abdominal cavity just cranial to the external genitalia. Extend the incision to the rib cage by cutting the abdominal musculature on both sides. Grasp the forestomach and cut the esophagus and immediately distal to the pylorus (proximal duodenum). Pull whole stomach out of the abdominal cavity and open along the greater curvature.

3. Wash the opened stomach with ice-cold DPBS.

4. Pin opened stomach (luminal side down) on the silicone-coated dish filled with ice-cold DPBS.

5a. *For isolation of fundic glands:* Under a dissecting microscope, strip the serosal muscle in the fundic region using micro-dissecting curved scissors and fine point curved forceps (Fig. 4A).

5b. *For isolation of antral glands:* Under a dissecting microscope, strip the serosal muscle in the antral region using two pairs of fine-point curved forceps (Fig. 4B).

6. Cut fundic or antral region from which the muscle was stripped and chop into <5 mm pieces.

7. Remove the tissue with forceps and place into a 15-ml conical tube filled with 5 ml of gastric gland chelating buffer.

   If there is trouble with tissue dissociation, use 10 mM EDTA in the gastric gland chelating buffer.

8. Bury the tube on ice horizontally. Gently shake the tube for 2 hr on an orbital shaker.

9. Gently invert the tube, allow the fragments to settle at bottom of tube, and discard the supernatant. Add 5 ml dissociation buffer.

10. With tube oriented perpendicular to the ground, shake by hand for 1 to 2 min at 2 cycles per sec to dissociate epithelium.

11. Follow steps 14 to 17 in Basic Protocol 1.

Gastroid culture

12. Mix the Matrigel with the growth factors on ice. Per 50 μl of Matrigel, add 0.5 μl of 100 μg/ml Wnt3a (100 ng/ml final), 0.5 μl of 1 mg/ml R-spondin 1 (1 μg/ml

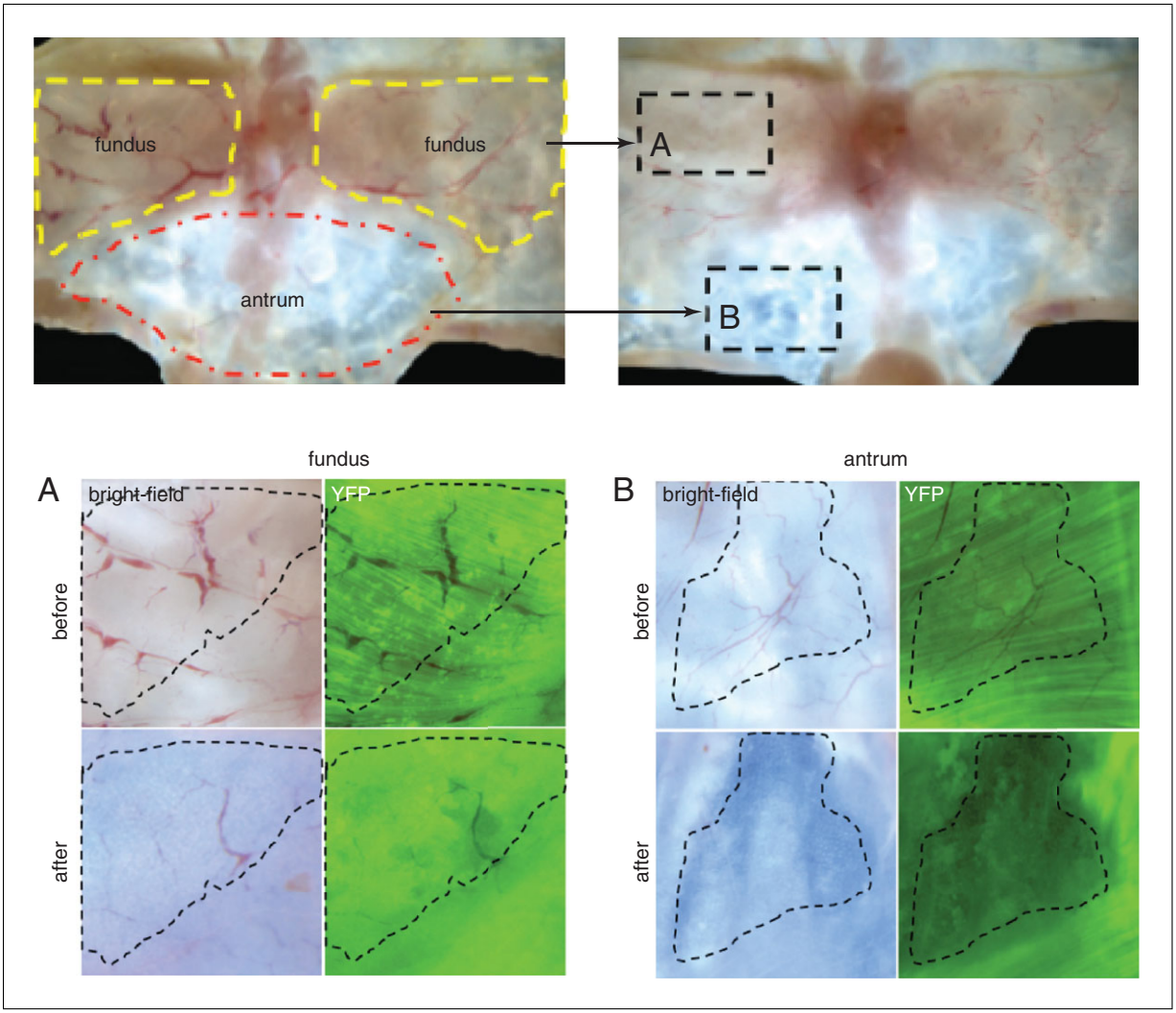

Figure 4 Dissection process for stomach. The stomach (from cytosolic YFP–expressing mouse) is opened lengthwise and stretched into a silicone-coated dish (top left). Fundic (outlined in yellow) and antral (outlined in red) regions are identified, and the muscle layer is dissected from the glands (top right). Fundus (**A**) and antrum (**B**) show magnified region before and after dissection. Dissected region is indicated by dotted outline. Under bright-field, glands can be observed following removal of the muscle layer as individual light spots. Loss of muscle structure can be seen using YFP fluorescence.

final), 0.5 µl of 100 µg/ml Noggin (100 ng/ml final), 0.5 µl of 100 µg/ml FGF10 (100 ng/ml final), 0.5 µl of 10 µM gastrin (10 nM final), 1 µl of 500 mM *n*-acetylcysteine (1 mM final), 0.05 µl of 500 µg/ml EGF (50 ng/ml final), and 0.5 µl of 10 mM Y-27632 (10 µM final; only add for fundus).

13. Using pre-chilled pipet tips, resuspend the gland pellet in the Matrigel supplemented with growth factors (200 to 500 glands/50 µl Matrigel).

14. Apply 50 µl of gland suspension in Matrigel suspension per well on the pre-warmed 24-well plate set up at step 1. Slowly eject the Matrigel into the center of each well.

15. Place the 24-well plate in a 37°C, 5% CO_2 incubator for 20 min to allow a complete polymerization of the Matrigel.

16. Overlay the Matrigel with 500 µl of basal minigut medium.

17. Culture the plate in the 37°C, 5% CO_2 incubator (Fig. 3A-B).

18. Every 4 days, remove medium and replace with fresh complete minigut medium supplemented with 100 ng/ml Wnt3a, 100 ng/ml FGF10, 10 nM gastrin, and 1 mM *n*-acetylcysteine.

Gastroid culture passaging

19. See Basic Protocol 1, steps 25 to 29.

Freezing and thawing the gastroids

20. See Basic Protocol 1, steps 30 to 40.

CULTURE OF COLONOIDS DERIVED FROM COLONIC CRYPTS

ALTERNATE PROTOCOL 2

In this second alternate protocol, we describe a method for the isolation and culture of primary colonic epithelial organoids (colonoids). This alternate protocol outlines the isolation process and culture of colonic crypts as well as the maintenance of the colonoids over time.

For materials, see Basic Protocol 1.

1. Perform steps 1 to 3 of Basic Protocol 1.
2. Make an incision into the abdominal cavity just cranial to the external genitalia. Extend the incision to the rib cage by cutting the abdominal musculature on both sides. Grasp the duodenum and cut the intestine from the stomach at the pyloric sphincter. Gently pull the intestine out of the abdominal cavity, cutting the mesentery with scissors as needed. Cut the proximal colon from the cecum and the distal colon at the anal margin.
3. Prepare the colon for crypt isolation as described in Basic Protocol 1, steps 5 to 10.
4. Shake the tube for 8 min. With tube oriented perpendicular to the ground, shake by hand at 2 to 3 cycles per sec to dissociate epithelium from the basement membrane.

   Wrap the tube with paper towels or use an insulated glove to keep it cool.

5. Use forceps to remove remnant intestinal tissue, freed of crypts, and follow steps 13 to 17 in Basic Protocol 1, except substitute a 100-μm filter in place of the 70-μm filter in step 13 (Fig. 2).

   The cell suspension can be observed under a microscope to check the crypts enrichment.

6. Mix the Matrigel with the growth factors on ice. Per 50 μl of Matrigel, add 0.5 μl of 100 μg/ml Wnt3a (100 ng/ml final), 0.5 μl of 1 mg/ml R-spondin 1 (1 μg/ml final), 0.5 μl of 100 μg/ml Noggin (100 ng/ml final) and 0.05 μl of 500 μg/ml EGF (50 ng/ml final).
7. Terminate the seeding of the colonic crypts according to Basic Protocol 1, steps 19 to 24 (Fig. 3D).

   Maintenance, passaging, and freezing procedures are listed in Basic Protocol 1.

Lgr5-GFP^{+ve} GASTROINTESTINAL STEM CELL SORTING AND CULTURE

BASIC PROTOCOL 2

In this section, we describe a protocol for the isolation and culture of Lgr5-GFP^{+ve} FACS-sorted cells. This approach allows the establishment of single cell–derived enteroids from small intestine crypts. This basic protocol outlines the isolation process and culture of single cells (Fig. 5). The strategy for isolating and culturing gastric (antral) and colonic Lgr5-GFP positive cells is identical to that for the small intestine, with the addition of tissue-specific growth factors as described in the alternate protocols, above.

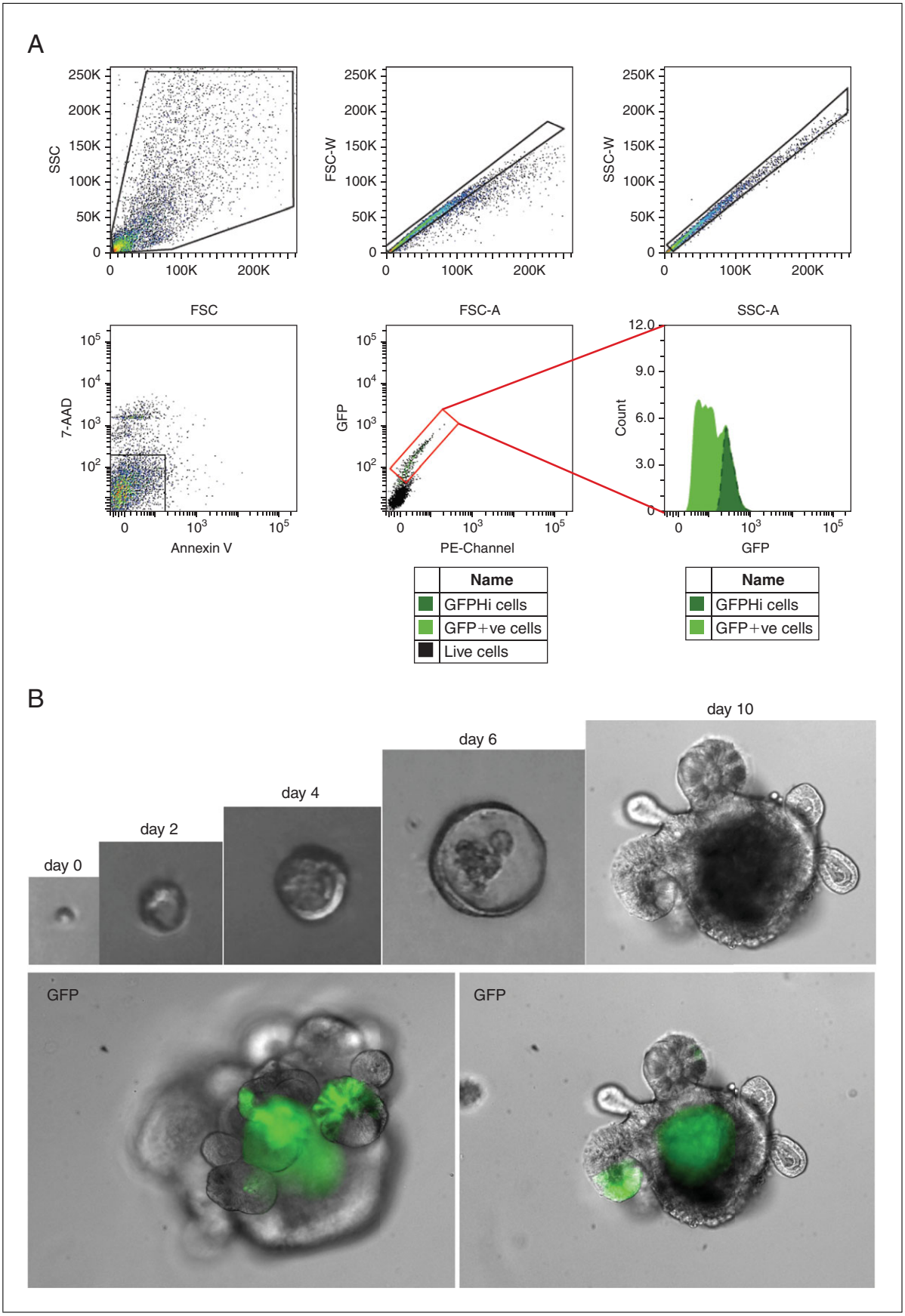

Figure 5 *(legend appears on next page)*

Materials

Lgr5-GFP^{+ve}-ires-CreER C57BL6/J mouse (The Jackson laboratory) aged 6 to 8 weeks
TryPLE Express (Invitrogen).
Y-27632 (Sigma-Aldrich, 10 mM in ultrapure H_2O, filter sterilized with 0.22-μm filter)
Basal minigut medium (see recipe)
N-acetylcysteine (Sigma-Aldrich, 500× stock; 500 mM in ultrapure water, filter sterilized with 0.22-μm filter)
Bovine serum albumin (BSA)
7-Aminoactinomycin D (100× stock; 500 μg/ml in sterile DPBS, Invitrogen, cat. no. A1310; Ex/Em (nm), 548/649)
APC-Annexin V (Invitrogen, cat. no. A35110; Ex/Em (nm), 650/660).
Matrigel, growth factor reduced (GFR), phenol red free (R&D Systems)
Jagged-1 Fc chimera peptide (R&D Systems, 1000× stock; 500 μg/ml in sterile DPBS)
Murine recombinant Wnt3a (R&D Systems, 1000× stock; 100 μg/ml in sterile DPBS/0.1%BSA)
Murine recombinant R-spondin 1 (R&D Systems, 1000× stock; 1 mg/ml in sterile DPBS/0.1% BSA)
Murine recombinant Noggin (R&D Systems, 1000× stock; 100 μg/ml in sterile DPBS/0.1% BSA)
Human recombinant EGF (R&D Systems, 10,000× stock ; 500 μg/ml in sterile DPBS/0.1% BSA)
CHIR99021 (4000× stock, 10 mM in DMSO; Stemgent, *https://www.stemgent.com*)
Thiazovivin (4000× stock, 10 mM in DMSO; Stemgent, *https://www.stemgent.com*)

MACS C-Tubes (Miltenyi Biotec)
GentleMACS Dissociator (Miltenyi Biotec)
50-ml conical polypropylene tubes (e.g., BD Falcon)
40-μm cell strainer
Refrigerated centrifuge
Hemacytometer
FACS tube with 35-μm mesh cap
Cell sorter (BD FACSAria II; Beckman-Coulter MoFlo XDP)
96-well plate

Additional reagents and equipment for isolation of crypts from mouse (Basic Protocol 1, steps 1 to 10), and counting cells using a hemacytometer and trypan blue exclusion test for cell viability (Sandell and Sakai, 2011)

Isolation of small intestinal crypts

1. Isolate crypts from Lgr5-GFP^{+ve}-ires-CreER mouse as described in Basic Protocol 1, steps 1 to 13.

   GFP-positive cells are most abundant in the proximal third of the small intestine; therefore, the proximal one-third of the small intestine is typically used for flow cytometry of this cell population.

Figure 5 *(image appears on previous page)* Intestinal murine Lgr5-GFP^{+ve} stem cell sorting. (**A**) Gating strategy to isolate Lgr5-GFP^{+ve} intestinal single cells from gastrointestinal tissues. (**B**) After the sorting, the single cells are plated into Matrigel. Here, a single Lgr5-GFP^{+ve} cell from the small intestine undergoes several divisions to give rise to an enterosphere. At day 10, a single cell–derived enteroid is formed with multiple buds. GFP expression is not always sustained during the growth of the epithelial organoids. GFP expression is epithelial organoids is dynamically mosaic.

2. With the tube oriented perpendicular to the ground, shake by hand at 2 to 3 cycles per sec to dissociate the crypt fraction another 3 min to promote the dissociation of the crypts.

3. Centrifuge the crypts fraction 5 min at 50 × *g*, 4°C.

   This step will eliminate the mucus and most blood cells in the supernatant.

4. Resuspend the pellet in 5 ml of pre-warmed TryPLE Express supplemented with 10 μM Y27632 (1:1000 stock dilution). Transfer the reconstituted crypts into a C-tube.

5. Run the pre-set program `m-intestine-1` on the GentleMACS dissociator at room temperature and incubate the tube for 5 min in a water bath at 37°C.

   The dissociation program consists of four rotation cycles of 15 sec each, anti- and clockwise. The manufacturer does not provide the program specifications.

   If a GentleMACS dissociator is not available, crypts can be incubated in TrypLE Express for 60 to 90 min, with gentle trituration every 10 to 15 min. Change the TrypLE solution once after 30 to 45 min.

6. After the incubation, again run the program `m-intestine-1` on the GentleMACS dissociator.

7. Transfer the dissociated crypts into a 50-ml conical tube filled with ice-cold basal minigut medium supplemented with 0.5 mM *N*-acetylcysteine (1:1000 stock dilution) and 10 μM Y27632 (1:1000 stock dilution).

8. Filter the cell suspension through a 40-μm cell strainer in a new 50-ml conical tube.

   This step removes any remaining cell clumps from the crypts dissociation.

9. Centrifuge the crypts suspension 5 min at 500 × *g*, 4°C, and gently aspirate the supernatant.

10. Resuspend the pellet in 1 ml ice-cold basal minigut medium supplemented with 0.5 mM *N*-acetylcysteine (1:1000 stock dilution), 10 μM Y27632 (1:1000 stock dilution), and 1% (w/v) BSA. Count the number of cells with a hemacytometer (Sandell and Sakai, 2011) and dilute the cell suspension to a concentration around 2 to 5 × 10^6 cells/ml.

    A trypan blue assay (Sandell and Sakai, 2011) can be done to assess the viability of the cells.

11. Stain the dead and dying cells with 7-aminoactinomycin D (1:100 stock dilution) and Annexin V (1:50 stock dilution) 15 min prior the sorting.

    An Annexin-binding buffer is not necessary, as the minigut medium contains 1 mM $CaCl_2$.

Lgr5-GFP^{+ve} cell sorting

12. Set up a 100-μm nozzle on the sorter. Set up the fluidics to reach at least 80% efficiency during the sort—i.e., flow rate and sample concentration.

    A compensation for correcting the spectral overlap from the fluorophores is necessary. Compensation controls, such as an unstained control, for the fluorescence background, and single-stained controls, one for each fluorochrome, have to be run to apply any compensation on the sample. The gating strategy consists first of doublet discrimination: the single cell population is plotted against forward scatter (FSC) versus side scatter (SSC) and SSC height versus area or FSC height versus area. When single cells pass through the laser beam, their FSC-area and FSC-height signals correlate linearly and plot along a relatively straight line. Clumps of cells will fall off the diagonal formed by single cells. Then, single cells are plotted against 7-aminoactinomycin D and Annexin

V where negative cells are gated. The green autofluorescence of the sample can be excluded by plotting the GFP channel against the phycoerythrin channel. In that case, the background signal triggered by the autofluorescence can be excluded from the GFP-positive gate. The Lgr5-GFP^{+ve} population is defined as the brightest population and is gated on the third part of the GFP histogram (Fig. 5A).

A nozzle <100 μm is detrimental for the intestinal stem cells. A 130-μm nozzle also could be used for this sorting.

Compensation is not necessary when the fluorophore panel does not present any spectral overlap.

13. Sort Lgr5-GFP^{+ve} cells into minigut medium supplemented with 0.5 mM *N*-acetylcysteine (1/1000 stock dilution) and 10 μM Y27632 (1:1000 stock dilution), refrigerated at 4°C.

    For RNA experiments, cells can be sorted directly into Trizol or RNA lysis buffer supplemented with 1% 2-mercaptoethanol.

Single cell–sorted culture

14. Centrifuge the sorted cells 5 min at 500 × *g*, 4°C, and gently pipet off the supernatant.
15. Mix the Matrigel with the growth factors on ice. Per 10 μl of Matrigel, add 0.1 μl of 500 μg/ml Jagged-1 Fc chimera peptide (500 ng/ml final), 0.1 μl of 100 μg/ml Noggin (100 ng/ml final), and 0.01 μl of 500 mg/ml EGF (50 ng/ml).
16. Using pre-chilled pipet tips, resuspend the cell pellet (from step 14) in the Matrigel supplemented with growth factors (200 to 500 cells/10 μl Matrigel).
17. Apply 10 μl of Matrigel suspension per well on a pre-warmed (37°C) 96-well plate. Slowly eject the Matrigel into the center of the well.

    To avoid any spreading of the Matrigel on the bottom of the well, 2 μl of plain Matrigel could be spotted before the Matrigel suspension.

18. Place the 96-well plate in a 37°C, 5% CO_2 incubator for 10 min to allow complete polymerization of the Matrigel.
19. Overlay the Matrigel with 100 μl of basal minigut medium supplemented with 2.5 μM CHIR99021 (1:4000) and 2.5 μM thiazovivin (1:4000).
20. Culture the plate in the CO_2 incubator.
21. Every 2 days, aspirate the medium and replace it with fresh complete minigut medium.

    Jagged-1 Fc chimera peptide is added to 500 ng/ml (final) on day 2 after sorting.

IMAGING OF THE GASTROINTESTINGAL EPITHELIAL ORGANOIDS

BASIC PROTOCOL 3

In this section, we describe the enteroid live-imaging procedure as well as 3-D whole-mount staining. Fluorescently tagged enteroids can be monitored in real time using this procedure.

Materials

Epithelial organoids (Basic Protocol 1)
Complete minigut medium (see recipe)
Phosphate-buffered saline (PBS; see recipe)
4% paraformaldehyde (PFA)
50 mM NH_4Cl in PBS
0.1% (v/v) Triton X-100 in PBS
5% (w/v) bovine serum albumin (BSA) or fetal bovine serum (FBS)

Primary antibody (E-cadherin for epithelial cells; see Table 1)
Secondary antibody (see Table 1)
10 μg/ml Hoechst 33342 (Invitrogen)
Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} (DPBS: Thermo Fisher Scientific, cat. no. SH3002802)
2% methylene blue in PBS
30% (w/v) sucrose
OCT compound (Tissue-Tek)
70% ethanol

8-well Lab-Tek chamber with #1.0 borosilicate coverglass (Thermo Scientific)
CO_2 module S/temperature module S/humidifier S unit (PeCon incubation system, *http://www.pecon.biz/*)
Heating insert P-Labtek S1 (PeCon incubation chamber, *http://www.pecon.biz/*)
Inverted confocal microscope (Zeiss LSM710)
EC Plan-Neofluar 10 × 0.3 (dry) or Plan-Apochromat 20×/0.8 (dry) objective lens
C-Achroplan NIR 40×/0.8 (water) objective lens
1 × 1–cm cryomold

Additional reagents and equipment for passaging epithelial organoids (Basic Protocol 1)

Live imaging

1. Passage epithelial organoids according to steps 25 to 28 of Basic Protocol 1.

2. Apply 25 μl Matrigel/epithelial organoid suspension to wells of 8-well chamber (split 1/2 well from original plate into 8 wells of this chamber).

   Do not put more than 20 organoids, in a single well, to keep medium fresh.

3. Add 400 μl complete minigut medium per well, and culture at in a 37°C, 5% CO_2 incubator until imaging.

4. Set the PeCon incubation chamber to 37°C and 5% CO_2 via the confocal microscope computer.

5. Insert 8-well chamber into the PeCon incubation unit.

   Do not remove cover from chamber, to avoid medium evaporation.

6. Set up optical configurations, example (ZO-1/RFP enteroids created from mouse gifted by Dr. Turner; Guan et al., 2011) as described below (see Videos 1, 2, and 3 at *http://www.currentprotocols.com/protocol/mo130179*).

7. Turn on 560-nm laser with 560-nm dichroic filter and set 565 to 650 nm for emission. Also turn on TPMT transmitted light channel.

8. Find an enteroid that has a bright RFP signal. Optimize laser power and detector gain while keeping the pinhole (30 to 50 μm for Plan-Apochromat 20×/0.8, dry) as small as possible.

   Usually, laser power is set low and detector gain high to avoid fluorescent bleaching during imaging.

   Avoid use of water/oil immersion lens unless imaging for short time period (less than 1 hr).

9. Set *z*-stack parameters by marking the first and last optical sections while adjusting the focus. Set 30 μm of blank space above and below the enteroid to allow for growth. Set slice interval 3 μm (see Troubleshooting).

10. Set time interval at 30 min, then start (see Troubleshooting).

Whole-mount staining

11. Passage epithelial organoids according to steps 25 to 28 in Basic Protocol 1.
12. Apply 25 μl Matrigel/epithelial organoid suspension to wells of 8-well chamber (split one well from original plate into four wells of this chamber).
13. Add 400 μl complete minigut medium per well, and culture in a 5% CO_2/37°C incubator until staining.
14. Remove medium, add 200 μl room temperature PBS, and leave for 5 min.

    Any solution applied to chamber must warm up to room temperature to avoid Matrigel dissolution.

15. Remove PBS, add 200 μl of 4% PFA at room temperature, and leave for 30 min.
16. Repeat step 14 (wash step) twice.
17. Add 200 μl NH_4Cl (50 mM in PBS: room temperature) and leave for 30 min.

    This step will quench autofluorescence (coming from shed cells and debris in the lumen, specifically at 488 nm excitation wavelengths), but if there is fluorescently tagged protein in the enteroid (e.g., ZO1-RFP), this should not apply.

18. Repeat step 14 (wash step) twice.
19. Add 200 μl Triton X-100 (0.1 % in PBS) and leave for 30 min (see Troubleshooting).
20. Repeat step 14 (wash step) twice.
21. Add 200 μl 5% BSA or serum and leave for 60 min.
22. Repeat step 14 (wash step) twice.
23. Add 200 μl primary antibody made in PBS (see Troubleshooting), and leave overnight at 4°C.

    Often, higher concentrations of antibodies are required than used for 2-D staining of tissue sections.

24. Repeat step 14 (wash step) five times.
25. Add secondary antibody made in PBS (see Troubleshooting), and leave overnight at 4°C.

    Often, higher concentrations of antibodies are required than used for 2-D staining of tissue sections.

26. Repeat step 14 (wash step) five times.
27. Add 200 μl Hoechst 33342 (10 μg/ml in PBS), and leave for 20 min.
28. Repeat step 14 (wash step) twice.
29. Observe staining by confocal microscope using a long-distance objective lens [C-Achroplan NIR 40×/0.8 (water) objective lens].

Processing for frozen and paraffin-embedded sections

30. Remove medium and resuspend the Matrigel containing epithelial organoids in ice-cold DPBS.
31. Transfer resuspended epithelial organoids to a microcentrifuge tube.

To prevent loss of epithelial organoids during manipulations, pipet tips have to be coated with FBS.

32. Microcentrifuge 1 min at 100 × *g*, and gently discard the supernatant.

33. Fix the epithelial organoids for 20 min at 4°C in 500 µl of 4% PFA.

34. Microcentrifuge 1 min at 100 × *g*, and remove the PFA.

35. Wash epithelial organoids with PBS and microcentrifuge 1 min at 100 × *g*.

36. Resuspend in 100 µl methylene blue solution for 20 min at room temperature.

    The methylene blue staining facilitates visualization of organoids in OCT or paraffin.

37a. *For frozen sections:* Wash with PBS, resuspend in 30% (w/v) sucrose, and incubate at 4°C overnight. Microcentrifuge 1 min at 100 × *g*, and remove sucrose-containing supernatant. Embed in OCT compound in a 1 × 1–cm cryomold.

    Let the epithelial organoids settle for 30 to 45 min before freezing.

37b. *For paraffin-embedded sections:* Wash with PBS and resuspend in 70% ethanol.

    Process the epithelial organoids manually through the dehydration steps. Spin at 100 × g for 1 min between solution changes. Embed the epithelial organoids in paraffin using a 1 × 1–cm mold and pre-warmed pipet tips.

    The dehydration will pack the epithelial organoids together without altering their morphology.

38. Proceed to the appropriate sectioning and staining suitable for your antibodies.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Basal minigut medium

Advanced DMEM/F12 medium supplemented with:
2 mM GlutaMax
10 mM HEPES
100 U/ml penicillin
100 µg/ml streptomycin
1× N2 supplement
1× B27 supplement
Divide into 10-ml aliquots in 15-ml conical tubes
Freeze

Thawed aliquots can be stored up to 5 days at 4°C without loss of activity.

Complete minigut medium

Basal minigut medium (see recipe) should be mixed with 1 µg/ml R-spondin 1 (1:1000 dilution of 1 mg/ml stock; R&D Systems), 100 ng/ml Noggin (1:1000 dilution of 100 µg/ml stock; R&D Systems), and 50 ng/ml EGF (1:10 000 dilution of 500 µg/ml stock dilution; R&D Systems). Prepare fresh immediately before crypt culture or medium change.

Complete minigut media can be stored up to 2 days at 4°C without loss of activity.

To maintain culture of gastroids and colonoids, 100 ng/ml Wnt3a (1:1000 stock dilution) must be added to supplement the complete minigut medium.

Crypt chelating buffer

EDTA stock solution: 0.5 M ethylenediamine tetraacetic acid (EDTA), pH 8 (Sigma-Aldrich), is prepared in ultrapure water and filter sterilized with a 0.22-μm filter. The EDTA stock solution is stored at room temperature indefinitely.

For intestinal crypt isolation: the following volumes of EDTA and DPBS, should be freshly mixed: 0.4 ml and 99.6 ml. The final 2 mM EDTA solution can be stored at 4°C.

For gastric gland isolation: The following volumes of EDTA and DPBS should be freshly mixed: 1 ml and 99 ml. The final 5 mM EDTA solution can be stored at 4°C.

Dissociation buffer

Dissolve 2 g D-sorbitol (54.9 mM final) and 3 g sucrose (43.4 mM final) in 200 ml DPBS and filter sterilize with a 0.22-μm filter. Store up to 1 month at 4°C.

Freezing medium

Combine 8 ml of Advanced DMEM/F12 (Life Technologies), 1 ml of DMSO (Sigma-Aldrich; 10% final), and 1 ml of complement-inactivated fetal bovine serum (FBS, Life Technologies; 10% final). Prepare fresh.

PBS

0.01 M sodium phosphate, pH 7.4
150 mM NaCl

COMMENTARY

Background Information

Primary culture of adult intestinal epithelium has been reported previously and has permitted the study of basic mechanisms involved in intestinal or pathological cellular mechanisms, but has been limited by the inability to maintain long-term growth and differentiation of primary cells. Colon cancer cell lines have been extensively used for their proliferative and metabolic properties but have extensive mutations and limited capacity for multilineage differentiation under standard culture conditions. Clonogenic growth of nontransformed intestinal epithelial cells has been reported in several different systems (e.g., IEC6, IEC18, MSIE, and YAMC) that allow growth and expansion of the cells, but without multilineage differentiation. In contrast, primary culture combining intestinal crypts and mesenchyme has been reported to retain the multiple cell types, but with limited cellular proliferation. Those models have limits, and may not fully reflect the normal physiology of the intestinal epithelium (Simon-Assmann et al., 2007). To address those problems, transplantation models have been developed to grow freshly isolated intestinal crypts (with attached mesenchyme termed "organoid units") subcutaneously or under the kidney capsule. These grafts have varied from cysts lined with a simple epithelium to multicellular and invaginated structures. However, the successful engraftment of intestinal crypt "organoid units," was dependent on use of fetal or neonatal intestine (Levin et al., 2013). Furthermore, the organoid units used for engraftment are unable to be expanded in vitro.

In 2009, Sato and colleagues described the development of a three-dimensional culture of small intestinal crypts and stem cells into epithelial organoids, termed "enteroids" (Sato et al., 2009). In this model, the intestinal crypts undergo continual crypt budding events and form villus-like epithelial domains that connect the crypts without any support from mesenchyme sources (see Video 4 at *http://www.currentprotocols.com/protocol/mo130179*). The crypt-derived enteroids generate a continuously expanding and self-organizing epithelial structure reminiscent of normal gut, continuously producing all cellular lineages of the intestinal epithelium (Sato et al., 2009). The transplantability of these organoids has been tested. Colon organoids ("colonoids") were instilled into DSS-damaged mouse colon, where they integrated into the recipient mouse colon and reconstituted part of the damaged epithelium to reform crypts within the healed mucosa (Yui et al., 2012). Gastrointestinal epithelial organoids constitute a system to study stemness and stem-cell-driven gastrointestinal

mucosal biology. This technique has been used to test the capacity of isolated single cells to function as stem cells in vitro, as initially used by Sato and colleagues (Sato et al., 2009; Barker et al., 2010; Yui et al., 2012). Several other studies have used fluorescent reporters of gene expression (e.g., Sox9, Dll1, Bmi1; Ramalingam et al., 2012; van Es et al., 2012; Yan et al., 2012) as well as cell surface antigens such as cluster of differentiation (CD) markers (e.g., $CD24^{lo}$, $CD44^{+}CD24^{lo}CD166^{+}$; von Furstenberg et al., 2011; Wang et al., 2013) to enrich for cells with organoid-forming capacity (stem cells). Together, those studies demonstrate the utility of epithelial organoid cultures for testing the stemness of isolated cells. Other investigators have used enteroids to study the fate and function of specific cells. Several studies demonstrated intestinal stem cell niche functions for Paneth or colonic goblet cells (Durand et al., 2012; Farin et al., 2012; Rothenberg et al., 2012; Sato et al., 2011). Similarly, enteroids deficient for Csf1r(-/-), which have a defect in Paneth cell production, showed defective enteroid formation (Akcora et al., 2013).

Physiological studies of intact gastrointestinal epithelium have been limited by problems of accessibility in vivo and dedifferentiation in standard primary culture. Epithelial organoids serve as a replenishable and novel experimental system to study both normal and abnormal gastrointestinal physiology. For example, Mizutani and colleagues used enteroids to evaluate the dynamics of intestinal drug transport. In this report, they investigated the physiological effect of the P-glycoprotein on the bioavailability of lumenally administered drugs to the intestinal epithelium (Mizutani et al., 2012). Other investigators have used enteroids to study the activity of the cystic fibrosis transmembrane conductance regulator (CFTR). Assessed by microelectrode analysis, enteroids exhibited CFTR expression and activity that recapitulated the intestinal epithelium in vivo (Liu et al., 2012). Together, these studies show that gastrointestinal epithelial organoids provide a primary culture model that is suitable for functional, cell biological, and physiological studies of regenerating GI epithelium.

Critical Parameters

Tissue handling and crypt preparation

Delayed crypt isolation and culture could be performed up to 24 hr after tissue collection. The tissue has to be maintained in DPBS at 4°C (Fuller et al., 2013). The delayed preparation allows tissue shipping. However, intestinal tissue has to be placed in a conical tube completely filled with DPBS to avoid any tissue disruption. An insulated box must be used to avoid any temperature variation during the transport.

Growth factors

Recombinant growth factors could be replaced by Wnt3a, R-spondin, and Noggin conditioned media. A Wnt3a-expressing L-cell line is commercially available (ATCC). Other groups have developed R-spondin 1– (Jung et al., 2011; Ootani et al., 2009), Noggin– (Farin et al., 2012), and Wnt3a/R-spondin3/Noggin– (Miyoshi et al., 2012) expressing cell lines.

Lgr5-GFP^{+ve} cell sorting

The experiment has to be carried out on ice as much as possible to avoid apoptosis. The washing and sorting buffers after the cell dissociation must contain an apoptosis inhibitor (Y27623).

Use of viability markers during the sorting process is needed in order to improve the efficiency of single-cell forming epithelial organoids.

For good efficiency of FACS staining, all antibodies should be titrated before the experiments.

Troubleshooting

Chelation and crypts isolation

The chelation step is critical, as it will determine the yield from the crypt preparation. Depending on the organ, the concentration of EDTA could vary from 2 mM to 30 mM. A balance must be achieved between stronger chelation that will release more crypts from the basal membrane, and disintegration of the tissue that will increase the cellular debris in the crypts fraction and contaminate the culture with a high number of apoptotic cells.

Live imaging

As long as low laser power is used, epithelial organoids should stay healthy. However, bleaching of fluorescence may easily occur during live imaging. If you observe bleaching of fluorescence, optimize as following:

Decrease laser power and increase detector gain or pinhole.

Decrease *z*-stack range to minimize imaging above and below the organoid. Even if nothing is in the field of view, any time the laser is turned on, there will be some light load

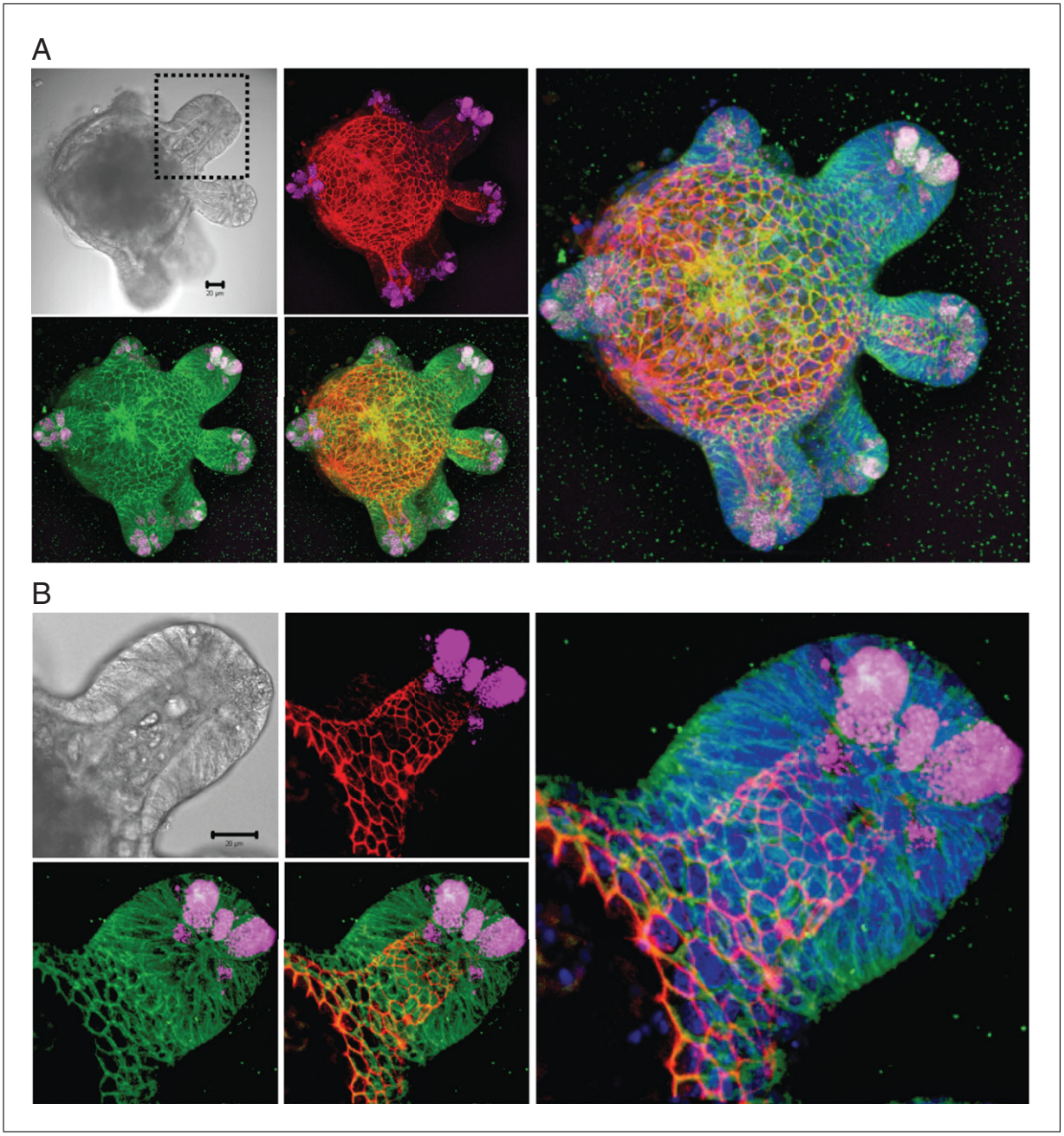

Figure 6 Imaging of the organoids. Confocal imaging and 3-D reconstruction of an enteroid at low (**A**) and high magnification (**B**: outlined area in A). Images show transmitted light, nuclei (blue), E-cadherin (green), Paneth cell (pink), and ZO1-RFP (red). RFP (ZO-1) is endogenously expressed, while E-cadherin is detected using a specific antibody. Paneth cells are marked by nonspecific staining of Alexa Fluor 488 F(ab')2 fragment of goat anti-rabbit IgG. Nuclei are labeled with Hoechst 33342. Scale bar = 20 μm.

that will hit the cell above or below your focal plane and potentially cause photobleaching.

Decrease number of slices taken through the organoid (increase slice interval, > 5 μm) to the number that is necessary to observe the phenomena you are seeking to capture (sometimes you need to have multiple slices through each cell, sometimes you only need to see every third cell).

Increase the time interval between images. Once you know how fast your biological phenomenon is, you can sample at a rate that minimizes light exposure but is sure to capture the biological events of interest.

Immunofluorescence

Some primary or secondary antibody will be taken up by the Paneth cells (Fig. 6), and may appear as nonspecific fluorescence. All antibody combinations should be tested. In most cases, reduced concentration of antibodies may improve specificity.

If staining appears weak, increase cell permeabilization by increasing Triton X-100 up to 0.5%. In addition, antibodies can be diluted in PBS containing 0.1 % Triton X-100 (and serum if there is high background).

Some antibodies appear to stay within the Matrigel and not reach the organoid well. In this case, Matrigel can be diluted with PBS (matrigel:PBS = 2:1) when the organoid is plated in the chamber (Basic Protocol 3, step 12) .

Anticipated Results

Basic Protocol 1

Figure 3 shows a typical example of freshly isolated crypts from the different regions. After isolation, the crypts will round up 3 to 4 hr after seeding in Matrigel. The crypt budding usually occurs 3 to 4 days after seeding. The passaging can be done after 7 days, depending on the organ considered. All the gastrointestinal epithelial organoids present all the differentiated lineages that can be observed by immunofluorescence imaging. Enteroid culture expands in a reproducible manner. However, differences in region and age affect enteroid growth (Fuller et al., 2013).

Basic Protocol 2

Figure 5B shows a typical example of isolated single cells from the different regions. After isolation, the single cell should balloon up after 48 hr post splitting. The first buds usually appear around day 10 and undergo extensive budding beyond this day. Sorted cells express GFP; however, the GFP expression may vary during the growth, and mosaic expression appears in established enteroids.

Basic Protocol 3: Live imaging

Videos 1 to 3 (at *http://www.currentprotocols.com/protocol/mo130179*) show growth of ZO1-RFP tagged enteroids from day 3 to 6, while Videos 5 to 7 (at URL above) show growth of YFP (cytosolic) enteroids from day 0 to 3. ZO1-RFP enteroids grow into spheres, before retraction and observation of budding. In the YFP enteroids, imaging started immediately after passage; the enteroid first seals itself and then begins budding.

Basic Protocol 3: Whole-mount staining

Figure 6 shows nuclei (blue)/E-cadherin (green)/ZO-1 (red)/Paneth (pink) immunofluorescence in enteroids. ZO-1 was endogenously tagged with RFP, while a high concentration of secondary antibody resulted in binding to Paneth cells.

Time Considerations

Crypts isolation

Preparing the solutions takes ∼15 min; dissection of mice ∼15 to 30 min depending on the number; crypt isolation from 30 min to 1 hr; and crypt seeding, 30 min.

Lgr5-GFP^{+ve} cell sorting

Preparing the solutions takes ∼15 min; dissection of mice ∼15 to 30 min depending of the number; crypt isolation ∼45 min; cell dissociation ∼30 min; antibody staining ∼45 min; sorting ∼25 min; and single-cell seeding, ∼20 min.

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Literature Cited

Akcora, D., Huynh, D., Lightowler, S., Germann, M., Robine, S., de May, J.R., Pollard, J.W., Stanley, E.R., Malaterre, J., and Ramsay, R.G. 2013. The CSF-1 receptor fashions the intestinal stem cell niche. *Stem Cell Res.* 10:203-212.

Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H.J., van Es, J.H., Sato, T., Stange, D.E., Begthel, H., van den Born, M., Danenberg, E., van den Brink, S., Korving, J., Abo, A., Peters, P.J., Wright, N., Poulsom, R., and Clevers, H. 2010. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 6:25-36.

Donovan, J. and Brown, P. 2006. Euthenasia. *Curr. Protoc. Immunol.* 73:1.8.1-1.8.4.

Durand, A., Donahue, B., Peignon, G., Letourneur, F., Cagnard, N., Slomianny, C., Perret, C., Shroyer, N.F., and Romagnolo, B. 2012. Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor

Math1 (Atoh1). *Proc. Natl. Acad. Sci. U.S.A.* 109:8965-8970.

Farin, H.F., Van Es, J.H., and Clevers, H. 2012. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* 143:1518-1529.

Fuller, M.K., Faulk, D.M., Sundaram, N., Mahe, M.M., Stout, K.M., von Furstenberg, R.J., Smith, B.J., McNaughton, K.K., Shroyer, N.F., Helmrath, M.A., and Henning, S.J. 2013. Intestinal stem cells remain viable after prolonged tissue storage. *Cell Tissue Res*. 354:441-450.

Guan, Y., Watson, A.J., Marchiando, A.M., Bradford, E., Shen, L., Turner, J.R., and Montrose, M.H. 2011. Redistribution of the tight junction protein ZO-1 during physiological shedding of mouse intestinal epithelial cells. *Am. J. Physiol. Cell. Physiol* 300:C1404-C1414.

Jung, P., Sato, T., Merlos-Suarez, A., Barriga, F.M., Iglesias, M., Rossell, D., Auer, H., Gallardo, M., Blasco, M.A., Sancho, E., Clevers, H., and Batlle, E. 2011. Isolation and in vitro expansion of human colonic stem cells. *Nature Med.* 17:1225-1227.

Levin, D.E., Sala, F.G., Barthel, E.R., Speer, A.L., Hou, X., Torashima, Y., and Grikscheit, T.C. 2013. A "living bioreactor" for the production of tissue-engineered small intestine. *Methods Mol Biol.* 1001:299-309.

Liu, J., Walker, N.M., Cook, M.T., Ootani, A., and Clarke, L.L. 2012. Functional Cftr in crypt epithelium of organotypic enteroid cultures from murine small intestine. *Am. J. Physiol. Cell Physiol.* 302:C1492-C1503.

Miyoshi, H., Ajima, R., Luo, C.T., Yamaguchi, T.P., and Stappenbeck, T.S. 2012. Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. *Science* 338:108-113.

Mizutani, T., Nakamura, T., Morikawa, R., Fukuda, M., Mochizuki, W., Yamauchi, Y., Nozaki, K., Yui, S., Nemoto, Y., Nagaishi, T., Okamoto, R., Tsuchiya, K., and Watanabe, M. 2012. Real-time analysis of P-glycoprotein-mediated drug transport across primary intestinal epithelium three-dimensionally cultured in vitro. *Biochem. Biophys. Res. Commun.* 419:238-243.

Noah, T.K., Donahue, B., and Shroyer, N.F. 2011. Intestinal development and differentiation. *Exp. Cell Res.* 317:2702-2710.

Ootani, A., Li, X., Sangiorgi, E., Ho, Q.T., Ueno, H., Toda, S., Sugihara, H., Fujimoto, K., Weissman, I.L., Capecchi, M.R., and Kuo, C.J. 2009. Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat. Med.* 15:701-706.

Ramalingam, S., Daughtridge, G.W., Johnston, M.J., Gracz, A.D., and Magness, S.T. 2012. Distinct levels of Sox9 expression mark colon epithelial stem cells that form colonoids in culture. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302:G10-G20.

Rothenberg, M.E., Nusse, Y., Kalisky, T., Lee, J.J., Dalerba, P., Scheeren, F., Lobo, N., Kulkarni, S., Sim, S., Qian, D., Beachy, P.A., Pasricha, P.J., Quake, S.R., and Clarke, M.F. 2012. Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice. *Gastroenterology* 142:1195-1205.

Sandell, A. and Sakai, D. 2011. Mammalian cell culture. *Curr. Protoc. Essen. Lab. Tech.* 5:4.3.1-4.3.32.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., and Clevers, H. 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459:262-265.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G., Van Es, J.H., Van den Brink, S., Van Houdt, W.J., Pronk, A., Van Gorp, J., Siersema, P.D., and Clevers, H. 2011. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 141:1762-1772.

Simon-Assmann, P., Turck, N., Sidhoum-Jenny, M., Gradwohl, G., and Kedinger, M. 2007. In vitro models of intestinal epithelial cell differentiation. *Cell Biol. Toxicol.* 23:241-256.

Spence, J.R., Mayhew, C.N., Rankin, S.A., Kuhar, M.F., Vallance, J.E., Tolle, K., Hoskins, E.E., Kalinichenko, V.V., Wells, S.I., Zorn, A.M., Shroyer, N.F., and Wells, J.M. 2011. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 470:105-109.

Stelzner, M., Helmrath, M., Dunn, J.C., Henning, S.J., Houchen, C.W., Kuo, C., Lynch, J., Li, L., Magness, S.T., Martin, M.G., Wong, M.H., and Yu, J. 2012. A nomenclature for intestinal in vitro cultures. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302:G1359-G1363.

Tait, I.S., Flint, N., Campbell, F.C., and Evans, G.S. 1994. Generation of neomucosa in vivo by transplantation of dissociated rat postnatal small intestinal epithelium. *Differentiation* 56:91-100.

van Es, J.H., Sato, T., van de Wetering, M., Lyubimova, A., Nee, A.N., Gregorieff, A., Sasaki, N., Zeinstra, L., van den Born, M., Korving, J., Martens, A.C., Barker, N., van Oudenaarden, A., and Clevers, H. 2012. Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. *Nat. Cell Biol.* 14:1099-1104.

von Furstenberg, R.J., Gulati, A.S., Baxi, A., Doherty, J.M., Stappenbeck, T.S., Gracz, A.D., Magness, S.T., and Henning, S.J. 2011. Sorting mouse jejunal epithelial cells with CD24 yields a population with characteristics of intestinal stem cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 300:G409-G417.

Wang, F., Scoville, D., He, X.C., Mahe, M., Box, A., Perry, J., Smith, N.R., Lei Nanye, N., Davies, P.S., Fuller, M.K., Haug, J.S., McClain, M., Gracz, A.D., Ding, S., Stelzner, M., Dunn, J.C., Magness, S.T., Wong, M.H., Martin, M., Helmrath, M., and Li, L. 2013. Isolation and characterization of intestinal stem cells based on surface marker combinations and colony-formation assay. *Gastroenterology*. 145:383-395.

Yan, K.S., Chia, L.A., Li, X., Ootani, A., Su, J., Lee, J.Y., Su, N., Luo, Y., Heilshorn, S.C., Amieva, M.R., Sangiorgi, E., Capecchi, M.R., and Kuo, C.J. 2012. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proc. Natl. Acad. Sci. U.S.A.* 109:466-471.

Yui, S., Nakamura, T., Sato, T., Nemoto, Y., Mizutani, T., Zheng, X., Ichinose, S., Nagaishi, T., Okamoto, R., Tsuchiya, K., Clevers, H., and Watanabe, M. 2012. Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5(+) stem cell. *Nat. Med.* 18:618-623.

Key References

Barker et al., 2010. See above.

This paper describes, for the first time, the establishment of gastric epithelial organoids (gastroids).

Sato et al., 2009. See above.

The authors developed the conditions for a long-term culture of intestinal crypt-derived enteroids as well as the establishment of single Lgr5+ve^{+ve} cell-derived enteroids. Methods described in this article are based on this paper.

Sato et al., 2011. See above.

In this study, colonic crypt-derived colonoids are generated based on the method developed by Sato et al. in 2009.